EXPERIMENTAL FINDINGS OF THE STUDY OF OZONE IN THE ANTITUMOR RESPONSE

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WHAT IS CANCER ?

Progressive molecular changes, leading to **cell clones arising with a malignant phenotype**, characterized by developing **adaptive survival advantage** over normal cells, which **proliferate chronically** and result in **clinically detectable** malignant tumors.

Cancer Progression

Evade molecular controls of cell growth, induction of senescence and programmed cell death.

The current knowledge about the origin and evolution of malignancies should contribute to the design of strategies for prevention, early diagnosis and treatment of advanced disease

THE ESCAPE TO THIS REGULATION INDUCE THE SUSTAINED PROLIFERATION OF TUMORS

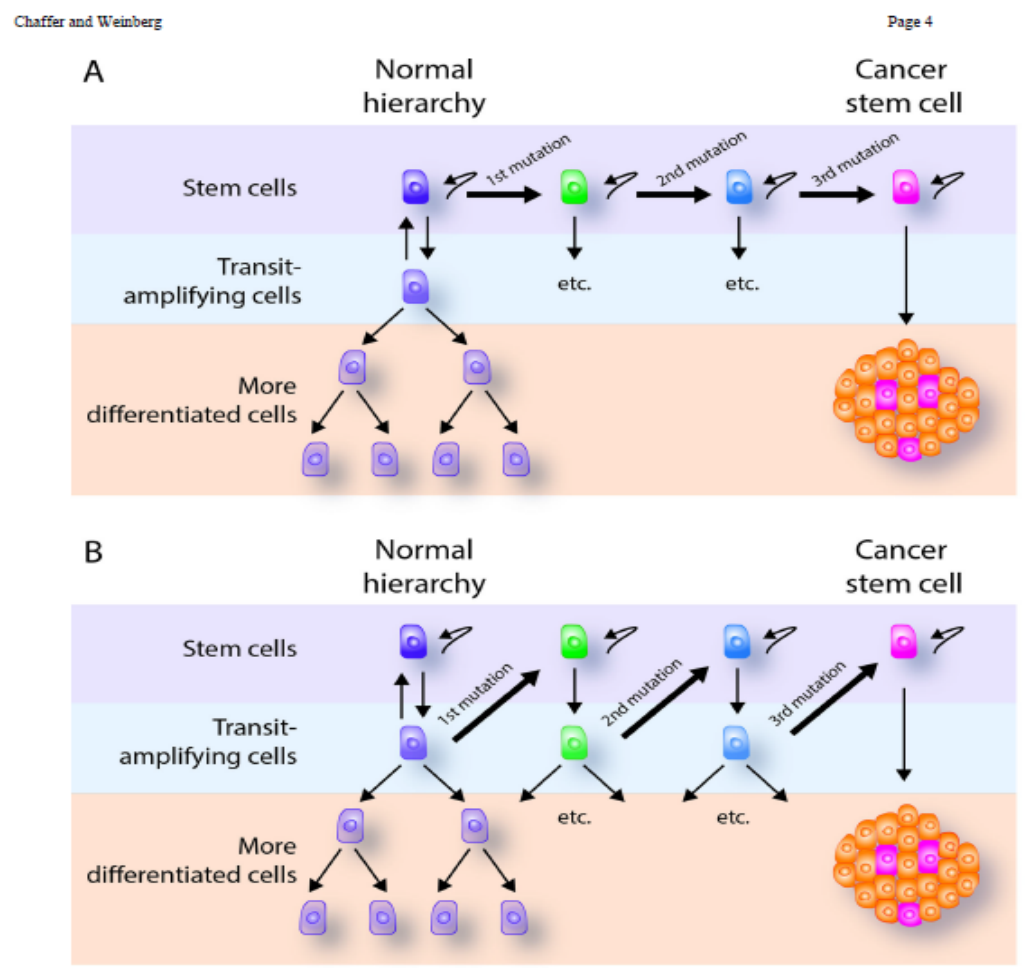
Signals factors growth

Cdk/C

Tumor suppressor genes

THIS IS CONSIDERED THE INITIAL EVENT OF TUMORIGENESIS

PLASTICITY OF CELL POPULATIONS ORGANIZED HIERARCHICALLY



- An emerging concept states that Cancer Stem Cells (CSC), key in cancer initiation and metastasis cells, arise when "transit amplifying" cells containing mutations are de-differentiate and enter a state of stem cell

- This model contrasts with the notion that CSC are derived from neoplastic transformation of normal cells mothers.

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Understand cancer as a genetic disease that generates a systemic inflammation that allows the metastatic spread of the disease.

HALLMARKS OF CANCER

Deregulation of cellular energy (metabolic Reprogramming)

Self-sufficiency in growth signals

Insensitivity to growth suppressive signals

Unlimited replicative potential

Sustained angiogenesis

Tissue invasion and metastasis

Evasion of apoptosis

Promoting inflammation

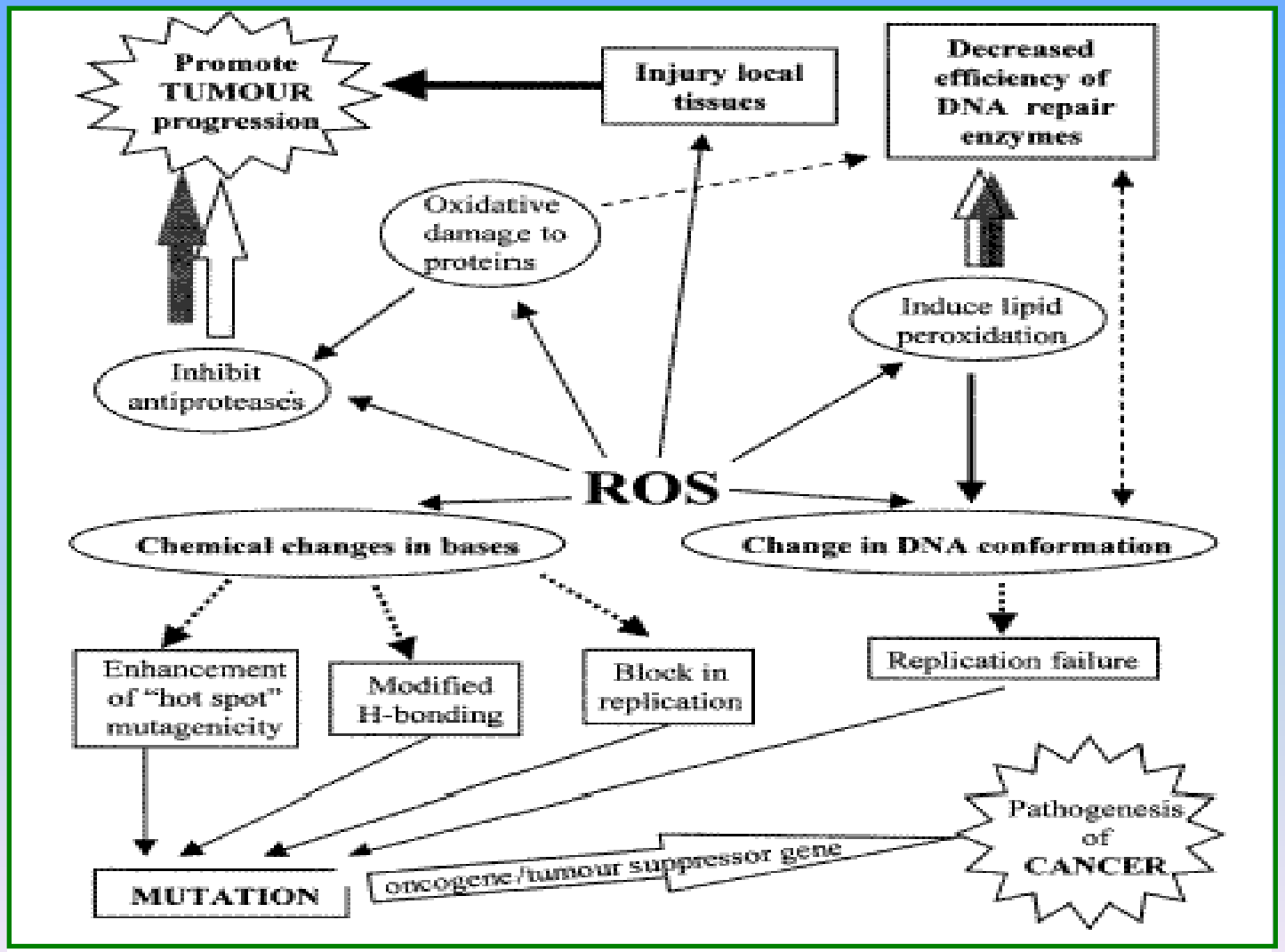
Immunosuppressant environment

Inflammation and genetic instability: enabling conditions

Dunn GP,. Old LJ,. Schreiber RD. The Three Es of Cancer Immunoediting. *Annu. Rev. Immunol.* 2004, 22:329-60

Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell* 2011;144: 646-72.

