Evaluation of the effects of ozone therapy and cisplatin in an experimental model in mice with Ehrlich carcinoma

[*Avaliação do efeito da ozonioterapia e cisplatina em modelo experimental de camundongos com carcinoma de Ehrlich*]

"Scientific Article/Artigo Científico"

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Abstract
Ehrlich carcinoma is an aggressive, rapidly-growing tumor used as an experimental model in female mice because it corresponds to mammary adenocarcinoma. The present objective was to evaluate Swiss albino mice (*Mus musculus*) with Ehrlich carcinoma treated with intrarectal ozone therapy and cisplatin. Twenty-four female mice, approximately 60 days of age, varying between 35g and 43g in weight were divided into four groups, Group 1 (G1): 1mL of cisplatin 2.5mg/kg, orally; Group 2 (G2): Ozone-oxygen mixture (OOM) 20 µg/mL, via rectal insufflation (RI); Group 3 (G3): 1mL of cisplatin 2.5mg/kg, orally+ 20 µg/mL (OOM RI), and Group 4 (G4): control with 1mL of saline 0.9%, orally. All animals underwent an eight-day adaptation period. Forty-eight hours after inoculation, the treatments were performed daily, for six days. The mice were euthanized at the end of the experiment, after treatment, and the tumor was removed. There were statistically significant differences between groups for tumor weight. Mean tumor weight was greater in G4 (3,83 ± 1.20), and lesser in G3 (0.79 ± 0.73). These significant differences were observed between the group G4 and other groups. On histopathology, there were no significant differences between groups. It is concluded that ozone therapy associated with cisplatin proved to be the treatment in which mice with Erhlich's carcinoma showed delay in tumor growth, therefore, the lowest tumor weight.

Keywords: cancer, oncology, ozone, rectal insufflation.

Resumo
O carcinoma de Ehrlich é um tumor de rápido crescimento e comportamento agressivo usado como modelo experimental por corresponder ao adenocarcinoma mamário. O objetivo deste trabalho foi avaliar os camundongos albinos Swiss (*Mus musculus*) com carcinoma de Ehrlich submetidos aos tratamentos com ozonioterapia intrarretal e cisplatina. Foram utilizados 24 camundongos, fêmeas, aproximadamente 60 dias, peso variando entre 35g e 43g. Divididos em quatro grupos, Grupo 1 (G1): 1 mL de Cisplatina 2,5mg/kg; Grupo 2 (G2): Mistura Ozônio Oxigênio via Insuflação Retal (MOO IR); Grupo 3 (G3): 1 mL de Cisplatina 2,5mg/kg + (MOO IR) e Grupo 4 (G4): Controle 1mL de soro Fisiológico 0,9%. Todos os animais foram submetidos a um período de adaptação de oito dias. Após 48h da inoculação se iniciou os diferentes tratamentos durante seis dias, ao fim do experimento, foi realizada a eutanásia dos animais e feita a remoção do tumor. O peso do tumor apresentou diferenças estatisticamente significativas entre os grupos. A média do peso do tumor foi maior no G4 (3,83 ± 1.20) e foi menor no G3 (0,79 ± 0,73). Essas diferenças significativas foram notadas entre o G4 com os demais grupos. No exame histopatológico não foram notadas diferenças estatisticamente significativas entre os grupos. Conclui-se que a ozonioterapia associada à cisplatina demonstrou ser o tratamento em que os camundongos com carcinoma de Erhlich apresentaram atraso no crescimento do tumor, portanto, o menor peso do tumor.

Palavras-chave: câncer, oncologia, ozônio, insuflação retal.

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Introduction

Ehrlich carcinoma is an aggressive, rapidly-growing tumor used as an experimental model in female mice because it corresponds to mammary adenocarcinoma. Thus, it can be a comparative model in cancer research (Santos, 2016; Badir El-Din et al., 2022).

In Brazil, the Unified Health System (SUS) offers the population 29 free complementary and integrative practices (PICs). This practice has a scientific basis and has demonstrated the benefits of an integrated treatment using traditional medicine and complementary and integrated practices; ozone therapy is included in this policy (BRASIL, 2020).

