www.centauro.it Rivista Italiana di Ossigeno-Ozonoterapia 5: 85-92 2006

Ozone Treatment Inhibits Proliferation in Human Neuroblastoma SK-N-SH Cells

B. BUCCI*, A. CANNIZZARO*, E. BRUNETTI*, M. MARTINELLI**

*AFAR-Centro Ricerca S. Pietro, Fatebenefratelli Hospital; Roma, Italy
** Dipartimento di Scienze Biomediche, S. Pietro, Fatebenefratelli Hospital; Roma, Italy

Key words: neuroblastoma, ozone therapy, O_3, hypoxia

SUMMARY - Tissue hypoxia results from an inadequate supply of oxygen (O_2) that compromises biologic functions. Recent experimental and clinical studies suggest that intratumoral oxygen levels may influence a series of biologic parameters affecting the tumor's malignant potential. Indeed, sustained hypoxia in a growing tumour may cause cellular changes that can result in a more clinically aggressive phenotype. During the last two decades it has been shown that ozonated autohemotherapy is therapeutically useful in arteriopathic patients because it increases oxygen delivery to hypoxic tissues, leading to normoxia. Although several oxygenation approaches have been tested, none is able to restore normoxia permanently in cancer. Since a prolonged cycle of ozonated autohemotherapy has been postulated to correct tumor hypoxia leading to a less aggressive behavior, we studied whether ozone treatment could affect cell growth and cell cycle perturbations on the human neuroblastoma SK-N-SH cell line. These data indicate that ozone acts as a real chemical drug capable of inhibiting cell growth suggesting its possible antineoplastic role in neuroblastoma cancer.

Introduction

Tissue hypoxia results from an inadequate supply of oxygen (O_2) that compromises biologic functions [1].

Traditionally, tumour hypoxia has been considered a potential therapeutic problem because it renders solid tumours more resistant to ionizing radiation [2-4]. More recent experimental and clinical studies [5-11] suggest that intratumoral oxygen levels may influence a series of biologic parameters affecting the tumor's malignant potential. Indeed, sustained hypoxia in a growing tumour may cause cellular changes that can result in a more clinically aggressive phenotype [12-16].

During the process of hypoxia-driven malignant progression, tumour may develop an increased potential for local invasive growth [17,18], perifocal tumour spreading [12,19] and regional and distant tumour spreading [13,14,20-22].

Experimental evidence indicates that hypoxia not only induces proteome changes influencing tumour propagation but also drives malignant progression through transient and persistent genomic changes in neoplastic cells [19,20,23,24-27]. Hypoxia promotes genomic instability (through point mutations, gene amplification, and chromosomal rearrangements) and may unveil pre-existing cryptic genetic variations, thus increasing the number of genetic variants.

Tumour hypoxia, when assessed by polarographic probes, is an independent prognostic factor for response to treatment and/or survival of patients with head and neck [21,28-30], uterine cervical tumour [12,14] and sarcoma [13,31]. As demonstrated by Overgaard's meta-analysis [32], improving tumor oxygenation can lead to better local control and increased overall survival rates following chemo-radiotherapy. Consequently, several strategies have been proposed to enhance tumor oxygenation inducing a constant restoration of normoxia. Ozone therapy is a technique that has been used in the treatment of ischemic syndromes [33-35].

Bernardino Clavo described a relationship between oxygenation in head and neck cancer and in anterior tibialis muscles [36], and an ozone therapy-induced improvement in the oxygenation of the 'most-hypoxic' anterior tibialis muscles, together with improvement in the most hypoxic tumors [37]. These data suggested that ozone therapy could have some positive effect during the treatment of head and neck cancer [38].

Nevertheless, the potential usefulness of ozone therapy alone or as an adjuvant in neoplastic

patients receiving chemo–radiotherapy warrants further investigation.

The aim of the present study was to evaluate whether O_3 treatment could affect cell growth and cell cycle in the SK-N-SH human neuroblastoma cell line.

Materials and Methods

Cell Culture

Human SK-N-SH cell line was maintained as a monolayer culture in EMEM supplemented with 10% heat-inactivated FCS (Life Technologies, Scotland, United Kingdom), antibiotics and L-glutamine (2 mM) at 37°C in a 5% CO_2/95% air atmosphere in a humidified incubator.

