

Levamisole in treatment of urethane-induced pulmonary carcinoma in rats

Levamisol no tratamento de carcinoma pulmonar induzido por uretano em ratos

André Luiz Costa e Silva¹, Lívia Medeiros Soares Celani², Ítalo Medeiros Azevedo³, Aldo Cunha Medeiros⁴

1. Graduate student, Medical School, Federal University of Rio Grande do Norte (UFRN), Natal-RN, Brazil.
2. Fellow Master degree, Postgraduate Program in Health Sciences, UFRN, Natal- RN, Brazil
3. Fellow PhD degree, Postgraduate Program in Health Sciences, UFRN, Natal- RN, Brazil
4. PhD, Full Professor, Chairman, Nucleus of Experimental Surgery, UFRN, Natal-RN, Brazil.

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Mailing Adress: Surgery Department, Federal University of Rio Grande do Norte, Av. Nilo Peçanha 620, Natal, RN, Brazil. E-mail: cirurgex.ufrn@gmail.com

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ABSTRACT

Objective: The potential antitumor effects of the levamisole immunomodulatory agent remain uncertain, and its beneficial effects with increased survival in the adjuvant treatment of malignant tumors are controversial. The present study aims to compare the effects of levamisole with cisplatin in the treatment of urethane-induced lung carcinoma in rats. **Methods:** Wistar rats were allocated into three groups (n = six each). Group A: mice with lung tumor + treatment with levamisole. Group B: mice with lung tumor + cisplatin treatment. Group C: mice with lung tumor + saline treatment. After 12 weeks of the tumor induction process, the results were validated by ex vivo fluorescence imaging, determining the mean fluorescent intensity in the animal's lungs. Serum dosages of cytokines and alkaline phosphatase were performed. **Results:** Mean fluorescence intensity (MFI) in the lungs (ex vivo) was measured in all animals subjected to urethane effects. In levamisole-treated, the intensity (245 +/- 15) was lower than in cisplatin-treated (277 +/- 28), but the difference was not statistically significant (p<0.05).

In those treated with saline, the MFI was 680 ± 57 , significantly higher than in the other groups ($p < 0.05$). Dosages of TNF- α (pg/ml), IL-1 β (pg/ml), IL-6 (pg/ml) and alkaline phosphatase (mg/dl) were significantly lower in levamisole-treated rats than in rats with cisplatin and saline ($p < 0.005$). **Conclusion:** In conclusion, this study demonstrates the positive influence of levamisole in treatment of urethane-induced lung tumors in rats.

Keywords: Lung carcinoma. Carcinogenesis. Urethane. Treatment. Levamisole. Cisplatin. Rats

RESUMO

Objetivo: Os potenciais efeitos antitumorais do agente imunomodulador levamisol permanecem incertos, e seus efeitos benéficos com aumento da sobrevida com tratamento adjuvante dos tumores malignos é controverso. O presente estudo tem o objetivo de comparar os efeitos do levamisol com os da cisplatina no tratamento do carcinoma de pulmão induzido pelo agente carcinogênico uretano, em ratos. Métodos: Ratos Wistar foram alocados em 3 grupos (n=6 cada). Grupo A: ratos com tumor de pulmão + tratamento com levamisol. Grupo B: ratos com tumor de pulmão + tratamento com cisplatina. Grupo C: ratos com tumor de pulmão + tratamento com solução salina. Após 12 semanas do processo de indução dos tumores, a validação dos resultados foi aferida através de exames de imagem de fluorescência ex-vivo, determinando a intensidade fluorescente média nos pulmões dos animais. Dosagens séricas de citocinas e fosfatase alcalina foram realizadas. Resultados: A intensidade média da fluorescência (IMF) nos pulmões (ex-vivo) foi medida em todos os animais submetidos aos efeitos do uretano. Nos tratados com levamisol a intensidade (245 ± 15) foi menor do que nos tratados com cisplatina (277 ± 28), porém a diferença não foi estatisticamente significativa ($p > 0,05$). Nos tratados com solução salina a IMF foi de 680 ± 57 , significativamente mais elevada do que nos demais grupos ($p < 0,05$). As dosagens de TNF- α (pg/ml), IL-1 β (pg/ml), IL-6 (pg/ml) e fosfatase alcalina (mg/dl) foram significativamente mais reduzidas nos ratos tratados com levamisol do que nos tratados com cisplatina e solução salina ($p < 0,05$). Conclusão: Em conclusão, este estudo demonstra claramente a influência positiva do levamisol no tratamento de tumores de pulmão induzidos por uretano em ratos.

