Experimental rat lung tumor model with intrabronchial tumor cell implantation[1]

Modelo experimental de tumor de pulmão em rato por via intrabrônquica

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ABSTRACT

Purpose: The objective of this study was to develop a rat lung tumor model for anticancer drug testing. **Methods**: Sixty-two female Wistar rats weighing 208 ± 20 g were anesthetized intraperitoneally with 2.5% tribromoethanol (1 ml/100 g live weight), tracheotomized and intubated with an ultrafine catheter for inoculation with Walker's tumor cells. In the first step of the experiment, a technique was established for intrabronchial implantation of 10^5 to 5×10^5 tumor cells, and the tumor take rate was determined. The second stage consisted of determining tumor volume, correlating findings from high-resolution computed tomography (HRCT) with findings from necropsia and determining time of survival. **Results**: The tumor take rate was 94.7% for implants with 4×10^5 tumor cells, HRCT and necropsia findings matched closely ($r=0.953$; $p<0.0001$), the median time of survival was 11 days, and surgical mortality was 4.8%. **Conclusion:** The present rat lung tumor model was shown to be feasible: the take rate was high, surgical mortality was negligible and the procedure was simple to perform and easily reproduced. HRCT was found to be a highly accurate tool for tumor diagnosis, localization and measurement and may be recommended for monitoring tumor growth in this model.**Key words:** Lung neoplasms; Walker-256 carcinoma; rats.

RESUMO

Objetivo: O objetivo foi desenvolver um modelo de tumor de pulmão em rato que permita o teste de fármacos no tratamento deste câncer. **Métodos**: Sessenta e dois ratos Wistar fêmeas, peso médio de 208±20 g, foram anesthesiados com tribromo-etanol 2,5% IP (1ml/100g de rato), traqueostomizados e intubados com cateter ultrafino para injetar células do tumor de Walker. Na 1ª etapa, estabeleceu-se a técnica do implante de células tumorais por via intrabrônquica e o índice de pega tumoral, usando-se de 10^5 a 5×10^5 células. Na 2ª, avaliou-se o volume tumoral e a correlação dos achados obtidos na tomografia computadorizada de alta resolução (TCAR) de tórax com os da necropsia e verificou-se a sobrevida. **Resultados**: O índice de pega foi de 94,7, com o implante de 4×10^5 células do tumor; as medidas do tumor feitas na TCAR e comparadas com as da necropsia foram semelhantes ($r=0, 953$, $p<0,0001$); a sobrevida mediana foi de 11 dias; e a mortalidade cirúrgica de 4,8 %. **Conclusão**: O modelo mostrou-se viável, com alto índice de pega, mortalidade cirúrgica desprezível, de execução simples e fácil reprodutibilidade. A TCAR revelou alta acurácia no diagnóstico, localização e mensuração das lesões tumorais, credenciando-se para a monitorização de crescimento tumoral nesse modelo.**Descritores:** Neoplasias Pulmonares; Carcinoma 256 de Walker; Ratos.