Studies show that ozone therapy may be an adjuvant therapy used in addition to conventional cancer treatments. Intratumoral, intrarectal, intraperitoneal, as well as major and minor autohemotherapy are reported as possible administration routes for oxygen-ozone mixture (OOM) during cancer treatment (Waked et al., 2013; Megele et al., 2018). The objective was to evaluate Swiss albino mice (*Mus musculus*) with Ehrlich carcinoma undergoing treatment with intrarectal ozone therapy and cisplatin.

Material and Methods

This study was performed at the Vivarium of the Experimental Pharmacology and Cancerology Laboratory of the Antibiotic Department of the Universidade Federal de Pernambuco (UFPE), Recife, PE.

Twenty-four female Swiss albino mice (*Mus musculus*), with approximately 60 days of age and weighing between 35g and 43g, that had been receiving the same commercial ration and water ad libitum were obtained from the animal laboratory of the Antibiotic Laboratory of the Federal University of Pernambuco. The mice were subjected to controlled lighting conditions (12-hour light-dark cycle). The mice and the feed were weighed daily. The mice included in the experiment were randomly divided into four groups:

• Group 1 – G1 (N=6): 1mL of cisplatin 2.5mg/kg, orally.

• Group 2 – G2 (N=6): Ozone-oxygen mix via rectal insufflation (RI).

• Group 3 – G3 (N=6): 1mL of cisplatin 2.5mg/kg, orally + Ozone-oxygen mixture via rectal insufflation.

• Group 4 – G4 (N=6): Control – 1mL of saline 0.9%, orally.

All animals underwent an eight-day adaptation period, during which ozone therapy via rectal insufflation was performed as pre-treatment in groups G2 and G3 over six days. After this period, Ehrlich carcinoma cells were inoculated in all mice subcutaneous rout in axillar region (Figure 1). Forty-eight hours after inoculation, treatments with 1mL of cisplatin 2.5mg/kg, orally (G1), 20 µg/mL (OOM IR) (G2), 1 mL of cisplatin 2.5mg/kg, orally+ 20 µg/mL (OMM IR) (G3) were performed for six days.

Viable Ehrlich ascitic carcinoma cells were obtained from mice tumors from the animal facility of the Pharmacology and Experimental Cancerology Laboratory of the Antibiotic Department at UFPE.

The mice underwent daily treatment for 12 days using ozone-oxygen mixture (95% of O2 and 5% of oxygen) produced using a medicinal ozone generator model (O&L1.5 RM Ozone&Life®). The concentration, 20 µg/mL, was chosen based on the Madrid Declaration on Ozone Therapy (Schwartz et al., 2020), using a gas volume of 1 mL (Kızıltan et al., 2015). The mice were sensitized six days, and subjected to a pre-treatment prior to implantation of the solid Ehrlich tumor and to a treatment six days after implantation of the solid Ehrlich tumor. For rectal administration of OMM, 24-caliber intravenous cannulas without the stillette were used.

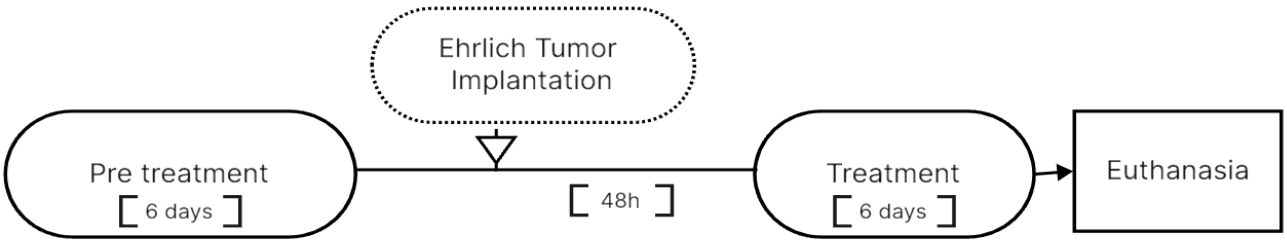

Figure 1. Methodology graphic scheme.