Reactive oxygen species (ROS) are involved in a diversity of important phenomena in the process of tumor development

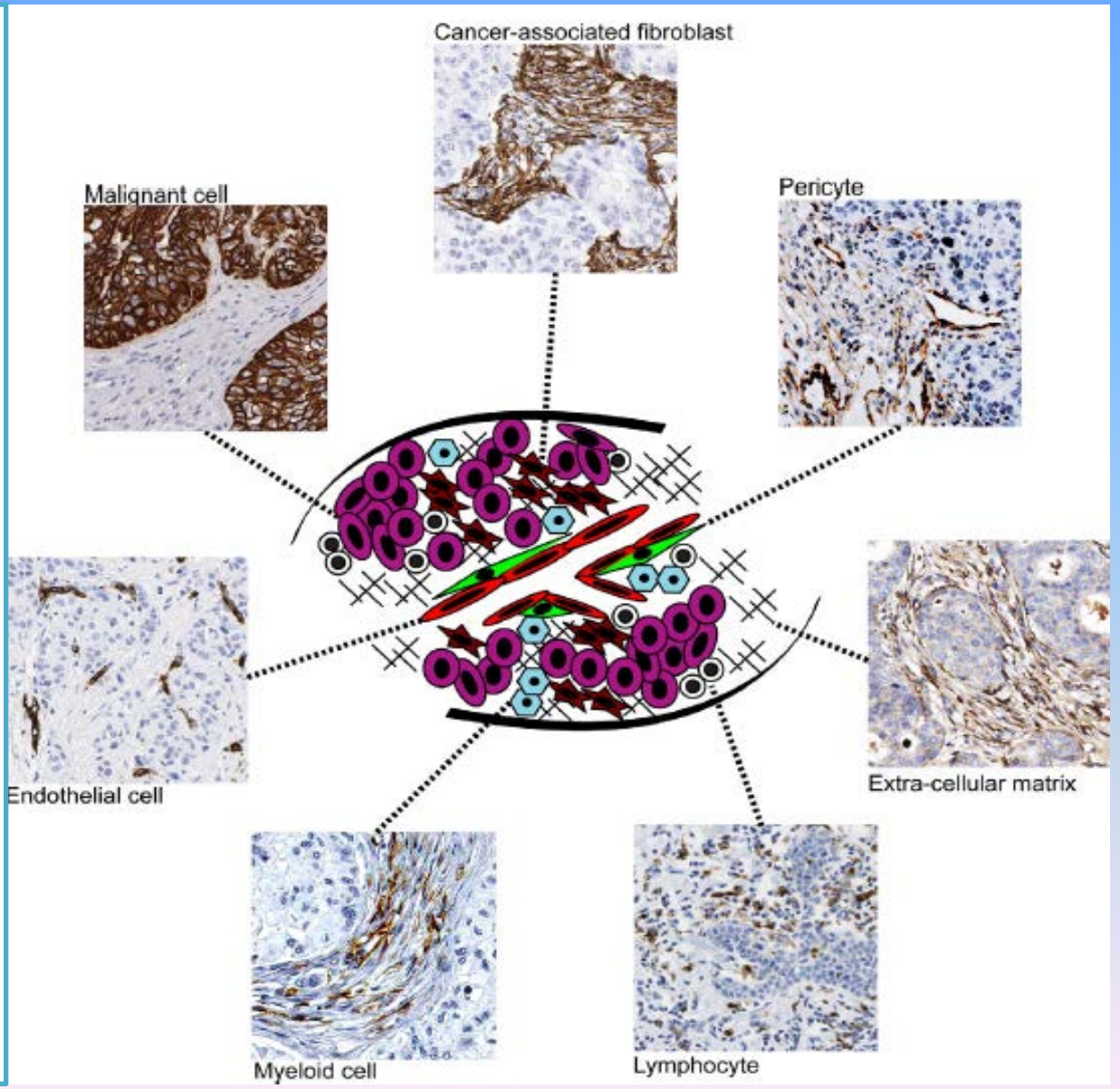


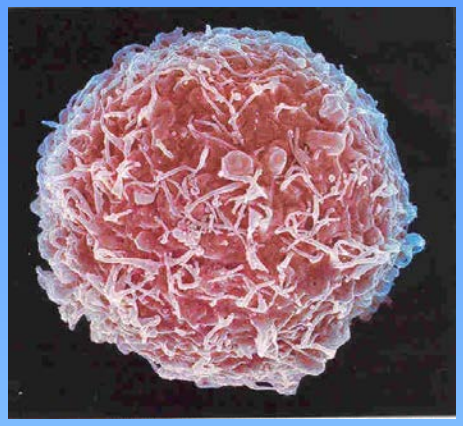
MECHANISMS ASSOCIATED TO CARCINOGENESIS MEDIATED BY ROS

TUMOR MICROENVIRONMENT (TME)

Essential elements:

- Tumor cells
- Fibroblasts (CAFs)
- Blood vasculature
  - Endothelial cells
  - Pericytes
  - Platelets
- Extracellular matrix (ECM)
- Immune cells
- Factors secreted





Mantains the cellular redox balance

Antioxidants Prooxidants

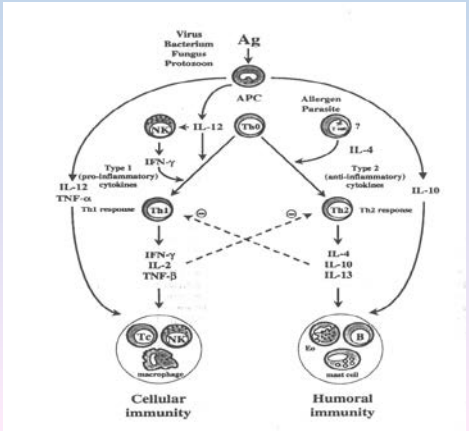
OZONE

Increases oxygen metabolism

(O_3 increases oxigenation in hypoxic tumors).

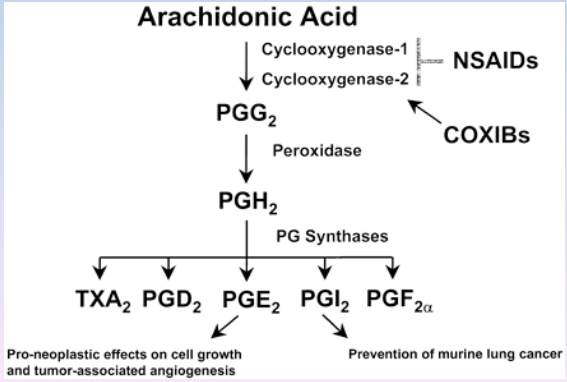
Tumor hypoxia increase resistance to radiotherapy and chemotherapy.