Introduction

Lung cancer has been the main cause of death from cancer worldwide over the past decade. Each year over 170,000 new cases are diagnosed in the U.S. alone leading to 160,000 deaths and representing approximately 28% of deaths from cancer[1]. In Brazil lung cancer was likewise the most frequent cause of death among men in the year 2000, although prostate cancer is expected to surpass the incidence of lung cancer in 2006[2]. Screening with low-dose helicoidal computed tomography, biological markers and other methods for early diagnosis of lung cancer currently represent an important field of study, especially since chances of cure are considerably greater when patients are submitted to surgery while the disease is still localized[3]. Apart from the newly introduced chemotherapy drugs, other treatment forms for patients with lung cancer are now becoming available, including immunomodulating, antiangiogenic and targeted drugs.[4,5] However, experimental models are necessary to study the biological behavior of lung tumors and the effects of novel anticancer drugs. Preclinical tests evaluating new drugs *in vivo* are usually performed with immunodeficient mice receiving an ectopic, subcutaneous graft of human cancer cells[4]. Lung cancer developing in murine models is genetically similar to human tumors, although tumor growth and response to treatment interventions depend on whether the tumor is implanted ectopically under the skin or orthotopically in the lung[5]. Therefore many authors have given attention to the development and validation of orthotopic tumor models using human tumor cells[6,7]. In such models, instead of inoculating tumor cells under the skin of rats and mice, tumors are implanted in the organ corresponding to the organ of origin. Moreover, orthotopic lung tumor models using human cancer cells require the use of cell lineages from primary lung tumors as well as immunosuppressed animals, since this type of tumor will not take or grow in immunocompetent animals[8]. In lung tumor models cells may be inoculated intrabronchially in the pulmonary parenchyma[7,8] or may be implanted directly by puncture during open thoracotomy[9,10].The orthotopic lung tumor model using intrabronchial inoculation of tumor cells was originally developed by McLemore *et al*.[11] and involved inserting a catheter by tracheal puncture. Later Howard *et al*.[8] and Johnston *et al*.[7] improved the technique by using cervical tracheotomy for the insertion of an ultrafine intrabronchial catheter, thus making it possible to implant cells on the periphery of the pulmonary parenchyma. None of the studies above used computed tomography (CT) to detect the presence of lung tumors in the animals, although a few other and more recent experimental studies have reported using CT scans with small animals[10,12,13]. The objectives of this study were a) to develop a technically simple rat lung tumor model with intrabronchial implantation of cells of Walker's carcinosarcoma by cervical tracheotomy, and b) to diagnose tumors *in vivo* using high-resolution computed tomography (HRCT) with subsequent correlation of findings from necropsy and histopathological examination.

Methods

Experimental animals

Experiments were carried out with 62 female Wistar rats weighing 208 ± 20 g reared at the laboratory of the Federal University of Ceará (UFC). During the study the animals were kept in cages at the physiology and pharmacology laboratory in groups of up to six individuals. The temperature was maintained at 24° C and the animals were exposed to a 24-hour circadian rhythm with free access to water and food. The study was previously approved by the UFC Ethics Committee for Animal Research (CEPA protocol #33/06), and all experiments were performed in accordance with sound ethical principles.

### *Origin and preparation of suspension of neoplastic cells*

The experiment used cells from the Walker-256 carcinosarcoma[14]. The neoplasm is cultivated at the laboratory by weekly intramuscular injections with a suspension of 10^6 tumor cells in the inner thigh of Wistar rats. The cells were prepared for the present study as described in the literature[10]. Tumor cell viability was assessed with trypan blue staining and the number of cells per 1 ml of suspension was determined using a Neubauer chamber. The suspension was subsequently kept at 4°C during the entire experiment.