Ozone Generation

Ozone therapy was administrated using clinical grade O_2, the O_3/O_2 gas mixture was prepared with an ozonosan α plus photonic device 1014/10 (Dr. Hansler, Germany) and sterilized by passage though a sterile 0.20 µm filter.

Treatment and Growth Inhibition Assay

Cells were seeded at a density of 1.0x 10^5 cells *per* T25 flask and after 24 hours of cell culture were treated at room temperature using an ozonosan α plus photonic device. Different O_3 doses (6, 12, 25, 50 and 80 µg/ml) were tested as a single-dose regimen. As repeated-dose regimen, O_3 was added at concentrations of 6, 12, 25, 50 and 80 µg/ml given 24 hours after seeding and repeated three hours after the first treatment. After O_3, the flasks were immediately put back in the incubator and cell viability, evaluated as trypan blue exclusion test, was determined daily from day 1 (four hours after treatment) to day 3 (48 hours after treatment) of culture. Data were evaluated as percentages of control (i.e., absolute treated cell number/absolute control sample). All the experiments were repeated four times and each experimental sample was seeded in triplicate.

Cell Cycle Analysis

The cell cycle was studied using Propidium Iodide (PI) staining. PI stained treated and untreated cells were harvested, washed in cold PBS, fixed in 70% ethanol for at least one hour, and, after removing alcoholic fixative, stained with a solution containing 50 µg/mL PI (Sigma Chemical) and 75 KU/mL RNase (Sigma Chemical) in PBS for 30 minutes at room temperature in the dark. Samples were then measured by using a FACScan cytofluorimeter (Becton Dickinson, Sunnyvale, CA).

Propidium Iodide Cytotoxicity Assay

The capacity of 6, 12, 25, 50 and 80 µg/ml O_3 used in a single or repeated-regimen, to produce cell death was determined by PI staining exclusion test and flow cytometry. Cytotoxicity was defined as the cellular damage identified by PI staining, which shows the loss of structural integrity of the plasma membrane, a typical event of necrotic cell death. SK-N-SH cell line was stained with 100 µl of the supravital PI (4 mmol/L) for three minutes. After incubation, cells were washed with 5% bovine serum albumin in PBS and kept on ice until flow cytometry analysis.

Results

The oxonation experiments were conducted to investigate whether O_3 could be considered an antineoplastic drug. First we determined the optimal schedule of ozone treatment. We exposed cells to 6, 12, 25, 50 and 80 µg/ml O_3 doses and examined the effects produced by treatment on cell growth and the cell cycle at different times after ozone exposure (4, 24, and 48 hours). At each time, cells were harvested and counted using the trypan blue dye exclusion test. After addition of O_3 given in a single dose (6, 12, 25, 50 and 80 µg/ml), a dose-dependent inhibition of cell proliferation was observed (figure 1A). Four hours after exposure of cells to 80ug/ml, a cell growth inhibition of 58% was observed. The inhibitory effect become 99% after 48 hours, whereas growth inhibitions of 33% and 29% were obtained after 4 hours of exposure to 50 and 25 µg/ml O_3 and persisting during the 48 hours following treatment. When cells were exposed to the lowest O_3 doses of 12 and 6 µg/ml the growth inhibitions were about 24% and 17%, respectively. These latter effects were partially lost during the following days, with cell growth inhibition values evaluated 48 hours after O_3 treatment of about 17% and 10%, respectively.

We also measured the effect of repeated doses of 6, 12, 25, 50 and 80 µg/ml O_3. Figure 1B shows that the different doses of 6, 12, 25, 50 and 80 µg/ml given 24 hours after seeding and repeated three hours after the first treatment (repeated dose-regimen) enhanced cell growth reduction. The