Immunological regulator



Influences in the synthesis / release of eicosanoids

PGI_2 PGE_2



Ozone may induce an adaptation to oxidative stress or an oxidative preconditioning, that under controlled doses, may stimulate the endogenous antioxidant mechanism, preparing the host to face physiopathological conditions mediated by reactive oxygen species

DEMOSTRATED PROTECTION MEDIATED BY O_3 /O_2

II

HEPATOTOXICITY (CCl_4) (EXPERIMENTAL)

ENDOTOXIC SHOCK INDUCED BY LPS (EXPERIMENTAL)

DIABETES (EXPERIMENTAL AND CLINICAL TRIAL)

HEPATIC DAMAGE (ISCHEMIA/REPERFUSION (EXPERIMENTAL)

ACUTE NEPHROTOXICITY INDUCED BY CISPLATIN (EXPERIMENTAL)

TOXIC GLOMERULONEPHRITIS INDUCED BY ADRIAMICINA (EXPERIMENTAL)

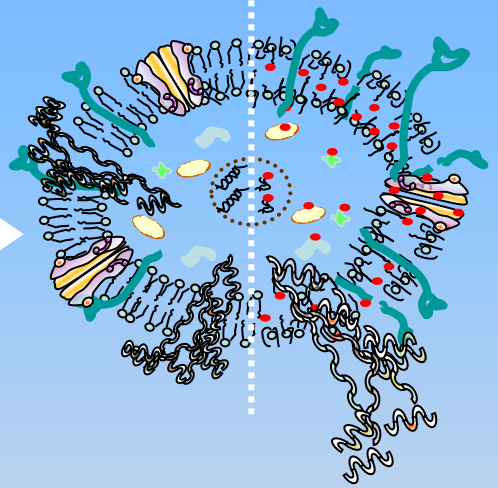
LETHAL PERITONITIS (EXPERIMENTAL)

BURN MICE

O_3

ROS

NORMAL CELL → CANCER CELL



LINK BETWEEN THE TISSULAR DAMAGE, INDUCED CHEMICALLY OR SURGICALLY, AND THE CANCER

GENERAL AIM

To determine the possible effects of ozone therapy in some experimental models of malignant tumors in mice.

SPECIFIC AIMS

1. **To evaluate the reiterated toxicity of the ozone therapy using different applications.**
2. **To evaluate the effect of ozone therapy on multiplicity of tumors.**
3. **To evaluate the effect of the ozone oxidative-preconditioning in tumor development.**

MATERIALS AND METHODS

OZONE GENERATOR OZOMED MOD 01

1. Reiterated Toxicity Assay:

Mice: NMRI males and females, 20-25 g.

Administration schedule:

O_3/O_2 during 15 days, rectal and intraperitoneal applications.

Rectal concentrations: 11, 35 y 71 mg/L,
Volume: 5, 40 y 80 mL/Kg in each case.

Intraperitoneal concentrations: 11, 20 y 35 mg/L, Vol 1 mL

MATERIALS AND METHODS

OZONE GENERATOR
OZOMED MOD 01

2. Evaluation of tumor multiplicity using different ozone concentrations.

Mice: NMRI and $B_6D_2F_1$ males, 18-20 g

Schedule: O_3/O_2 rectal application cycle of 12 days

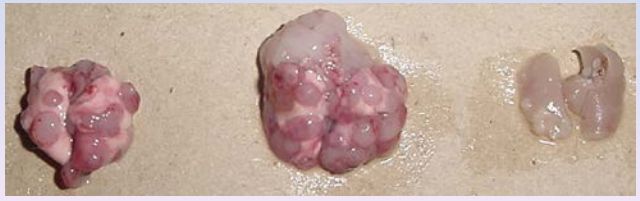
Concentrations: 19, 26 y 42 mg/L (n=10)
(1 mL in each case)

Tumors:

- Erlich Ascitic Tumor
- Sarcoma 37

(1 x 10^6 cells implanted by ocular plexus)

The hematogenic dissemination of the neoplastic cells present in the lung was evaluated.



MATERIALS AND METHODS

OZONE GENERATOR
OZOMED MOD 01

3. Effects of ozone oxidative-preconditioning in tumor development.

Mice: $B_6D_2F_1$ males, 18-20 g

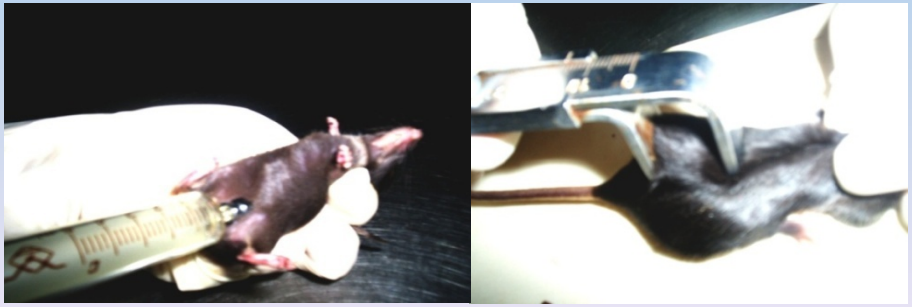
Schedule: 15 sessions daily O_3/O_2
Pre- treatment, intraperitoneal application.

Concentrations: 4, 11, 20 y 35 mg/L
volume 80 mL/Kg in each case. (n=10)

Tumor: Lewis Lung Carcinoma.
1 x 10^6 cells

(0.25 mL subcutaneous,

24 h after the ozone treatment)



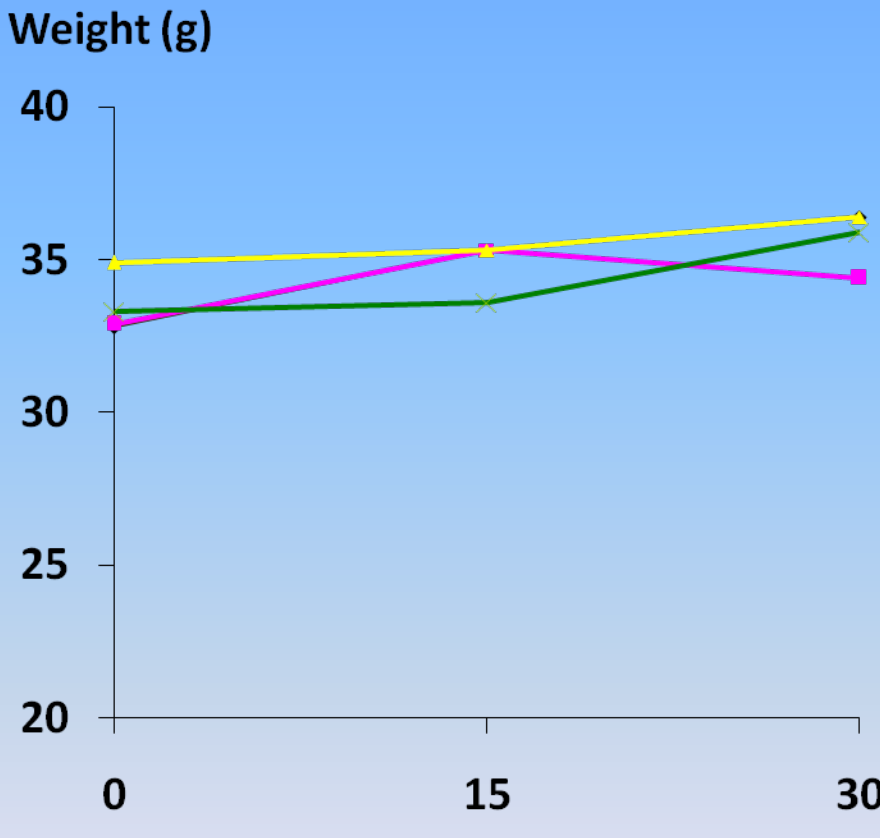
Tumoral volume calculation:

$V\ (mm^3) = a^2 \times L/2$
(a- wide, L- large)

Animal weights and tumor volume measurements.

Toxicity assays in mice

Table 1. Results of body weight in NMRI mice using rectal ozone application.



Groups	Body weight gained (g/day)	
	After 15 sessions	End of experiment (30 days)
Control	0.17 ±0.07	0.12 ±0.04
O3/O2 11 mg/L	0.16 ±0.06	0.07 ±0.01
O3/O2 35 mg/L	0.03 ±0.01	0.06 ±0.02
O3/O2 71 mg/L	0.02 ±0.02	0.09 ±0.01

Body weight gained during rectal ozone treatment was less in groups treated with higher concentrations, but there was not significant differences between groups at the end of the assay.