### *Technique of intrabronchial tumor cell implantation*

The animals were anesthetized intraperitoneally with 2.5% tribromoethanol at a concentration of 1 ml/100 g live weight, then placed in dorsal decubitus and submitted to hair clipping and antisepsis with Povidine® (polyvinylpyrrolidone) in the cervical area. Cervical tracheotomy was performed as described by Howard *et al*. (1991)[8], beginning with a skin incision just above the manubrium sterni and dissecting the muscle layers until uncovering the trachea (Figure 1). A small incision was made with a size 11 blade into the trachea by the 2nd and 3rd ring to allow for the introduction of a size 16G polyethylene catheter. By tilting it to the right, the catheter was led through the trachea into the left bronchus. A size 22G ultrafine polyethylene catheter (Figure 2) was then guided through the first catheter and advanced until detecting resistance from the lung periphery. At this location 70—100 µl of tumor cell suspension was inoculated. The catheters were subsequently removed and the trachea and skin were closed with a single stitch of Prolene® 7-0 thread and Mononylon® 4-0 thread, respectively (Figure 3). The procedure lasted 5–6 minutes, after which the animal was placed in left lateral decubitus until emergence from anesthesia, in order to confine the inoculum to the site of implantation, as recommended by Wang *et al*. (1997)[9].
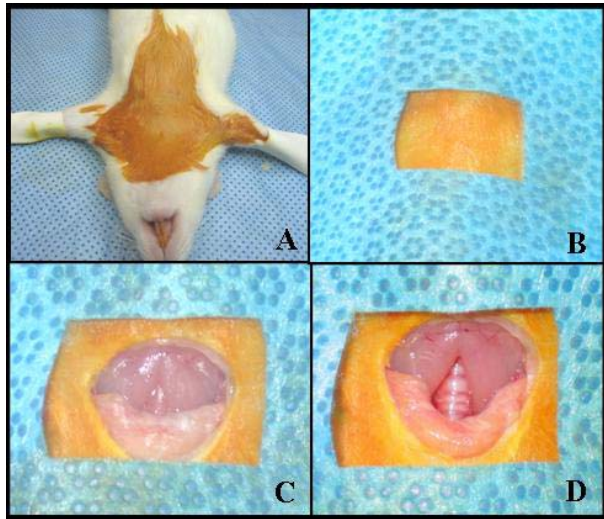
FIGURE 1 – A) animal in dorsal decubitus; B) surgical site; C) cross-sectional cervicotomy above the manubrium sterni and D) exposed trachea.
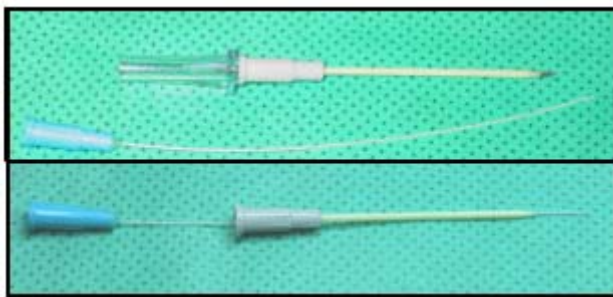
FIGURE 2 - A) Size 16G polyethylene catheter and size 22G ultrafine polyethylene catheter; B) ultrafine catheter emerging from inside 16G catheter at the site of inoculation.
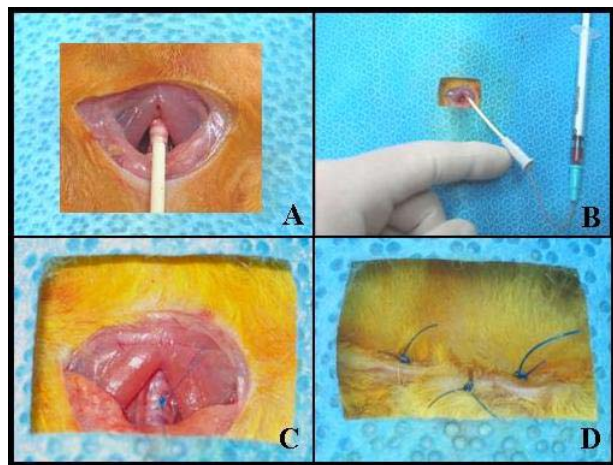
FIGURE 3 - A) trachea intubated with size 16G catheter; B) catheter in position of intrabronchial inoculation with tumor cells; C) stitched trachea; D) skin suture.

High-resolution computed tomography (HRCT)

The animals were anesthetized intraperitoneally with 10% chloral hydrate at a dosage of 0.1 ml/30 g live weight in order to maintain hypnosis during HRCT. During the scan the animals were kept in ventral decubitus with the aid of a cloth, avoiding the use of adhesive tape. Following scanning with HRCT the animals were placed in cotton-wadded boxes to keep them warm until emergence from anesthesia and transference to cages. Scanning was performed with a Siemens tomograph (SOMATON AR.TX: 130 KV, 50 mA, average FOV 5 cm, high-resolution filter for 2-mm sections, scanning time 3 seconds per section [150 mAS]). On the average, six 2-mm sections separated by 2-mm intervals were made of the lower half of the chest where the tumor was located. Images were captured in wide window mode for the lung study and in narrow window mode for the study of the mediastinum. Tumors were measured in two dimensions (axial and perpendicular) in wide window mode (Figure 4).

FIGURE 4 - Measurement of tumor (arrows) on high-resolution computed tomography of the chest performed in wide window mode (lung study) on the 5th day of tumor implantation.

Experimental design

The experimental model was developed in two steps with the animals assigned to experimental groups at random. Animals that died during follow-up of non-tumor-related causes or presented no tumors upon necropsia and histopathological examination were excluded from the analysis.
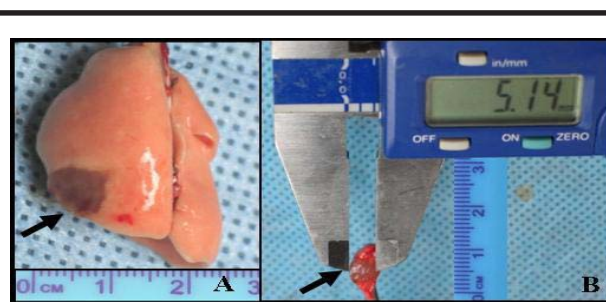
Step 1 (n=32): Establishment of intrabronchial tumor cell implantation technique and subsequent take rate.