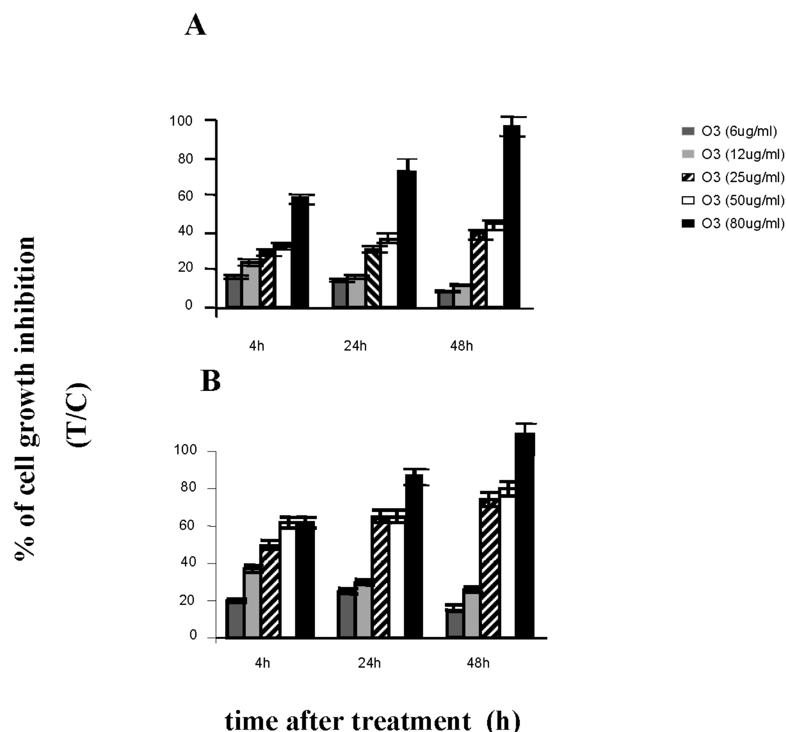

Figure 1 O_3-induced growth inhibition in SK-N-SK human cell line. 1×10^5 cells were seeded in T25 flasks and O_3 exposure was administered 24 h later. Cells were seeded at a density of 1.0×10^5 cells *per* T25 flask and, after 24 hours of cell culture, cells were exposed to increasing doses of O_3 6■, 12■, 25 ▨50 □and 80■ µg/ml in a single dose regimen A) and a repeated dose regimen B). The analysis was performed 4, 24 and 48 hours after treatment. At each time point the cells were harvested and counted by trypan blue exclusion test. Cell counts are reported as percentages of control. Data are representative of three different experiments. Values are means of triplicate samples.

maximum antiproliferative effect was observed at 80 µg/ml dose with similar values to those obtained with the previous regimen of O_3 treatment.

Four hours after the start of 50 and 25 µg/ml exposure we already observed cell growth inhibition of about 61% and 50% respectively (versus 33% and 29% obtained with the single-dose regime) and it was maintained up to the last time point of the cell growth experiment (days 3 = 48 hours after start of O_3 treatment). The percentage of inhibition was about 37% and 20% in the 12 and 6 µg/ml O_3-treated cells in comparison with the single-dose regimen (24% and 17%, respectively) and it was partially recovered during the following 48 hours.

To evaluate whether O_3-induced cell growth inhibition could be related to cell cycle perturbations, PI staining and fluorescence-activated cell sorting analysis were done on SK-N-SH cells exposed to 6, 12, 25, 50 and 80 µg/ml O_3 in single or repeated-dose regimen (table 1A and B). This analysis, performed at different times after 6, 12, 25 and 50 µg/ml O_3 exposure, given in a single dose-regimen, revealed an accumulation of the cells in the S-phase (41%, 44%, 46%, 50% and 34% in O_3 treated and untreated cells, respectively) suggesting a prolonged stay of the O_3-treated cells in S-phase. However, this S-phase accumulation was completely overcome in the 48 hours following 6 and 12 µg/ml O_3-treated cells. No detectable changes in the PI percentage toxicity was observed after 6, 12, 25 and 50 µg/ml O_3 exposure being less than 10% at 48h. Conversely the PI percentage toxicity was more than 75% after the highest dose indicating 80 µg/ml as a lethal dose.