Table 2. Relative weights (%) of the organs in NMRI mice using rectal ozone application

Groups		Spleen	Liver	Heart	Lung
Control		0.58	6.17	0.72	0.60
O_3/O_2	11 mg/L	0.74	5.55	0.64	0.63
	35 mg/L	0.54	5.69	0.62	0.62
	71 mg/L	0.52	5.39	0.54	0.64

These results are with the highest volume (80 mL/Kg) for all the ozone concentrations studied.

The pathology study of the organs did not show toxicity alterations in the groups at the maximum doses of ozone treatment.

Table 3. Hematological parameters evaluated in NMRI mice using rectal ozone application.

Time (days)	Control	O_3/O_2		
Beginning (t=0)		c=11 mg/L	c= 35 mg/L	c=71 mg/L
Hematocrite	0.42 ±0.029	0.43 ±0.057	0.51 ±0.012	0.47 ±0.064
Leucocytes(x 10^9)	5.13 ±0.15	4.23 ±0.12	4.23 ±0.12	5.13 ±0.15
Polymorph	25.0 ±1	18.33 ±8.50	16.33 ±6.11	16.67 ±5.51
Lymphocytes	75.0 ±1	81.67 ±8.50	83.67 ±6.11	83.33 ±5.51
End of treatment (t=15)				
Hematocrite	0.52 ±0.029	0.53 ±0.029	0.50 ±0	0.49 ±0.036
Leucocytes(x 10^9)	6.17 ±0.12	7.17 ±0.12	9.13 ±0.15	7.20 ±0.26
Polymorpho	25 ±5	27 ±3.61	20 ±2	39.67 ±1.53
Lymphocytes	75 ±5	73 ±3.61	80 ±2	60.33 ±1.53

These results are with the highest volume (80 mL/Kg) for all the ozone concentrations studied.

Table 4. Results of body weight in NMRI mice (both sex) using intraperitoneal ozone application.

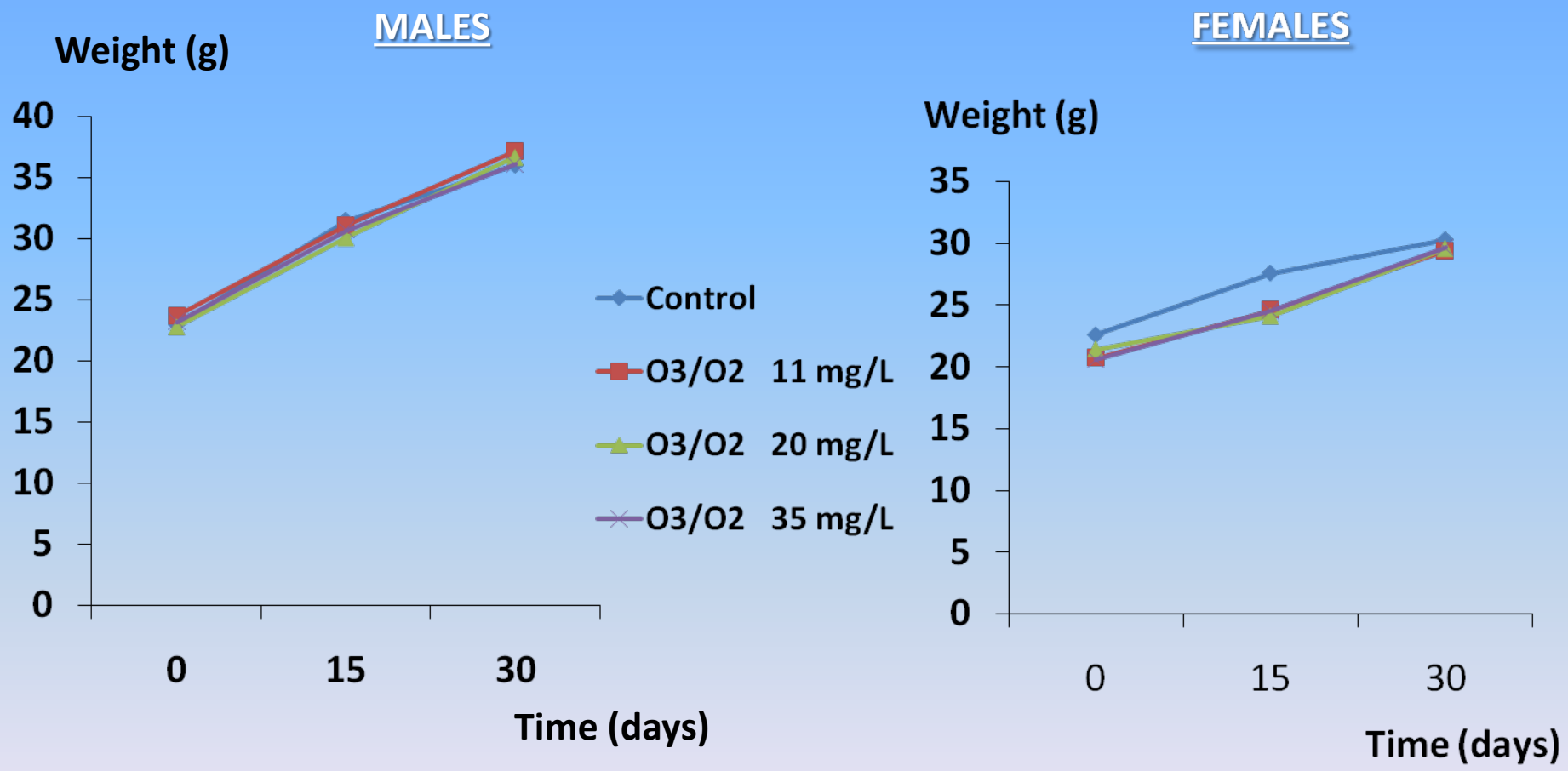


Table 5. Relative weights (%) of the organs in NMRI mice (both sex) by intraperitoneal ozone application

Groups		Spleen		Liver		Heart		Lung		Kidney	
		F	M	F	M	F	M	F	M	F	M
Control		0.69	0.53	6.57	7.01	0.55	0.51	0.88	0.75	1.27	1.61
O_3/O_2 (mg/L)	11	0.68	0.68	6.50	6.54	0.58	0.58	0.98	0.76	1.28	1.61
	20	0.81	0.77	6.81	7.34	0.56	0.66	1.08	0.84	1.33	1.66
	35	0.77	0.67	7.08	5.23	0.79	0.43	1.19	0.89	1.99	1.09

Table 6. Hematological parameters evaluated in NMRI mice (both sex) by intraperitoneal ozone application.

Time (days)	Control		O_3/O_2					
			c=11 mg/L		c= 20 mg/L		c=35 mg/L	
Beginning (t=0)	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**
Hematocrite	0.51	0.49	0.51	0.49	0.46	0.47	0.46	0.42
Leucocytes (x 10^9)	7.4	7.4	8.13	4.23	7.93	8.16	8.46	8.3
Polymorph	27.0	22.3	13.7	16.33	29	14.7	31.33	17.33
Lymphocytes	71.6	76.3	85.7	83.67	70.3	85	59.38	82.7
End of treatment (t=15)								
Hematocrite	0.6	0.52	0.52	0.45	0.51	0.51	0.52	0.49
Leucocytes (x 10^9)	8.23	9.13	8.7	7.85	9.23	7.8	8.9	9.26
Polymorph	11.3	20.3	12.7	28.5	21.3	18.33	10.7	14.66
Lymphocytes	86.6	77.6	86.6	70	77	80.7	88.3	83.65

The hematogenic dissemination of the Ehrlich Ascitic tumor in mice lung using rectal ozone application