The animals were randomly assigned to four groups and, using the technique described above, inoculated intrabronchially with different concentrations of Walker's tumor cells in order to establish the number of cells required for tumor take: Group 1 (n=8), 10^5 cells; Group 2 (n=8), 2×10^5 cells; Group 3 (n=10), 4×10^5 cells; and Group 4 (n=6), 5×10^5 cells. On the sixth day the animals were euthanized with chloral hydrate and submitted to necropsis through median sternotomy and laparotomy for the joint excision of trachea, lungs and heart in order to verify the presence of tumors in the chest and abdomen (liver and adrenal gland tumors). Lung sections were fixed in buffered isotonic formaldehyde (100 mL of 37% formaldehyde solution, 900 mL distilled water, 4 g monobasic sodium phosphate and 6.5 g dibasic sodium phosphate). Twenty-four hours later samples were immersed in 70% alcohol, stained with hematoxylin-eosin and examined histopathologically by a blinded pathologist.

Step 2 (n=30): Assessment of tumor volume, correlation of HRCT findings with findings from necropsia, and determination of time of survival.

The animals were randomly assigned to one of two protocols, A and B, both of which with intrabronchial inoculation of 4×10^5 tumor cells. Although the highest take rate in Step 1 was observed after inoculation with 5×10^5 tumor cells, a smaller number of cells was used in Step 2 in order to make tumor cells available for a greater number of experiments. The animals assigned to Protocol A (n=16) were HRCT scanned on the 5th day of implantation and subsequently euthanized with chloral hydrate and submitted to necropsis. HRCT sectioning was set to 2 mm and tumors were measured in two dimensions, as described above. At necropsy tumors were measured manually, registering the two largest diameters with a digital caliper (Figure 5). Tumor volumes were calculated in cm³ using Steel's formula: (Dxd2)/2, where D is the largest diameter and d the smallest. The animals assigned to Protocol B (n=14; survival group) were weighed daily until spontaneous death and then submitted to necropsis as described above.

FIGURE 5 - A) necropsy sample showing tumor in left lung (arrow); B) digital caliper measurement of

Statistical analysis

Tumor volumes obtained from HRCT and necropsy and expressed as average values ± standard error were compared using simple linear regression. Survival rates were determined with the Kaplan-Meier test. The statistical analysis was carried out with the SPSS software and the level of statistical significance was set at 5%.

Results

Step 1 (n=32): Establishment of intrabronchial tumor cell implantation technique and subsequent take rate.

The procedure posed no technical difficulty and lasted 5-6 minutes per animal on the average, making it possible to inoculate 12 animals in one hour. Two surgical deaths were registered during Step 1, corresponding to a mortality rate of 6.2% (2/32). On the sixth day, when the animals were euthanized and submitted to necropsy, macroscopic nodules were observed in the lower left lung (Figure 6) at a development rate proportional to the number of cells inoculated (Table 1). The histopathological tests confirmed macroscopic findings in all cases. Most tumors formed a dense mass around the bronchus or bronchiole and were characterized by the presence of polygonal cells, visible nucleoli, loose chromatin and widespread atypical mytosis (Figures 7 and 8).