The O_3 repeated dose-regimen induced an accumulation of cells in the G2 phase of the cell cycle compared with untreated cells and evaluated four hours after treatment (the G2 phase were 25%, 33%, 40%, 50% and 19% in 6, 12, 25 and 50 µg/ml and untreated cells, respectively). Cells treated by 6 and 12 µg/ml O_3 progressively decreased the G2 phase percentages to 17% and 23%, respectively within 48 hours after O_3 treatment and concomitantly increased the G1 phase (49% and 46%, respectively). Conversely, 48 hours after 25 µg/ml treatment, the G2 accumulation was still evident (40%). The cell cycle effect induced by 6 and 12 µg/ml O_3 doses was associated with a moderate cytotoxicity, evaluated as percentage of PI stained cells, being <13% and 15%, respectively, while a more consistent toxicity was observed at 25 µg/ml O_3 dose (more than 25%) within the same time interval (48 hours from treatment). Twenty-four hours after 50 µg/ml treatment, the cell cycle profile demonstrated that O_3 led to irreversible cellular damage promoting cell killing. Consistent with this hypothesis is the increasing toxicity percentages observed, the percentages of PI-positive cells was 50% at 48 hours. Moreover, these data

Table 1. Effect of O_3 on cell cycle distribution of SK-N-SH human neuroblastoma cells

A *Single dose-regime*

		G1 (%)	S (%)	G2 (%)	Toxicity (%)
O_3 4 hours	Control	47	34	19	2
	6μg/ml	45	41	14	3
	12μg/ml	42	44	14	4
	25μg/ml	40	46	14	3
	50μg/ml	39	50	11	5
	80μg/ml	nv	nv	nv	69
O_3 24 hours	Control	44	34	22	2
	6μg/ml	45	37	18	3
	12μg/ml	41	40	19	3
	25μg/ml	39	40	21	5
	50μg/ml	40	41	19	8
	80μg/ml	nv	nv	nv	78
O_3 48 hours	Control	49	39	12	2
	6μg/ml	46	41	13	5
	12μg/ml	43	41	16	8
	25μg/ml	42	42	16	9
	50μg/ml	43	42	15	9
	80μg/ml	nv	nv	nv	80

B *Repeated dose-regime*

		G1 (%)	S (%)	G2 (%)	Toxicity (%)
O_3 4 hours	Control	47	34	19	3
	6μg/ml	45	30	25	5
	12μg/ml	32	35	33	7
	25μg/ml	30	30	40	15
	50μg/ml	22	28	50	30
	80μg/ml	nv	nv	nv	70
O_3 24 hours	Control	43	34	22	3
	6μg/ml	41	31	28	8
	12μg/ml	44	31	25	12
	25μg/ml	20	40	40	25
	50μg/ml	nv	nv	nv	50
	80μg/ml	nv	nv	nv	80
O_3 48 hours	Control	49	39	12	3
	6μg/ml	49	34	17	13
	12μg/ml	46	31	23	15
	25μg/ml	20	40	40	30
	50μg/ml	nv	nv	nv	50
	80μg/ml	nv	nv	nv	88

FCM cell cycle analysis of untreated and O_3-treated SK-H-SH cells at the 4, 24 and 48 after the treatment. The cell cycle phase percentages were estimated by applying the MODFIT software to the PI/DNA histograms. Data are means of three separate experiments with similar results.

confirmed that 80 μg/ml O_3 is a lethal dose. These findings indicate that ozone acts as a real chemical drug capable of inhibiting neuroblastoma cell growth suggesting its possible antineoplastic role in neuroblastoma cancer.

Discussion

In solid tumours, oxygen delivery to the respiring neoplastic and stromal cells is frequently reduced or even abolished by a deteriorating diffusion geometry, severe structural abnormalities of tumour microvessels, and impaired microcirculation [39]. In an increasing number of reports on tumour oxygenation, the addition, anemia and formation of methemoglobin or carboxyhemoglobin reduce the blood's capacity to transport O_2. As a result, areas with very low (down to zero) oxygen partial pressure exist in solid tumours, occurring either acutely or chronically. These microregions of very low or zero O_2 partial pressures are heterogeneously distributed within the tumour mass and may be located adjacent to regions with normal O_2 partial pressure. In contrast to normal tissue, neoplastic tissue can no longer fulfil physiologic functions. Thus, tumour hypoxia cannot be defined by functional deficits, although areas of necrosis, which are often found in tumour tissue on microscopic examination, indicate the loss of vital *nv*= not evaluable cellular functions. Actually hypoxia (defined as the fraction of measured O_2 partial pressures of <5 mmHg) is a statistically significant adverse prognostic factor of disease-free survival. A Kaplan–Meier analysis showed statistically significantly shorter survival and recurrence-free survival for patients with hypoxic tumors. The results were consistent with the hypothesis that radiobiologically hypoxic tumors (i.e., tumors with a reduced radiosensitivity at critically low O_2 levels) are less curable [40], even though the role of hypoxia in conventional antineoplastic drug resistance could not be excluded.