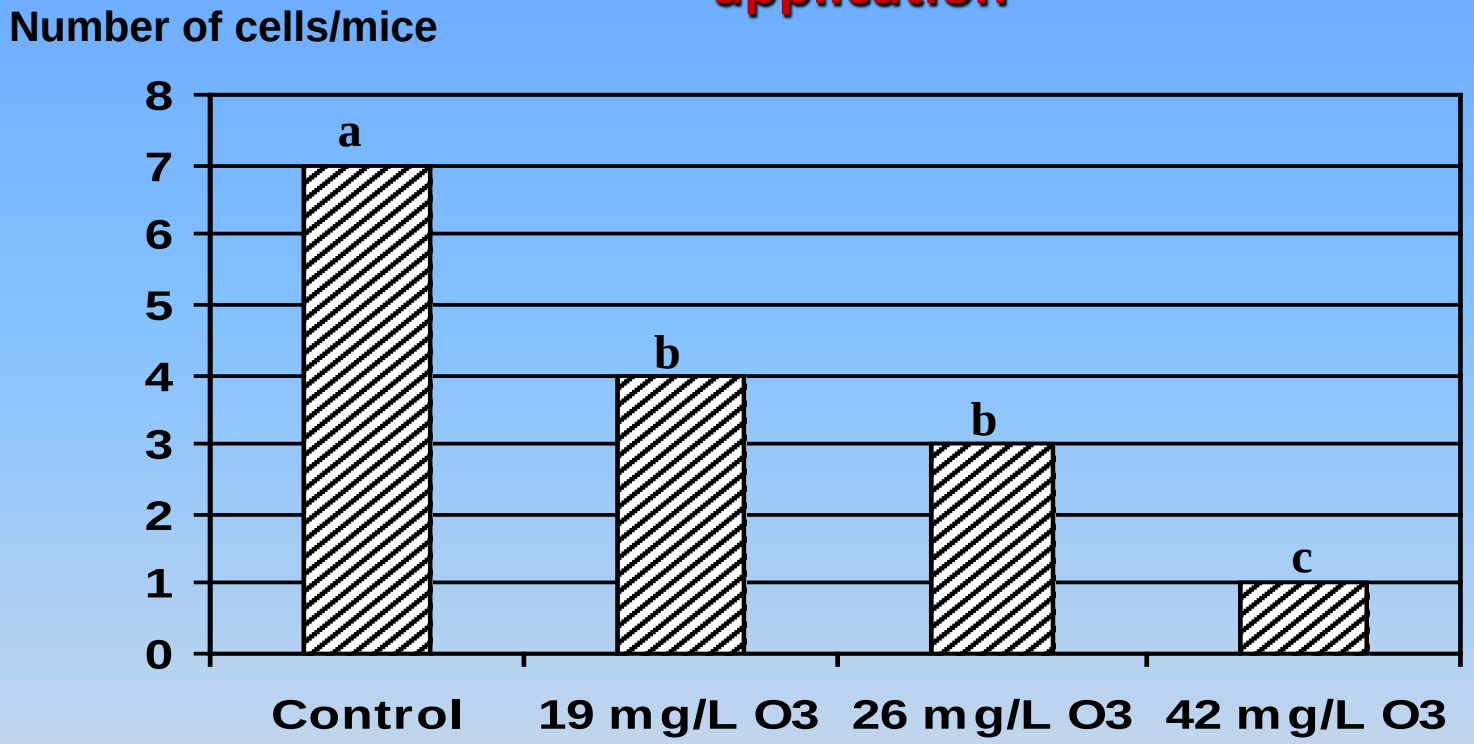


Figure 1. Tumor multiplicity in mice inoculated by the ocular plexus with 1 million cells of Ehrlich Ascitic Tumor, using 1 mL of ozone at different concentrations (19, 26 and 42 mg/L) by rectal application, during 12 sessions after the inoculation. Data are mean ± SEM. Means having different letters indicate significant difference ($p < 0.05$) between groups.

The hematogenic dissemination of the Sarcoma-37 tumor in mice lung using rectal ozone application.

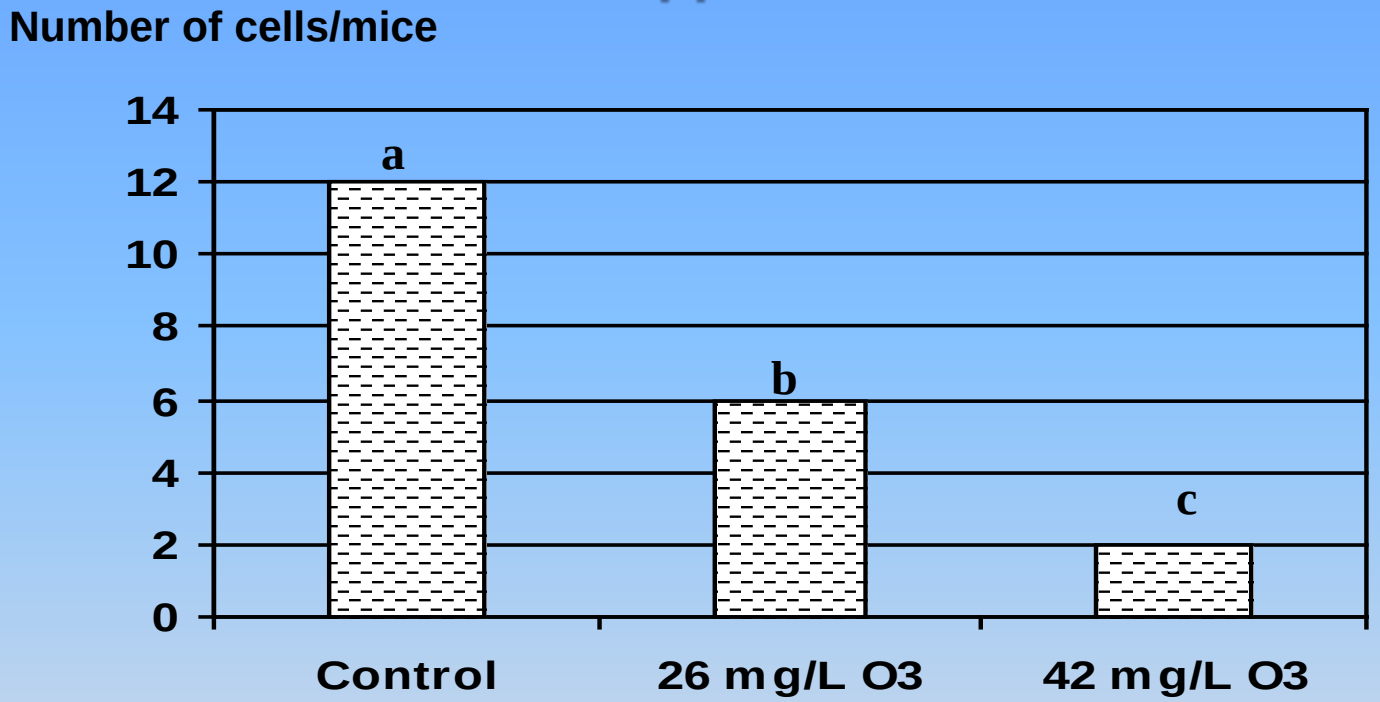


Figure 2. Tumor multiplicity in mice inoculated by the ocular plexus with 1 million cells of Sarcoma-37, using different ozone concentrations (26 and 42 mg/L) by rectal application, during 12 sessions after the inoculation. Data are mean ± SEM. Means having different letters indicate significant difference ($p < 0.05$) between groups.