Descritores: Carcinoma pulmonar. Carcinogênese. Uretano. Tratamento. Levamisol. Cisplatina. Ratos.

INTRODUCTION

Animal model systems have allowed significant advances in the study of carcinogenesis. Urethane is a chemical carcinogen that specifically promotes the development of lung tumors¹. In vivo, urethane transforms into the intermediate vinyl carbamate epoxide compound in a reaction mediated by CYP2E1, a cytochrome P450 enzyme, which acts on the bronchiolar cells of the lung. Vinyl carbamate epoxide binds to DNA, causing genetic changes involved in the development of lung tumors². Some male rodent strains have a higher overall sensitivity to urethane carcinogenic effects than female rodents³. It has been shown that the carcinogenic effect of urethane is twice as high in males as in females⁴.

Levamisole (LEV) is a synthetic imidazole-phenyl-thiazole widely used as anthelmintic and works as an immunomodulator preventing the action of suppressor T cells. LEV has been shown to increase delayed hypersensitivity reactions, mitogenic response and in vitro lymphocyte proliferation. It is effective in cellular immunity and stimulates T lymphocytes more than B-lymphocytes. LEV induces stronger Th1 immune responses by activating dendritic cells or inducing toll-like receptor expression^{5, 6}. In studies conducted on animals infected with enterotoxigenic *Escherichia coli*, it has been shown that LEV influenced T cell proliferation in ileal villi by stimulating differentiation and proliferation of lymphocytes in the lamina propria and Peyer's plaques of intestinal mucosa⁷.

LEV can also affect M cell differentiation and proliferation, increasing their number in intestinal villi. Lately, attention has been focused on M cells and their interactions with Peyer's plaque immune cells, the main inductive defense sites in gut. It has been shown that stimulation of M cell proliferation in ileal villi may have relevance to the process of cellular immunity exerted by the intestinal lamina propria^{8, 9}.

A large-scale clinical trial showed that adjunctive treatment of 12-month-old colon cancer with 5-fluoracil (5-FU) associated with LEV significantly improved survival compared with LEV or surgery alone^{10, 11}. The treatment combining 5-FU + LEV was subsequently recommended as standard by the European Consensus Conference and

the NIH. While the potential antitumor effects of the LEV immunomodulatory agent remain uncertain, its beneficial effect with the adjuvant treatment of colon tumors has been proven¹². LEV has been shown to be effective in some studies and not others. However, studies in which no beneficial effects were observed, especially on survival, were related to terminal melanomas and other advanced tumors¹³. In the present protocol, we study the effects of LEV on the evolution of newly induced lung tumors, therefore not advanced or metastatic.

OBJECTIVE

The present Project aims to compare the effects of levamisole with cisplatin effect on the treatment of urethane-induced lung carcinoma in rats.

METHODS

Rats (*Rattus norvegicus*, Wistar) with an average weight of 275 +/- 23g, from the Health Sciences Center Biotery of the Federal University of Rio Grande do Norte (UFRN), Brazil, were used. This protocol was submitted to the institutional Animal Ethics Committee (CEUA) and approved by protocol 01/2018. Care in the use of animals followed the rules in force in the Brazilian Legislation for the scientific use of animals (Law No. 11794/2008). The study was conducted at the Experimental Surgery Center-UFRN.

Experimental design

The animals were randomly selected and divided into three groups, with six rats each.

Group A: lung tumor rats + treatment with levamisole.

Group B: lung tumor rats + cisplatin treatment.

Group C: lung tumor rats + saline treatment.

Rats were kept in individual polypropylene cages with 12-hour light-dark cycles, room temperature 22°C and particle control. Initially, the animals underwent a 7-day acclimatization period in the laboratory, with ad libitum access to water and rodent food (Presence®).

Induction of pulmonar neoplasia

The animals were handled in the experimental rooms of the Experimental Surgery Center-UFRN, Brazil, and during the observation period were kept in a postoperative control room. The rats were randomly distributed in the levamisole (n = 6), cisplatin (n = 6) and control (n = 6) groups. All rats received treatment with a weekly intraperitoneal injection of 50 mg/kg urethane (Sigma Chemical Company, St. Louis, MO) for four weeks. Drug treatment started in week 6, when the onset of adenomas and neoplastic lesions was expected to begin, according to a study by Umemura et al, 1999¹⁴.