**TABLE 1 -** Tumor take rate *versus* number of cells implanted in lung

Group	Number of tumor cells implanted	Animals inoculated	Animals with tumor	Tumor take rate (%)
Group 1	1×10^5	7	5	72
Group 2	2×10^5	7	6	86
Group 3	4×10^5	10	9	90
Group 4	5×10^5	6	6	100
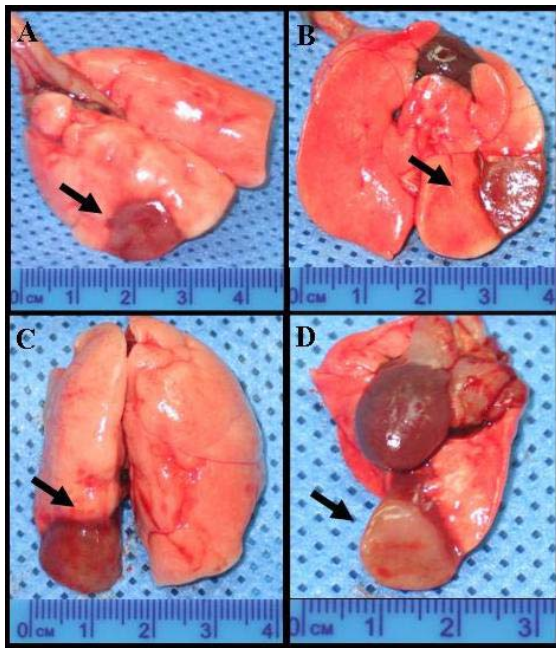
FIGURE 6 - Fifth day following intrabronchial implantation with Walker's tumor cells. Rat *A* with tumor (arrows) located in the lateral segment of the left lower lobe, showing the lateral face (A) and medial face (B). Rat *B* with tumor (arrows) located in the posterior segment of the left lower lobe, showing the posterior face (C) and medial face (D).
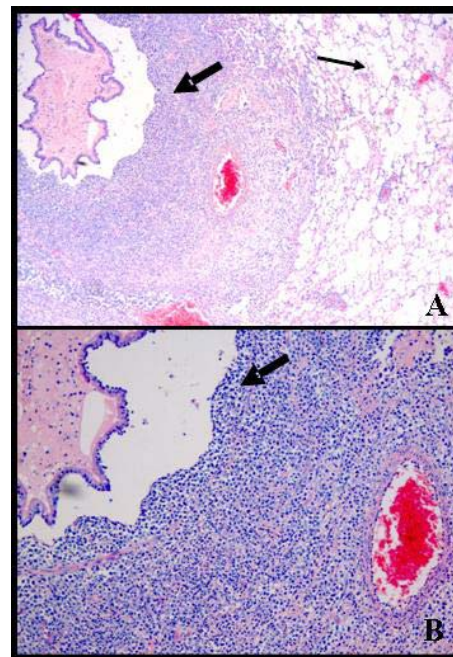
FIGURE 7 - Histopathological test with hematoxylin-eosin staining. A) Tumor on the sixth day of implantation forming a peribronchiolar mass (thick arrow) with adjacent lung showing signs of swelling and congestion (thin arrow). Magnified 40X. B) Tumor infiltrating bronchiolar wall (arrow). Magnified 100X.
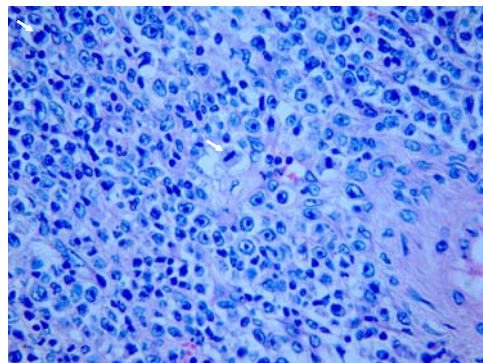

FIGURE 8 - Histopathological test with hematoxylin-eosin staining (400X), showing tumor on the sixth day of implantation, characterized by polygonal cells with large nuclei, visible nucleoli, loose chromatin and widespread atypical mytosis (arrows) affecting most of the lung parenchyma in this area.

Step 2, Protocol A (n=16): Assessment of tumor volume and correlation of HRCT findings with findings from necropsy.