These data were supported by Cox regression analysis which revealed tumor oxygenation as the strongest independent prognostic factor, followed by FIGO stage [12]. Of special interest was the fact that the disadvantage in outcome for patients with hypoxic tumor was independent of primary treatment (radiation therapy or radical surgery). Sundfor et Al [41] reported a poor outcome associated with low oxygen tension in 40 patients with advanced squamous cell carcinoma of the uterine cervix. These findings are in agreement with

results obtained by Fyles et Al [42] who found that the pretreatment oxygenation status of tumors can predict disease-free survival in patients with cervical cancer. In addition, Knocke et Al [43] confirmed the prognostic relevance of pretreatment tumor oxygenation status by studying 51 patients with cancer of the uterine cervix after radiation treatment. The pretreatment tumor oxygenation status was also assessed in patients with soft-tissue sarcoma: patients with hypoxic tumor were associated with a poorer survival when compared with patients with normoxic tumor resulting from local treatment failure or distant metastases [13,44,45,46].

Recent work has led to the hypothesis that tumor hypoxia may be associated with malignant progression by locoregional and distant tumor propagation. This observation was confirmed by Hochel et Al who reported that hypoxia may not only counteract O_2-dependent forms of therapy but may also advance tumor progression *per se* independently of treatment [12]. Since modification of the levels of hypoxia with therapy has been shown to improve therapeutic outcomes [32], several strategies have been proposed to enhance tumor oxygenation inducing a constant restoration of normoxia.

A special workshop sponsored by the National Cancer Institute, established the need to investigate methods to overcome tumor hypoxia [47]. Clavo reported that ozone therapy could have an important role in increased oxygenation restoring normoxia in the most poorly oxygenated head and neck tumors [38].

Ozone (O_3) is the allotropic form of oxygen with three atoms and two unpaired electrons, which has a higher oxidizing capacity than oxygen. Nevertheless, the potential usefulness of ozone therapy as an adjuvant in chemo–radiotherapy for neoplastic lesions warrants further investigation. Our findings demonstrated that O_3 affects cell growth and the cell cycle in the neuroblastoma SK-N-SH cell line. Our data are in agreement with Sweet et Al [48] who reported ozone as a possible antineoplastic drug because of its capacity to inhibit the growth of human cancer cells. Since the therapeutic window for ozone concentration ranges from 20 to 80 µg/ml [49], we utilized 6, 12, 25, 50 and 80 µg/ml O_3 doses to verify its possible antitumoral effect on the SK-N-SH human neuroblastoma cell line.

We found that the 6, 12, 25, 50 and 80 µg/ml O_3 doses produced different cell cycle effects when administered in single or repeated-dose regime. We showed that tumor cells exposed to the lowest O_3 doses of 6 and 12 µg/ml when given in single dose-regime partially recovered the O_3-induced S-phase accumulation while 6 and 12 µg/ml given in a repeated dose-regimen produced a different cell cycle perturbation, inducing a G2 arrest. However, this G2 accumulation was overcome in the 48 hours following treatment. Whereas the 25 µg/ml O_3, given in the repeated dose-regime abolished the ability of the cells to overcome the G2 block compared to the lowest doses (6 and 12 µg/ml). Indeed, this G2 block seems to be permanent, because at 48 hours after the start of treatment a fraction of the cells was still in G2 phase (40%). On the contrary, 25 µg/ml O_3, in a single dose-regime produced a slight S-phase accumulation. The highest dose of 80 µg/ml in both single and repeated dose-regimens produced irreversible cellular damage, indeed the cells were not able to repopulate the cell cycle and died from necrosis, whereas 50 µg/ml O_3 given in a single dose-regime did not produce any significant toxic effect. Our hypothesis is that different molecular events occur following O_3, which affect the cell cycle and can lead in turn to DNA damage repair, cell cycle perturbation or death. If the damage is too massive it is followed by death in an attempt to eliminate such severely damaged cells.