Animals treatment

In the first week after the initiation of tumor induction, 25 mg/kg of levamisole (Zeneca) was injected intraperitoneally (i.p.) into group A rats, with one dose being repeated once a week. Group B animals were treated with cisplatin at a dose of 5 mg/kg i.p., single dose¹⁵.

Group C: animals were treated with saline i.p.

Ex vivo imaging exams by fluorescence

After the 12th week of neoplasia induction and treatments, the animals were anesthetized (Ketamine 70 mg/kg and Xylazine 10 mg/kg i.p.) and intravenous injection of 0.5 ml green indocyanine (25 mg diluted in 2 ml of saline solution). After 24-hours a thoracotomy was performed, the lungs fully excised, washed in saline solution and wiped with sterile gauze. Fluorescence images of ex vivo lungs were obtained using the Kodak-FX Image Station. The emission and excitation filters were 700 and 540 nm respectively. The imaging protocol (60-second exposure time, 4x binning, f-stop 2.5, 160 mm field of view and 9 mm focal plane) was maintained for all examinations. The images were analyzed by Kodak Molecular Imaging software (version 5.0) and qualified according to a color scale. A region of interest (ROI) has been created and determined around the lungs. Average signal intensities of the region of interest were expressed as arbitrary units of fluorescent signal intensity. Fluorescence grayscale images were artificially colored for representation purposes according to a scale set for the highest and lowest levels of mean fluorescence intensity (red and purple indicated maximum and minimum light intensity, respectively).

Dosage of cytokines and alkaline phosphatase

At the end of the observation period, under anesthesia, blood was collected by cardiac puncture to measure tumor necrosis factor-alpha (TNF- α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6). The blood remained in vials without anticoagulant for 30 minutes and the serum was separated by centrifugation at 3000 rpm and stores at -40°C for subsequent dosing. Cytokines were measured by enzyme-linked immunosorbent assay (ELISA). The reagents used were from PeproTech, USA. Alkaline phosphatase was measured on the Konelab60i automated analyzer, Kits Wiener Lab.

Statistic

Statistical evaluation was performed by analysis of variance (ANOVA) followed by Tukey test, considering significant differences when $p < 0.05$. BioEstat 5.0 software was used, Belém, PA, Brazil.

RESULTS

Visual representations of changes in fluorescence signal intensity were obtained by applying a series of colors grading the fluorescence signal intensity. Quantitation of ex vivo fluorescence intensity of regions of interest of affected lungs peaked 10 hours after intravenous administration of green indocyanine, remaining up to 24 hours. The fluorescence signal was significantly higher in the lung cancer regions of urethane and saline-treated rats. Besides, the fluorescence signal was lower or absent in lung areas not affected by lung tumor. The fluorescence signal was anatomically located in the presence of the lung tumor. This demonstrates the preferential localization of green indocyanine in lung tumors (figure 1).

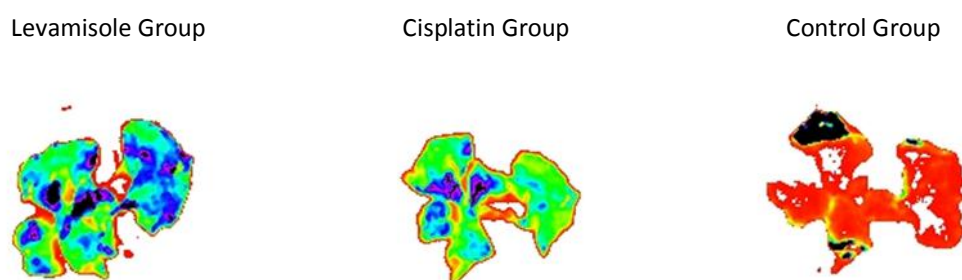


Figure 1. Representative images of the ex vivo lung fluorescence of animals of the respective groups. The lungs of levamisole and cisplatin groups exhibit rare low-intensity fluorescent red areas. In the control group, there is intense disseminated fluorescent activity in both lungs.

Mean fluorescence intensity (MFI) in the lungs (ex vivo) was measured in all animals subjected to urethane effects. In levamisole-treated, the intensity (245 +/- 15) was lower than in cisplatin-treated (277 +/- 28), but the difference was not statistically significant ($p > 0.05$). However, in those treated with saline, the MFI was 680 +/- 57, significantly higher than in the other groups ($p < 0.05$). Data summarized in Table 1.

Table 1. Mean fluorescence intensity (MIF) values and their statistical interpretation.