On the fifth day following implantation animals inoculated with 4×10^5 tumor cells and submitted to HRCT and necropsy presented a tumor take rate of 100%. One surgical death was registered. The correlation between findings of tumor volume (cm^3) obtained with HRCT and necropsy was positive (r=0.953; *p*<0.0001) (Figures 9 and 10). The necropsy confirmed HRCT findings with regard to tumor volume and location (lateral or posterior segment of the left lower lobe). The microscopic examination yielded findings similar to those obtained in Step 1.
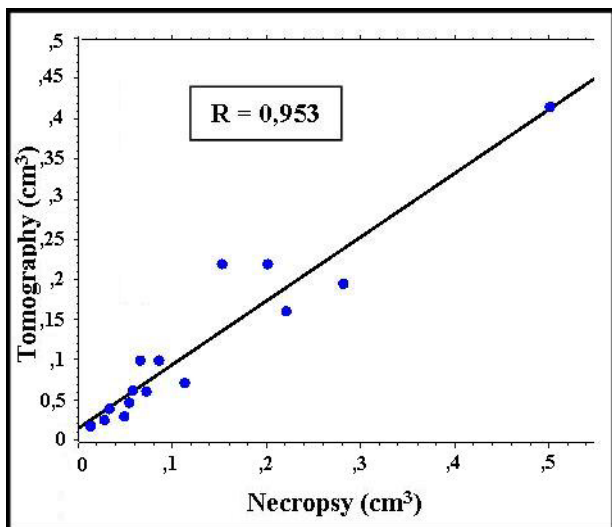
FIGURE 9 - Simple linear regression test showing positive correlation between HRCT and necropsy findings for tumor volume (cm³) (R^2= 0.908; p=0.0001).
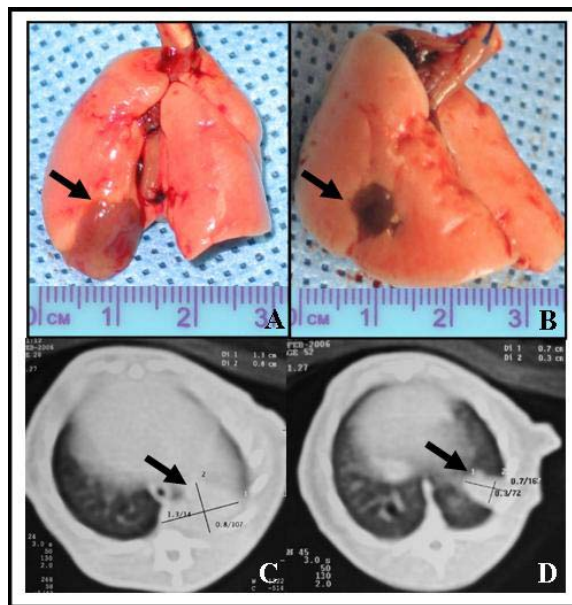

FIGURE 10 - Rat lung on the fifth day following implantation with 4×10^5 cells of Walker's carcinosarcoma. Rat *A* with tumor (arrows) located in the posterior segment of the left lower lobe. Rat *B* with tumor (arrows) located in the lateral segment of the left lower lobe. Surgical samples (A and B); HRCT wide window mode (lung study) (C and D).

Step 2, Protocol B (n=14): Time of survival for rats inoculated with 4×10^5 cells of Walker's carcinosarcoma.

An animal which died of an unknown cause on the third day following tumor cell implantation was excluded from the analysis. Of the remaining 13 animals submitted to necropsy after spontaneous death in consequence of the neoplasm, only one presented no tumor. Tumors confirmed by necropsy and histopathological examination displayed the same level of differentiation as

tumors examined during Step 1. In almost all cases, the tumor occupied most of the lung with large areas of necrosis and hemorrhagic foci (Figure 11). Eight animals (66%) presented loco-regional tumor dissemination either towards the mediastinum (n=6; 49.5 %) or the pleura (n=2; 16.5%). No distant metastases were detected in the lung, liver or adrenal glands, as opposed to what is commonly observed with lung cancer in humans. All animals died within two weeks; the median survival time was 11.00±0.38 days (Figure 12).
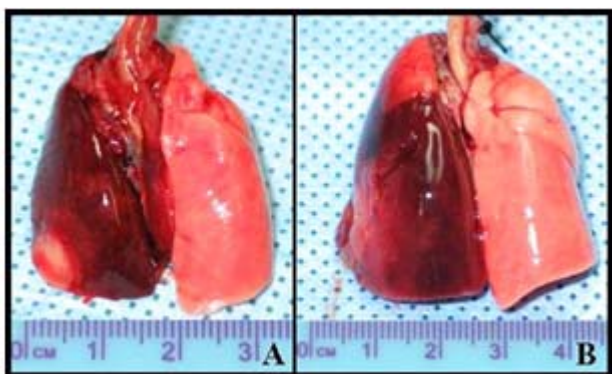
FIGURE 11 - Animals of the control group with tumor mass occupying most of the left lung and invading the mediastinum. The right lung is preserved.
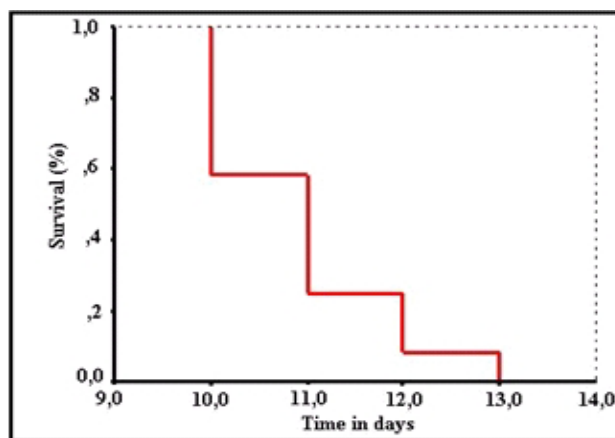
FIGURE 12 - Average survival time of rats in the control group: 10.92±0.29 days (CI 95%; range 10.35—11.48). Median survival time: 11.00±0.38 days (CI 95%; range 10.27—11.74).