After the sixth day of treatment, at the end of the experiment, the animals were euthanized using 5 mg/kg xylazine and 75 mg/kg ketamine, given intraperitoneally. The tumors were weighed using a Bell® analytic scale and was sent for histopathology at the anatomic pathology laboratory of the Department of Veterinary Medicine at Universidade Federal Rural de Pernambuco (UFRPE). The tumor was fixed in 10% neutral buffered formaldehyde and sent for histopathologic analysis using routine methods for dehydration, clearing, and inclusion in paraffin prior to being cut into 4µ transverse slices with a microtome and stained with hematoxylin and eosin (H&E). In histopathological exam, coagulation necrosis, hemorrhage, adipose tissue, anisocytosis, anisokaryosis and vascularization were evaluated and classified as intense, moderate and mild.

Statistical analysis for comparison between groups used the Kruskal-Wallis test. The margin of error used for statistical tests was 5%. In weight evaluation, values were expressed as mean ± standard deviation (SD) Data were entered into an EXCEL spreadsheet and the software used for statistical calculations was IMB SPSS, version 25.

Results

Table 1 shows the statistical differences for tumor weight between the groups. For these variables, it should be noted (P=0,002) that mean tumor weight was greater in G4 (3.83 g± 1.20) and lesser in G3 (0.79 g ± 0.73). These significant differences were noted between the control (G4) and the other groups.

Different letters in parenthesis indicate a significant difference between groups determined via the aforementioned paired test. Table 2 shows the variation in histopathology results for parameters coagulation necrosis, hemorrhage, adipose tissue, anisocytosis, anisokaryosis and vascularization was evaluated in tumors; no significant difference was observed between groups (P>0,05).

Discussion

The use of the ozone-oxygen mixture (95% of O2 and 5% of oxygen) *in vitro* at a dose of 20 µg/mL with chemical therapy agents against tumor cells showed a significant decrease in viability of the tumor cells, because they reduced the increase in IL-8, IL-6, and cellular levels of IL1-β by approximately 16, 10, and 21%, respectively, suggesting a possible anticancer treatment (Simonetti et al., 2017).

Ozone therapy helps oxygenate hypoxic tissues as a possible adjuvant in treatments using chemical and radiation therapy (Clavo et al., 2018). The decrease in tumor size in G3 was possibly related to the increased perfusion of the chemical therapy agent along the tissue following improved oxygenation generated by the use of ozone rectally.

Group 2, which received only ozone therapy, had a significant reduction of tumor size when compared with G4 (control). This is an important result to show that ozone, administered via rectal insufflation, has systemic effects that preclude tumor development.

Treatment with MOO provides cellular protection by reestablishing the redox intracellular state, induced by the nuclear transcription gene Nrf2 (Re et al., 2014). This is one of the means by which MOO may reduce the tumor cell. Therefore, the tumor may be inhibited by the activation mechanism of various tumor-suppressing proteins such as superoxide dismutase (SOD) (Rodríguez et al., 2017).

These actions inhibit tumor growth via an antioxidant mechanism, leading to an increase in hydrogen peroxide and decrease of superoxide anion, causing changes in the intracellular redox state, thus resulting in sublethal damage or changes to the physiological pathways, which in turn reduces tumor growth (Rodríguez et al., 2017).

Ehrlich carcinoma is a highly malignant, fast-growing tumor (Segura et al., 2000), as demonstrated by the high incidence of necrosis found in the groups. Less areas of tumoral necrosis were observed in group 3, which may be associated to better oxygenation, as described by Clavo et al. (2018).

However, so far, few studies still exist or were able to prove the improvement due to ozone therapy, which was evaluated in most of the studies is that there may be a modulation of free radicals and antioxidants by ozone therapy associated with a decrease in toxicity induced by chemotherapy (Clavo et al., 2018).

It should be noted that, for coagulative necrosis, the greatest differences occurred in the G4 with changes in four animals classified as intense. In G1, G2, and G4, there was a larger area of coagulative necrosis and the presence of large zones with necrosis in bundles. Being the G3 the

one that presented a lower incidence of animals with intense coagulation necrosis.

When investigating adipose tissue, one mouse from G3 had the presence of adipose tissue classified as intense. The tumor grow induce the presence of intense adipose like observed in G3, feature formed by mostly round cells and arranged in cords infiltrating the adipose tissue. This behavior may be directly associated with tumor growth and tumor development in G3, formed by mostly round cells and arranged in cords infiltrating the adipose tissue.