Tumor development kinetics of Lewis' Lung carcinoma with ozone oxidative-preconditioning by intraperitoneal application

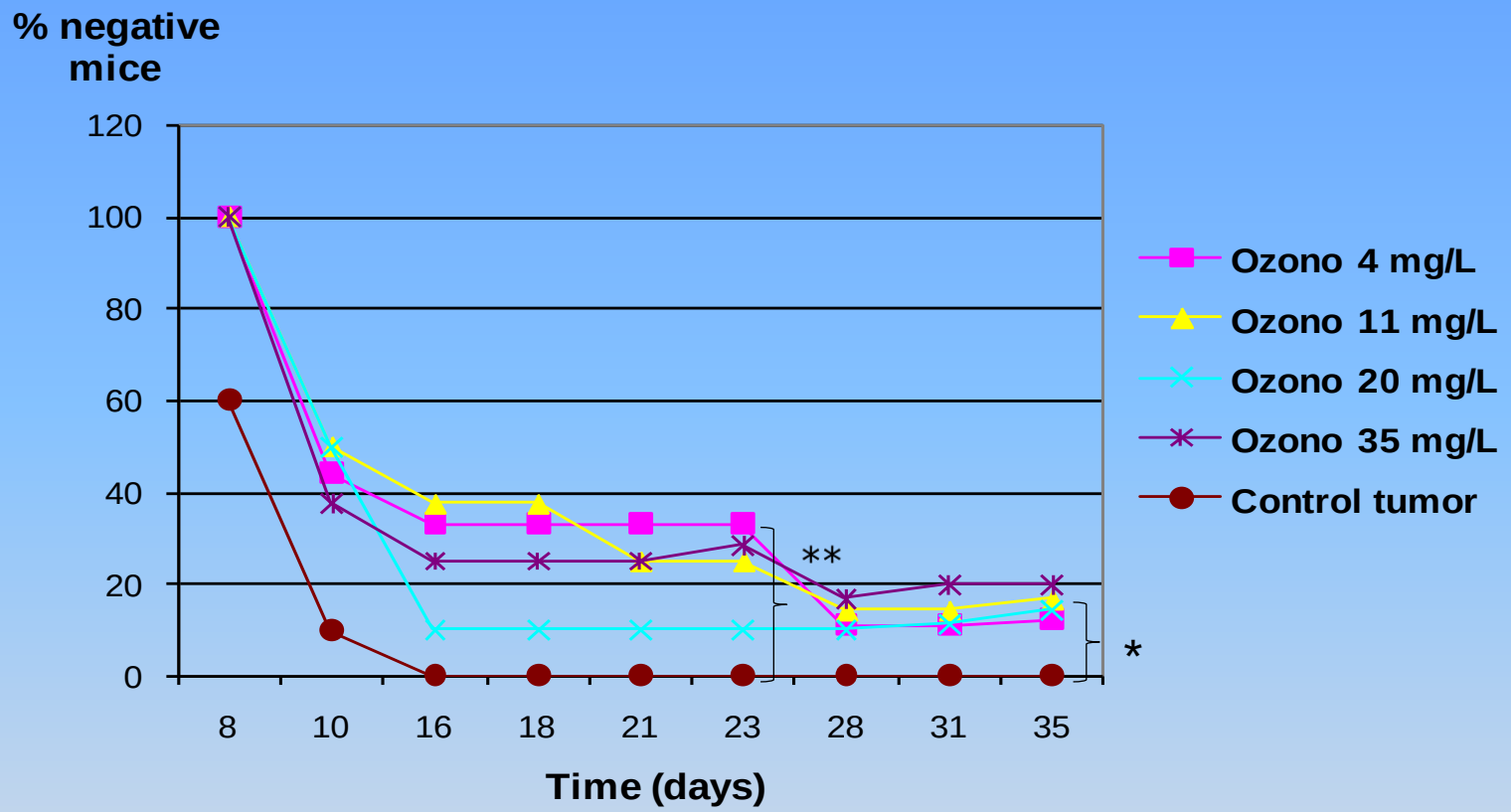


Figure 3. A significant delay ($p \leq 0.05$) in the beginning kinetics of Lewis´lung carcinoma was observed in ozone treated groups respect control group. Ozone was applied to mice intraperitoneally at concentrations of 4, 11, 20, 35 mg/L and a volume of 80 ml/kg, daily for 15 days. Twenty four hours after the last ozone treatment, animals were inoculated with 1 million cells of tumor by subcutaneous way (0.25 mL).

Tumor volume increase of Lewis' lung carcinoma with ozone oxidative-preconditioning by intraperitoneal application.

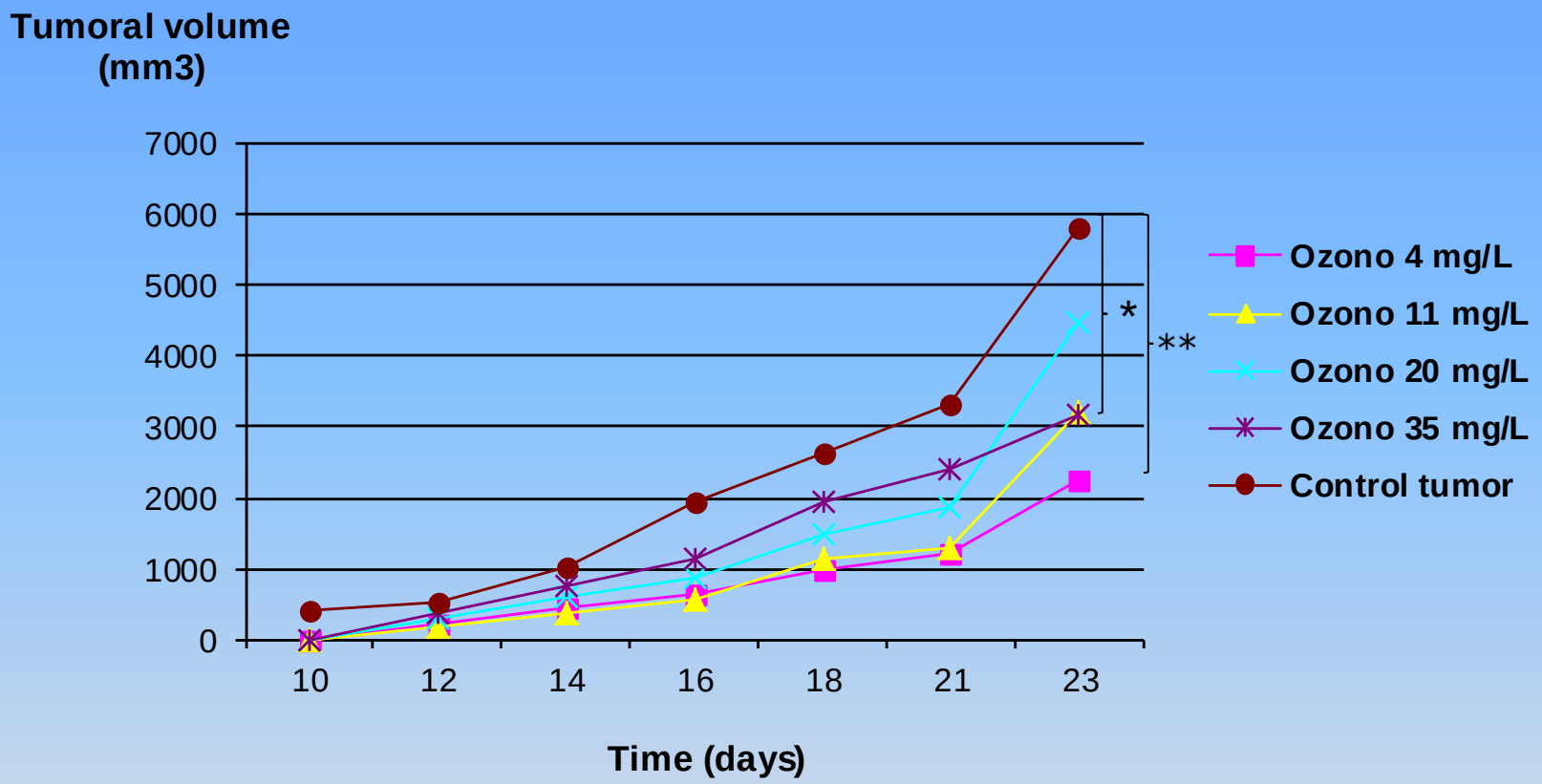
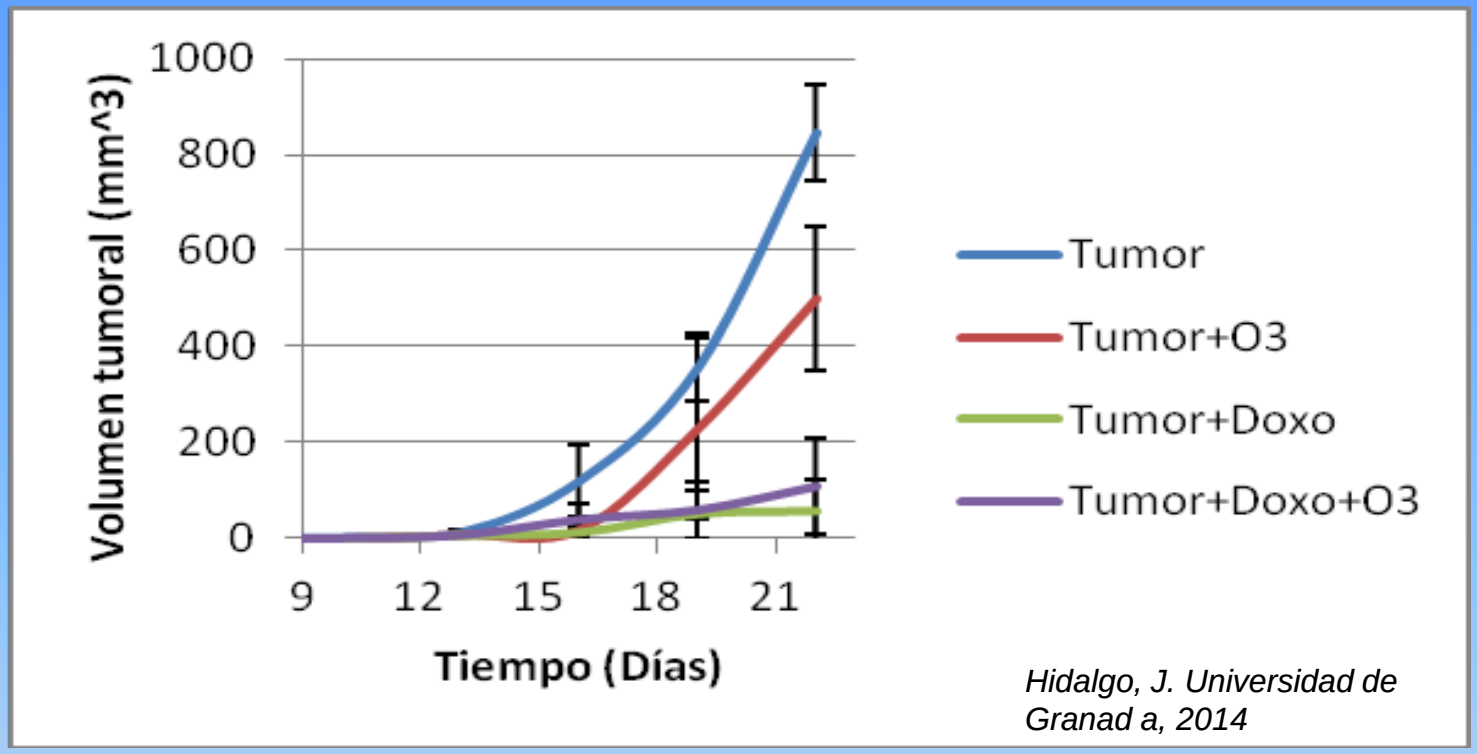