The overall surgical mortality was 4.8 % (3/62) with a variation of 0% (Step 2, Protocol B) to 6.2% (in both Step 1 and Step 2, Protocol A) as shown in Table 2, indicating that the intrabronchial lung tumor implantation model tested in this study is simple to reproduce.

TABLE 2 - Surgical mortality of animals submitted to intrabronchial implantation with Walker's tumor cells.

Step/ Protocol	Animals inoculated	Surgical deaths	Mortality %
1	32	2	6.2 %
2 / A	16	1	6.2 %
2 / B	14	0	0.0 %
Total	**62**	**3**	**4.8 %**

The histopathological examination confirmed the presence in the left lower lobe of a nodule or large tumor mass occupying most of the lung in 36 of the 38 animals submitted to intrabronchial implantation with 4×10^5 cells of Walker's carcinosarcoma, thus yielding an overall tumor take rate of 96% (Table 3).

TABLE 3 - Overall tumor take rate in animals submitted to intrabronchial implantation with 4×10^5 cells of Walker's carcinosarcoma.

Step/ Protocol	Animals inoculated	Animals presenting tumors	Tumor take rate
1	10	9	90 %
2 / A	15	15	100 %
2 / B	13	12	92 %
Total	**38**	**36**	**94.7 %**

Discussion

The lung tumor model described in the present study was shown to be simple to use and easy to reproduce. The procedure demanded 5–6 minutes for each animal, making it possible to inoculate 20–24 animals in 2 hours. Tracheotomy and intrabronchial cell implantation caused minimal trauma and mortality was negligible (4.8%) compared to the mortality rates reported in the literature (5–10%) for similar models[8,9,11]. Our model was developed with cells from Walker's carcinosarcoma, which may be described as an adenocarcinoma of the rat mammary gland, although tumor cells from the lungs of rodents and humans may also be used. However, experiments with human tumor cells require the use of athymic mice or immunosuppressed rats. With experimental animals of this type it is possible to develop orthotopic models in which tumor cells are implanted in the organ corresponding to the organ of origin[7,8]. The fact that orthotopic models may be developed with any lineage of human tumor cells regardless of phenotype makes it easier to measure response to anticancer therapy. The authors are presently developing an orthotopic rat lung tumor model based on the model described in this study. During Step 1, tumors turned into nodules in 90% of the animals, as documented by HRCT and necropsy performed on the 6th day following intrabronchial implantation with 4×10^5 cells.

The overall tumor take rate with this number of cells was 94.7 %. This is similar to the tumor take rate (94.5%) observed for an earlier model developed in our laboratory[10] and inoculated via thoracotomy with 2×10^5 cells of the same lineage. In a study by Wang *et al.*[9], tumor cells implanted by direct puncture of the parenchyma via thoracotomy yielded a higher take rate (100%) than cells inoculated intrabronchially (95%), illustrating the influence of the microenvironment upon tumor development. Other workers implanting cells in the lung transtracheally and intrabronchially found tumor take rates of 80–100% and very low mortality[8], lending support to the overall tumor take rate observed in the present study (94.7%). Models based on implantation via thoracotomy may present high tumor take rates, but require a high level of surgical skill, are more difficult to reproduce and are associated with higher rates of surgical mortality. The thoracotomy-based model developed by Gomes-Neto *et al.*[10] presented a relatively high surgical mortality rate (14.3%), in contrast with the present model of intrabronchial implantation (4.8%). The aggressive nature of Walker's carcinosarcoma is evident in the high tumor take rates observed and the rapid growth of the neoplasm characterized by the presence of large nodules in the lung (average volume: 0.118 ± 0.108 cm^3) as early as the 5th day following cell implantation. In a study inoculating rats with different lineages of tumor cells intrabronchially, Howard *et al.*[8] reported no tumor growth