Table 1. Statistical results for tumor weight in an experimental model using mice with Ehrlich carcinoma.

	Groups				
Weight	**G1 (Cisplatin)**	**G2 (Ozone therapy)**	**G3 (Ozone therapy + Cisplatin)**	**G4 (Control)**	**P value**
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Tumor	1.54 ± 0.55 (A)	1.66 ± 0.58 (A)	0.79 ± 0.73 (A)	3.83 ± 1.20 (B)	p(1) = 0.002*

The letters A and B indicate statistically significant difference at $p < 0.05$ within each row comparison between groups.
(1) Using the Kruskal Wallis test.

Table 2. Evaluation of the histopathological changes of the tumors according to group in the experimental model of Swiss albino mice (*Mus musculus*) with Ehrlich carcinoma.

	Groups					
Tumor	**G1 (Cisplatin)**	**G2 (Ozone therapy)**	**G3 (Ozone therapy + Cisplatin)**	**G4 (Control)**	**TOTAL**	**P value**
	N(%)	N(%)	N(%)	N(%)	N(%)	
TOTAL	**6 (100.0)**	**6 (100.0)**	**6 (100.0)**	**6 (100.0)**	**24 (100.0)**	
Coagulation Necrosis						p (1) = 0,332
Mild	1 (16.7)	1 (16.7)	1 (16.7)	-	3(12.5)	
Moderate	3 (50.0)	3 (50.0)	4 (66.7)	2 (33.3)	12 (50)	
Intense	2 (33.3)	2 (33.3)	1 (16.7)	4 (66.7)	9 (37.5)	
Hemorrhage						p (1) = 0,547
Absent	5 (83.3)	6 (100.0)	5 (83.3)	4 (66.7)	20 (83.4)	
Discret	-	-	1 (16.7)	2 (33.3)	3 (12.5)	
Moderate	1 (16.7)	-	-	-	1 (4.1)	
Adipose tissue						p (1) = 0,297
Absent	1 (16.7)	1 (16.7)	1 (16.7)	2 (33.3)	5 (20.80)	
Discret	5 (83.3)	4 (66.7)	2 (33.3)	4 (66.7)	15 (62.5)	
Moderate	-	1 (16.7)	2 (33.3)	-	3 (12.5)	
Intense	-	-	1 (16.7)	-	1 (4.1)	
Anisocytosis/ Anisokaryosis						p (1) = 1,000
Intense	6 (100.0)	6 (100.0)	6 (100.0)	6 (100.0)	24 (100.0)	
Vascularization						p (1) = 0,242
Absent	-	3 (50.0)	4 (66.7)	4 (66.7)	11 (45.8)	
Discreet	2 (33,3)	-	-	-	2 (8,3)	
Intense	4 (66,7)	3 (50,0)	2 (33,3)	2 (33,3)	11 (45,8)	

(1) Using the Kruskal Wallis test.

This differs from tumor cell characteristics observed in the other groups. Regarding treatments, G3 had the least developed tumors due to combination of cisplatin plus OOM, increasing oxygen, delivery drug more homogenously due to increase perfusion.

Hemorrhage was classified as absent in 84.0% of G1 and G3, which may indicate that there was a better response to treatment in these two groups. Vascularization was classified as 66.7% absent in G3 and G4 and 66.7% intense in G1. Neovascularization, however, is normally present in inflammatory processes and is already described as a fundamental change in tumor development (Onuchic and Chammas, 2010). It is worth noting that a longer duration of treatment (>six days) would be important to verify the results obtained in this study. However, this was not possible for the chosen protocol because of concerns related to animal welfare. Due to the importance of ozone therapy within integrative health practices, continuation of this study is warrant.

Conclusion

It is concluded that ozone therapy associated with cisplatin in an experimental model in mice was the treatment which resulted the lowest tumor weight in mice with Ehrlich carcinoma. Possibly this combination treatment inhibits tumor growth via an antioxidant mechanism induced by ozone therapy, leading to an increase in hydrogen peroxide and decrease of superoxide anion, causing changes in the intracellular redox state.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethics Committee

The study was approved by the Ethics Committee for the Use of Animals (CEUA/UFPE) under process n. 0018/2020.

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