Variable	Levamisole	Cisplatin	Control	P-value
MIF	245±15 ^B	277±28 ^A	680±57 ^{AB}	<0,05

MIF – mean fluorescence intensity. Mean +/- standard deviation. Values in the same row followed by equal letters mean statistically significant differences. Tukey test.

Dosages of TNF- α (pg/ml), IL-1 β (pg/ml), IL-6 (pg/ml) and alkaline phosphatase (mg/dl) were significantly lower in levamisole-treated rats than in rats with cisplatin and saline solution ($p < 0.005$). Data summarized in Table 2.

Table 2. Cytokine and alkaline phosphatase dosage values and their statistical interpretation.

Variable	Levamisole	Cisplatin	Control	P-value
TNF- α (pg/ml)	172,9±15 ^A	193,5± 25,7 ^B	371,95± 42,9 ^{A,B}	<0,05
IL-1 β (pg/ml)	41,61± 7,0 ^A	58,2±6,3 ^A	83,5±10,2 ^A	<0,05
IL-6 (pg/ml)	77,3± 9,0 ^A	98,6±10,4 ^A	230,5±13,2 ^A	<0,01
AP (mg/dl)	79,4± 11,2 ^A	118,7±15,4 ^A	187,5±18,7 ^A	<0,01

TNF- α , Tumor necrosis factor-alpha; IL, Interleukin; AP, Alkaline phosphatase. Mean \pm standard deviation. Values in the same row followed by equal letters mean statistically significant differences. Tukey test.

DISCUSSION

Levamisole (LMS) is an anthelmintic imidazothiazole that has been used for many years. Besides, it is effective in reducing the risk of tumor recurrence when adjunctly administered to patients with surgically resected stage III colon cancer^{10, 16}, although it is no longer used clinically in this setting. It is believed to act by stimulating the cellular immune system. In patients, LMS has been shown to increase the number of circulating NK (natural killer) cells¹⁷, the expression of CD25 (IL2 receptor) on the membrane¹⁸ and to increase serum IL-2 levels¹⁹.

Fluorescent imaging acquisition times were chosen based on the work of Ishizawa et al (2009)²⁰, who reported that fluorescence imaging is preferably at least 24

hours after administration of green indocyanine. As the maximum fluorescence penetration depth is up to 1 cm, the identification of non-deeply situated lung tumors in preclinical studies is challenging. Knowing this, in our study, we performed ex vivo imaging exams. Improvements in imaging systems, new fluorescence contrast agents, more accurate software, and reduction in background fluorescence can facilitate fluorescence imaging of deeper localized tumors and metastases.

Levamisole (LMS), which is an immunostimulator, has been studied as synergistic to 5-fluorouacil in adjuvant therapy in patients with stages III colon cancer. Immunological parameters included lymphocyte antigen expression and serum cytokine levels. It has been suggested that LMS is immunomodulatory and well-tolerated. Holcombe et al (2001) observed that LMS is a potential chemopreventive agent of colon cancer²¹.

Clarke et al (1997)²² examined the immunomodulatory effect of levamisole on NC-1.1 + cell-mediated natural cytotoxicity against BALB / c WEHI-164 murine fibrosarcoma. Levamisole administration to BALB / c rats significantly increased in vitro splenic natural cytotoxic against tumor cells. The effect was most pronounced 48 hours after treatment with levamisole at a dose of 10 mg/kg body weight. Levamisole (10 mg/kg) significantly reduced WEHI-164 fibrosarcoma growth and this effect was observed in the pretreatment of rats, indicating an antitumor effect of levamisole²². The effect of Levamisole was observed on CD138 expression and IL-6 secretion, proving once again that Levamisole is an antineoplastic drug²³. Serum IL-6 levels are associated with disease activity in myeloma and elevated levels indicate severity²⁴. Besides, IL-6 appears to have antiapoptotic activity in various B²⁵ cell tumors. Given its effect on tumor cell growth, survival, adhesion, and invasion, IL-6 may be a potential beneficial regulator of myeloma pathobiology. Therefore, drugs that affect IL-6 function are expected to be useful as therapeutic agents in myeloma patients. In our study, we observed significant reduction in IL-6 levels in the levamisole treated group.

Levamisole has the property of inhibiting alkaline phosphatase (AP)^{26, 27}. Increased AP activity correlates with tumor cell proliferation. Our results showed that in levamisole-treated rats, AP levels were significantly lower than in the other groups.

In conclusion, this study demonstrates the positive influence of levamisole in the treatment of urethane-induced lung tumor in rats.

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