This hypothesis is supported by the capacity of O_3 when administered as autotransfusion in cancer patients, to react with organic compounds (hydrosoluble and lipophilic antioxidants, unsaturated fatty acids, etc) generating a number of messengers acting on various blood components and procuring early (by ROS) and late (by LOP) biological effects. Bocci et Al [49] showed that ozone, *via* the transitory action of hydrogen peroxide, acts as a mild inducer of cytokines in leukocytes. Therefore, by releasing cytokines in lymphoid microenvironments primed lymphocytes and monocytes may slowly bring about a concerted activation of the immune system usually suppressed by tumor growth.

In contrast to the claim of a possible O_3 toxic effect on blood cells, it has been reported that O_3-induced toxicity is overcome using the appropriate dose. Indeed, the range of the therapeutic window has been determined between 20 and 80 µg/ml *per* ml of blood cells (0.42-1.68 mM) which did not produce any toxic effect. Indeed, patients undergoing ozone therapy do not have adverse effects and most patients reported a feeling of wellness and euphoria. Normally a cycle of 14-15 treatments (twice weekly) significantly improves visual acuity in about 70% of patients with the atrophic form of age-related macular degeneration: (ARMD) [50] and in most patients with chronic limb ischemia (stage II) [34,51,52]. Our data are in partial agreement with results obtained by Bocci et Al [49] on O_3 cytotoxicity. We did not find toxicity at doses of 6, 12, 25 and 50 µg/ml when O_3 was administered in a single

dose-regime while toxicity was obtained when O_3 was given in a repeated dose-regime. The highest dose of 80 μg/ml produced an elevated cytotoxicity in both single and repeated-dose regime and was considered a lethal dose. We observed that the O_3 induced a dose dependent cell growth inhibition and that the highest dose was able to activate necrotic cell death by producing irreversible cellular damage inhibiting the progression of cells through the cell cycle. In agreement with Bocci et Al. [40] we also believe that ozone acts as a real chemical drug.

Conclusion

Ozone directly inhibited neoplastic cell growth following injection into the neoplastic nodule or *via* reinfusion of ozonated blood in patients bearing neuroblastoma cells. In accordance with Overgaard [32], we think that improving tumor oxygenation can lead to better local neoplastic control, increased patient survival rates and a better quality of life. Indeed ozone therapy could be a new therapeutic approach targeted to specifically bypass the resistance implemented by hypoxic selection reducing the risk of hypoxia-mediated treatment failures and improving survival and disease-free recurrence in neuroblastoma cancer patients. Besides the normalization of hypoxia, ozone therapy displays other interesting biological effects that may enhance the therapeutic outcome.

Acknowledgements

This work was supported by AFaR (Associazione Fatebenefratelli per la Ricerca).