until the third week following implantation. Other workers implanting human cancer cell lineages intrabronchially in nude rat lung tumor models reported observing the first small lung nodules (<1–3 mm diameter) during the 5th week following inoculation with 20 x 10^{6} cells.[15] Using Walker's carcinosarcoma, our model presented lung nodules on the 5th day following inoculation, and in less than two weeks all animals in the control group had died. Howard *et al.*[16] developed an orthotopic nude rat lung tumor model with endobronchial implantation of human lineages of lung carcinoma (NCI-H460) and found distant metastases in several organs, the earliest of which in the mediastinal lymph nodes. The most severely affected areas were the contralateral lung, kidneys, brain, bones and (less frequently) the adrenal glands. However, no metastases were observed until the 14th day following implantation. Dissemination to the mediastinal lymph nodes was detected on the 21st day and distant metastases only by the 28th day**.** In a study using the same lineages in nude rats and the same model, Johnston *et al.*[7] found metastases in the mediastinal lymph nodes in 100% of the animals in the control group, as well as systemic metastases in the bones (95%), kidneys (83%), brain (48%) and contralateral lung (82%). The authors found the lung cancer model useful, in spite of the aggressiveness of the tumor causing the animals to die by the 5th week of implantation by way of local and systemic dissemination. In our model tumors developed quickly and disseminated to the mediastinum, but no distant or systemic metastases were found, perhaps because of the early death of the animals (median survival time: 11 days) in consequence of the aggressive nature of the cell lineage inoculated. Not even the contralateral lung displayed metastases, proving that no dissemination or endobronchial leakage of tumor cells occurred at the moment of implantation, a possibility discussed by some authors[7,16]. The massive mediastinal dissemination observed in the present study resembles findings from human patients with small-cell carcinoma. In fact, small-cell carcinoma behaves very aggressively in humans, usually with early dissemination to the mediastinum, although distant metastases are also frequently observed[17]. Presently oncological research tends to employ mice inoculated subcutaneously when testing new anticancer drugs[18]. However, the validity of the results obtained with these models is questionable due to differences in the pharmacodynamic aspects of subcutaneous tumor grafts and tumors in their organ of origin. Because chemosensitivity and response to therapy with anticancer drugs depend on the microenvironmental interaction of stroma and tumor, the choice of anatomical site of implantation is of considerable importance[6]. Thus, the development of orthotopic animal models is likely to increase our ability to anticipate human response to therapy with anticancer drugs[19]. The results obtained in the present study demonstrate the feasibility of our rat lung tumor model based on intrabronchial inoculation of tumor cells in the lower left lung and may contribute to the development of an orthotopic lung tumor model using immunosuppressed rats inoculated with human tumor cells. The HRCT technique employed to diagnose lung tumors in rats inoculated with

Walker's tumor cells was shown to be time-saving, non-invasive and capable of early tumor detection. HRCT findings regarding tumor size were validated by histopathological examination and correlated closely with necropsy findings (r=0.953; *p*<0.0001), suggesting that HRCT scanning may be used in the future to evaluate tumor growth and volume without euthanasia, thus avoiding time and resource-consuming procedures. Other recently published studies using experimental models[10,12,13] found the HRCT scanning technique to be an efficient tool for diagnosing lung nodules and to be less costly than magnetic resonance imaging[20]. The efficacy of HRCT scanning in the detection and measurement of tumors, as demonstrated by our study, makes it a suitable and non-invasive technique for diagnosing lung tumors, monitoring tumor growth and evaluating response to anticancer drugs *in vivo* in animals submitted to implantation with tumor cells.

Conclusion

1. Model of intrabronchial tumor implantation proved feasible: the take rate was high, surgical mortality was negligible and the procedure was simple to perform and easy to reproduce.

2. High-resolution computed tomography was found to be a highly accurate tool for tumor diagnosis, localization and measurement and may be recommended for monitoring tumor growth in this model.

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