Figure 4. A significant delay (p≤0.05) in tumor volume increase was observed in ozone treated groups respect control group. Ozone was applied to mice intraperitoneally at concentrations of 4, 11, 20, 35 mg/L and a volume of 80 ml/kg, daily for 15 days. Twenty four hours after the last ozone treatment, animals were inoculated with 1 million cells of the Lewis' lung carcinoma by subcutaneous way (0.25 mL).



Hidalgo, J. Universidad de Granad a, 2014

Effect on tumor volume of different treatments by IP route (O3 50 mg/L, Doxorubicin 2 mg/Kg and its combination), post-inoculation of lung carcinoma 3LL (5x 10^5 cells),

The intraperitoneal treatment with ozone induced reduction of tumor volume in treated animals compared to the control, but there was not a better response of the combination compared to the treatment with doxorubicin.

Int. J. Cancer: **122,** 2360–2367 (2008)

Treatment with ozone/oxygen-pneumoperitoneum results in complete remission of rabbit squamous cell carcinomas

Siegfried Schulz[1], Ulrich Häussler[2], Robert Mandic[2], Johannes T. Heverhagen[3], Andreas Neubauer[4], Anja A. Dünne[2], Jochen A. Werner[2], Eberhard Weihe[5] and Michael Bette[5]*

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Head and neck squamous cell carcinomas (HNSCC) represent a group of metastasizing tumors with a high mortality rate in man and animals. Since the biomolecule ozone was found to inhibit growth of various carcinoma cells *in vitro* we here applied the highly aggressive and lethal VX2 carcinoma HNSCC tumor model of the New Zealand White rabbit to test whether ozone exerts antitumorous effects *in vivo*. Therapeutic insufflation of medical ozone/oxygen (O_3/O_2) gas mixture into the peritoneum (O_3/O_2-pneumoperitoneum) at an advanced stage of tumor disease led to a survival rate of 7/14 rabbits. Six of the seven surviving rabbits presented full tumor regression and the absence of local or distant lung metastases. Insufflation of pure oxygen (O_2) resulted in a survival rate of 3/13 animals accompanied by full tumor remission in 2 of the 3 surviving animals. Of the 14 sham-treated animals only 1 had spontaneous tumor remission and survived. No adverse effects or changes in standard blood parameters were observed after repeated intraperitoneal insufflations of the O_3/O_2 or O_2 gas. Animals with O_3/O_2-induced tumor eradication developed tolerance against reimplantation of the VX2 tumor. This could be reversed by immune suppression with a combination of dexamethasone and cyclosporin A suggesting an antitumorous effect of O_3/O_2-mediated activation of the body's own immunosurveillance. Although the exact mechanisms of action are still unclear the present data point to O_3/O_2-pneumoperitoneum as a promising new strategy in anticancer therapy.