Bibliography

1 West JB: Respiratory physiology-the essentials. 6th ed. Baltimore (MD), London (U.K.), and Los Angel (CA): Williams & Wilkins, 1999.
2 Gray LH, Conger AD, Ebert M et Al: The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. Br J Radiol 26: 638-648, 1953.
3 Hall EJ, editor: Radiobiology for the radiologist. 4th ed. Philadelphia (PA): Lippincott, 1994.
4 Hill RP: Cellular basis of radiotherapy. In: Tannock IF, Hill RP (Ed): The basic science of oncology. McGraw-Hill, New York 1992: 259-279.
5 Vaupel P, Kelleher DK, editors. Tumor hypoxia: pathophysiology, clinical significance and therapeutic perspectives. Stuttgart (Germany): Wissenschaftliche Verlagsgesellschaft, 1999.
6 Molls M, Vaupel P, editors. Blood perfusion and microenvironment of human tumors. Implications for clinical radiooncology. Berlin and Heidelberg (Germany) and New York (NY): Springer 2000.
7 Raleigh JA, editor. Hypoxia and its Clinical Significance. Semin Radiat Oncol 6: 1-70, 1996.
8 Brown GM, Giaccia AJ: The unique physiology solid tumors: opportunities (and problems) for cancer therapy. Cancer Res 58: 1408-1416, 1998.
9 Semenza GL: Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. Crit Rev Biochem Mol Bio 35: 71-103, 2000.
10 Semenza GL: HIF-1 mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 88: 1474-1480, 2000.
11 Sutherland RM: Tumor hypoxia and gene expression-implications for malignant progression and therapy. Acta Oncol 37: 567-574, 1998.
12 Hockel M, Schlenger K, Aral B et Al: Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 56: 4509-4515, 1996.
13 Brizel DM, Scully SP, Harrelson JM et Al: Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. Cancer Res 56: 941-943, 1996.
14 Sundfor K, Lyng H, Rofstad EK: Tumor hypoxia and vascular density as predictors of metastasis in squamous cell carcinoma of the uterine cervix. Br J Cancer 78: 822-827, 1998.
15 Hockel M, Schlenger K, Hockel S et Al: Tumor hypoxia in pelvic recurrences of cervical cancer. Int J Cancer 79: 365-369, 1998.
16 Walenta S, Wetterling M, Lehrke M et Al: High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancer. Cancer Res 60: 916-921, 2000.
17 Cuvier C, Jang A, Hill RP: Exposure to hypoxia, glucose starvation and acidosis: effect on invasive capacity of murine tumor cell and correlation with cathepsin (L+B) secretion. Clin Exp Metastasis 15: 19-25, 1997.
18 Graham CH, Forsdike J, Fitzgerald CJ et Al: Hypoxia mediated stimulation carcinoma cell invasiveness via upregulation of urokinase receptor expression. Int J Cancer 80: 617-623, 1999.
19 Hockel M, Schlenger K, Hockel S et Al: Hypoxic cervical cancers with low apoptotic index are highly aggressive. Cancer Res 59: 4525-4528, 1999.
20 Young SD, Marshall, Hill RP: Hypoxia induces DNA over replication and enhances metastatic potential of murine tumor cells. Proc Natl Acad Sci USA 85: 9533-9537, 1988.
21 Brizel DM, Sibley GS, Prosnitz LR et Al: Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. Int J Radiat Oncol Biol Phys 38: 285-289, 1997.
22 Jang A, Hill RP: An examination of the effects of hypoxia, acidosis, and glucose starvation on the expression of the metastasis-associated genes in murine tumor cells. Clin Exp Metastasis 15: 469-483, 1997.
23 Rice GC, Hoy C, Schimke RT: Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells. Proc Natl Acad Sci USA 83: 5978-5982, 1986.
24 Reynolds TY, Rockwell S, Glazer PM: Genetic instability induced by the tumor microenvironment. Cancer Res 56: 5754-5757, 1996.
25 Stoler DL, Anderson GR, Russo CA et Al: Anoxia-inducible endonuclease activity as a potential basis of the genomic instability of cancer cells. Cancer Res 52: 4372-4378, 1992.
26 Cheng KC, Loeb LA: Genomic instability and tumour progression: mechanistic considerations. Adv Cancer Res 60: 121-156, 1993.
27 Russo CA, Weber TK, Volpe CM et Al: An anoxia inducible endonuclease and enhanced DNA breakage as contributors to genomic instability in cancer. Cancer Res 55: 1022-1028, 1995.
28 Gatenby RA, Kessler HB, Rosenblum JS et Al: Oxygen distribution in squamous cell carcinoma metastases and its relationship to outcome of radiation therapy. Int J Radiat Oncol Biol Phys 14: 831-838, 1988.
29 Nordsmark M, Overgaard J: A confirmatory prognostic study on oxygenation status and loco-regional control in advanced head and neck squamous cell carcinoma treated by radiation therapy. Radiother Oncol 57: 39-43, 2000.
30 Clavo B, Lloret M, Perez JL et Al: Association of tumour oxygenation with complete response, anemia and erythropoietin treatment. J Clin Oncol 21: 443, 2002.
31 Nordsmark M, Alsner J, Keller J et Al: Hypoxia in human soft tissue sarcomas: adverse impact on survival and no association with p53 mutations. Br J Cancer 84: 1070-1075, 2001.
32 Overgaard J, Horsman MR: Modification of hypoxia-induced radioresistance in tumours by the use of oxygen and sensitizers. Semin Radiat Oncol 6: 10-21, 1996.
33 Rovira G, Galindo N: La ozonoterapia en el tratamiento de las úlceras crónicas de las extremidades inferiores. Angiologia 2: 47-50, 1991.
34 Romero A, Menéndez S, Gómez M et Al: La Ozonoterapia en los estadios avanzados de la aterosclerosis obliterante. Angiologia 45: 146-148, 1993.
35 Giunta R, Coppola A, Luongo C et Al: Ozonized autohemotransfusion improves hemorheological parameters and oxygen delivery to tissues in patients with peripheral occlusive arterial disease. Ann Hematol 80: 745-748, 2001.
36 Clavo B, Perez JL, Lopez L et Al: Influence of haemoglobin concentration and peripheral muscle pO2 on tumour oxygenation in advanced head and neck tumours. Radiother Oncol 66: 71-74, 2003.
37 Clavo B, Perez JL, Lopez L et Al: Effect of ozone therapy on muscle oxygenation. J Altern Complement Med 9: 251-256, 2003.
38 Clavo B, Perez JL, Lopez L et Al: Ozone therapy for tumor oxygenation: A pilot study. eCAM 1: 93-98, 2004.
39 Vaupel P, Kallinowski F, Okunieff P: Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumours: a review. Cancer Res 49: 6449-6465, 1989.
40 Hockel M, Knoop C, Schlenger K et Al: Intratumoral pO2 predicts survival in advanced cancer of the uterine cervix. Radiother Oncol 26: 45-50, 1993.
41 Sundfor K, Lyng H, Trope CG et Al: Treatment outcome in advanced squamous cell carcinoma of the uterine cervix: relationships to pretreatment tumor oxygenation and vascularization. Radiother Oncol 54: 101-107, 2000.
42 Fyles AW, Milosevic M, Wong R et Al: Oxygenation predicts radiation response and survival in patients with cervix cancer. Radiother Oncol 48: 149-156, 1998.
43 Knocke TH, Weitmann HD, Feldmann HJ et Al: Intratumoral pO2–measurements as predictive assay in the treatment of carcinoma of the uterine cervix. Radiother Oncol 53: 99-104, 1999.
44 Nordsmark M, Hoyer M, Keller J et Al: The relationship

between tumour oxygenation and cell proliferation in human soft tissue sarcomas. Int J Radiat Oncol Biol Phys 35: 701-708, 1996.
45 Nordsmark M, Hoyer M, Keller J et Al: Predictive assays in radiotherapy, the Aarhus experience. Oxygenation, cell kinetics and 31P MR spectroscopy in human soft tissue sarcomas. Radiother Oncol 46: 107, 1996.
46 Nordsmark M, Keller J, Hoyer M et Al: Hypoxia in human soft tissue sarcomas associated with poor survival. Radiother Oncol 48: 162, 1998.
47 Stone HB, Brown GM, Phillips TL et Al: Oxygen in human tumours: correlations between methods of measurement and response to therapy. Radiat Res. 136: 422-434, 1993, Review.
48 Sweet F, Kao MS, Lee SC et Al: Ozone selectively inhibits growth of human cancer cells. Science 209: 931- 933, 1980.
49 Bocci V, Larini A, Micheli V: A constant restoration of normoxia may control neoplastic growth. A reappraisal of an old approach. J Altern Complement Med 11: 257-265, 2005, Review.
50 Bocci V, Diadori A, Larini A et Al: Ozonetherapy and age related macular degeneration: a pilot study. Eur. J. Ophthalmol, 2003, in press.
51 Rokitansky O, Rokitansky A, Steiner J Trubel et Al: Die Ozontherapie bei peripheren, arteriellen Durchblutungsstörungen; klinik, biochemische und blutgasanalytische Untersuchungen. In Wasser IOA, Ozon-Weltkongress, Berlin 1981: 53-75.
52 Matassi R, D'Angelo F, Bisetti P et Al: Terapia con ozono per via parenterale nelle arteriopatie obliteranti periferiche: meccanismo biochimico e risultati clinici. Il Giornale di Chirurgia VIII: 109-111, 1987.

Dr Barbara Bucci
AFAR - Centro Ricerca S. Pietro
Fatebenefratelli Hospital
Via Cassia 600
00189 Rome
ITALY
Ph: 0039633582873
Fax: 0039633251278
E-mail: bucci.barbara@fbfrm.it