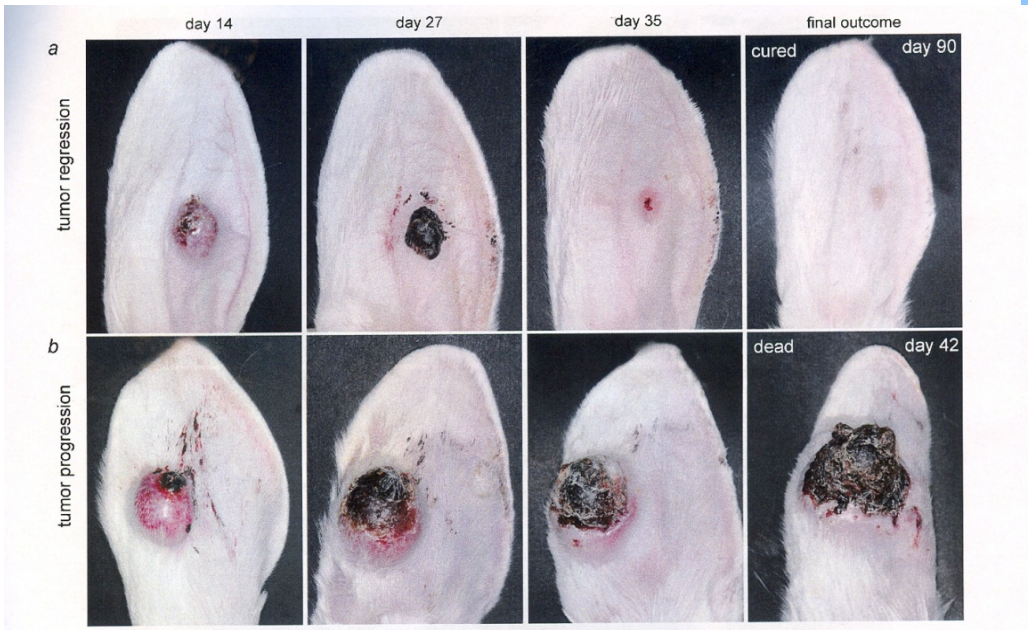
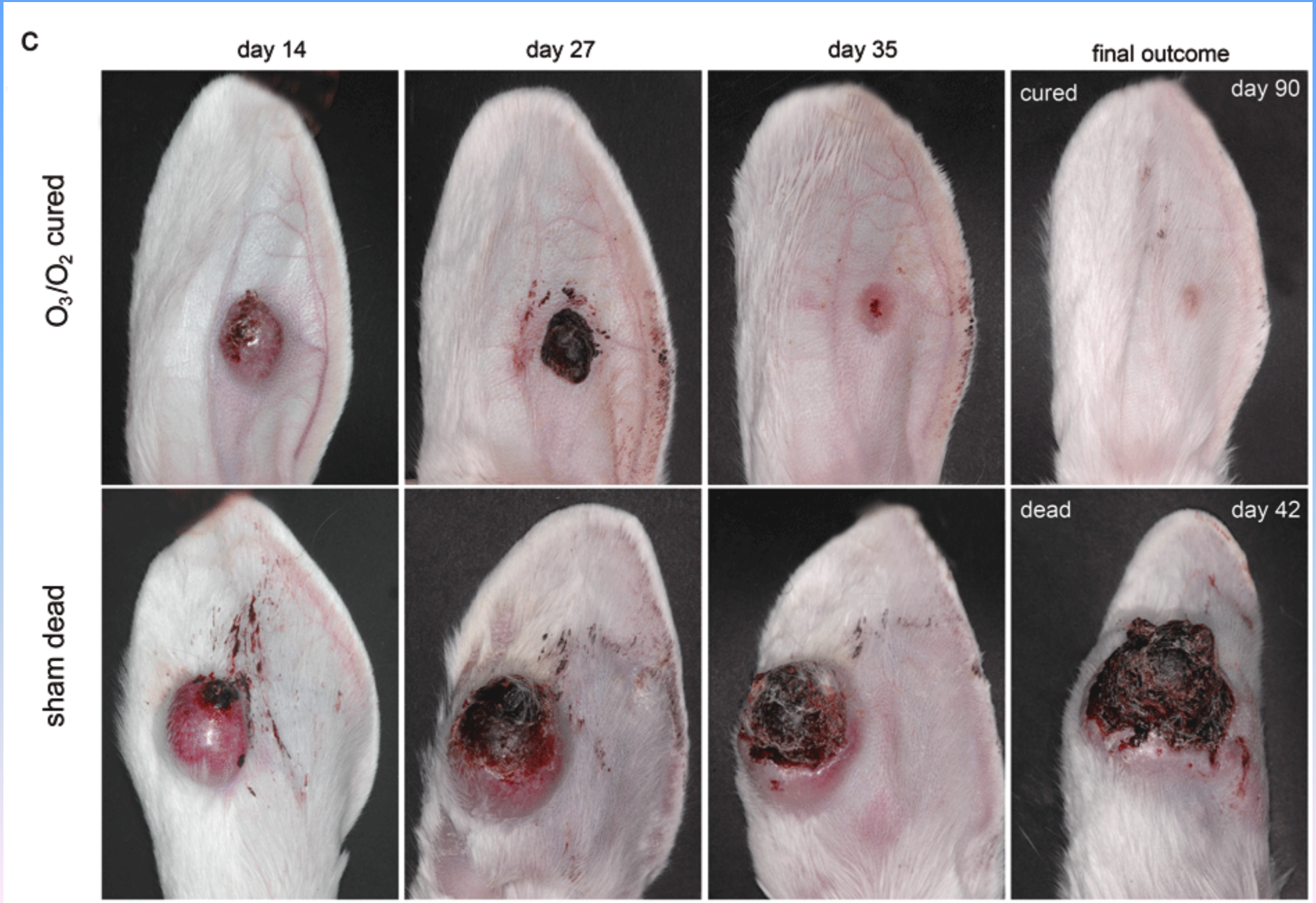


FIGURE 3 – Growth and development of the VX2 tumor cells in the right ear of NZW rabbits after inoculation. Panel (*a*) shows representative macroscopic views of a solid auricular VX2 tumor in the right ear of a rabbit on Day 14 after tumor cell inoculation and different stages of remission after O_3/O_2 therapy (O_3/O_2-pneumoperitoneum). The spontaneous tumor remission observed in one sham-treated rabbit and the 2 remissions after O_2 gas insufflation were similar (macroscopic views are not shown). Note, that only a small scar of the remitted auricular tumor remained on Day 90, the end point of our observation period. In sharp contrast, in rabbits that succumbed to tumor progression the auricular tumor continued growing resulting in severe ulcerations associated with massive bleeding and onset of local infections introducing the final stage of this tumor disease (*b*, representative stages of the same rabbit are shown). Depicted are tumor stages on Days 14, 27, 35, and at the end of the observation period (90 days or 42 days in case of death).

Tumor development



CONCLUSIONS

- ✓ **The ozonetherapy by rectal and intraperitoneal application did not induce toxicity manifestations in the reiterated assay in mice.**
- ✓ **There was obtained a significant decrease in the number of lung metastasis in Ascitic Erlich Tumor and Sarcoma 37 by rectal ozone application at the used doses.**
- ✓ **Ozone pre-conditioning by intraperitoneal application produced a retard effect in the beginning kinetics of Lewis´lung carcinoma and in tumor volume increase.**

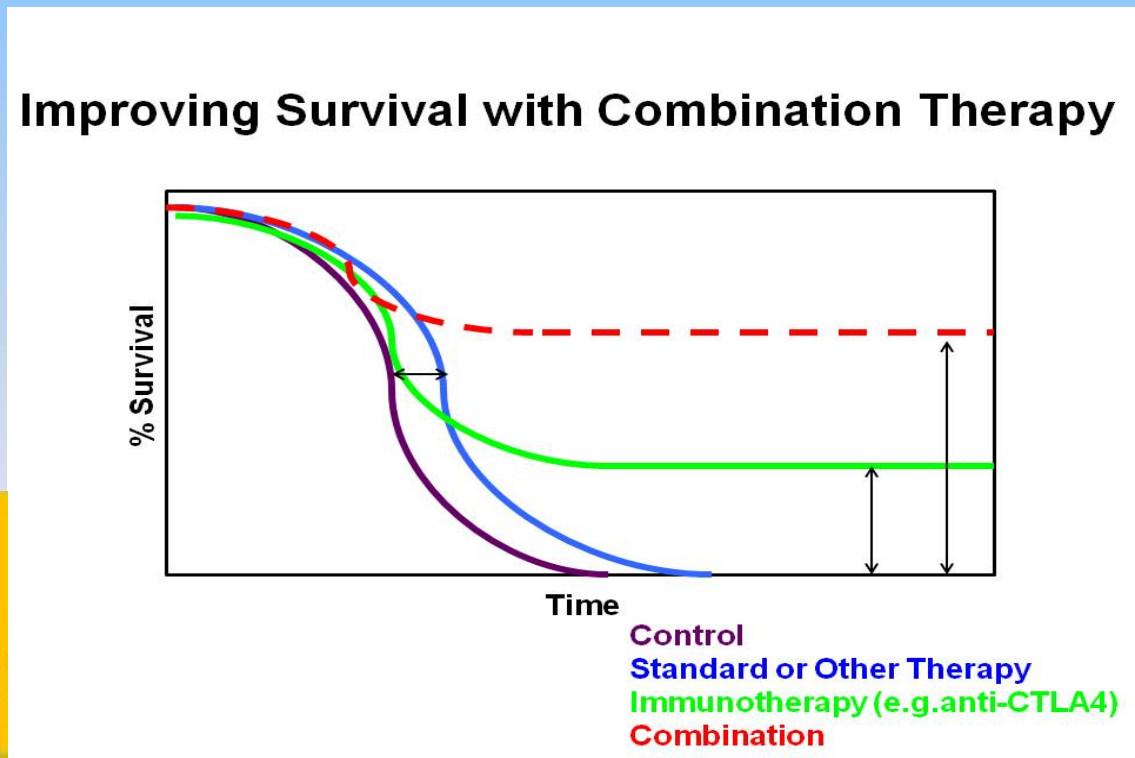
CONCLUSIONS

- ✓ In spite of the positive ozone biological effects and its potential usefulness observed in this experimental tumors, further preclinical researches are necessary to be performed, in order to reaffirm the ozone therapy as complementary therapy for cancer.

NEW TRENDS

- To move to more advanced stages of the disease.
- Inserted in initial treatment regimens in combination with standard therapies (chemo and radiation treatments)
- Using combinations of different forms of therapy

Expectations of therapeutic combinations in Cancer



INOR

“The better strategies for cancer treatment could be based on the combination of complementary therapies with different mechanisms of action and acceptable toxicity”

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