

Action of tacrolimus on Wistar rat kidneys implanted with Walker 256 carcinosarcoma¹

Estudo da ação do tacrolimus em rins de ratos Wistar implantados com carcinossarcoma de Walker

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ABSTRACT

Purpose: To evaluate the development of Walker 256 tumor in male Wistar rats treated with tacrolimus using an experimental kidney tumor model. **Methods:** 40 male Wistar rats were divided into four groups: Tumor group (TU) (n=10), Tacrolimus-Tumor group (TT) (n=10), Tacrolimus group (TC) (n=10) and Control group (C) (n=10). Treatment with tacrolimus was performed in groups TT and TC. Under anesthesia, the right kidney of each animal of TU and TT was accessed through a supraumbilical incision and inoculated with a 0.1mL solution containing 2×10^6 tumor cells (Walker 256 carcinosarcoma tumor cells). Group TC was treated with a saline solution. All the animals of groups TC and TT were treated with tacrolimus (5mg/kg/day) by gavage for 15 days. TU group animals received saline by gavage for 15 days. On the 15th postoperative day, all animals were submitted to euthanasia and blood sampling for analysis of serum creatinine (Cr) and blood urea nitrogen (BUN). Abdominal gross examination was performed, the right kidney removed and prepared for histological analysis by hematoxylin-eosin staining. The resulting data were submitted to statistical analysis by ANOVA. **Results:** Statistical significance was found when comparing creatinine level between groups TU, TT and TC -TT group culminated with a marked increased in creatinine levels (Cr=1.013 ± 0.3028 mg/mL), TU group (Cr=0.5670 ± 0.03536 mg/dL) P=0.00256, TC group (Cr =0.711 ± 0.1653 mg/mL) P= 0.02832. Statistical significance was found when comparing BUN levels in TT group (71.32 ± 17.14 mg/mL), compared with TU group (45.83 ± 5.046 mg/dL), P=0.000318. There were no statistically significant differences between groups TT and TC (61.23 ± 9.503 mg/mL) P=0.7242. Histological analysis showed a poor evolution in TT group with multiple foci of hemorrhage and cortical invasion by the Walker tumor. **Conclusion:** The Tacrolimus-treated group developed a more aggressive tumor and a drug-related nephrotoxic effect.

Key words: Carcinoma 256, Walker. Tacrolimus. Tacrolimus Binding Proteins. Rats.

RESUMO

Objetivo: Avaliar as alterações na evolução do carcinosarcoma 256 de Walker, inoculado no rim de ratos Wistar, sob tratamento imunossupressor com o tacrolimus. **Métodos:** Foram utilizados 40 ratos Wistar, machos divididos em quatro grupos de 10: grupo Tumor (TU), Tumor-Tacrolimus (TT), Tacrolimus (TC) e Controle (C). Os ratos dos grupos TU e TT foram inoculados com 0,1 mL de solução contendo 2×10^6 células do tumor de Walker no parênquima do rim direito. Os dos grupos TC e TT receberam tratamento com tacrolimus na dose de 5mg/kg de peso, via gavagem orogástrica durante 15 dias. Os ratos do grupo TU receberam solução salina isotônica pH 7,2. Ao 15º dia de evolução, todos foram submetidos à eutanásia. Amostras de sangue eram coletadas para dosagem de creatinina (Cr) e uréia (Ur) e posteriormente realizada nefrectomia para avaliação histológica. **Resultados:** As dosagens séricas de creatinina foram maiores no grupo TT (Cr = 1,013±0,3028 mg/mL), que diferiu significativamente dos grupos TU (Cr=0,5670 ± 0,03536 mg/dL) com p=0,00256 e do TC (Cr=0,711 ± 0,1653 mg/mL) com p=0,02832. As dosagens séricas de uréia foram maiores no grupo TT (71,32 ± 17,14 mg/mL), que diferiu significativamente do grupo TU (45,83 ± 5,046mg/dL) com p=0,000318, mas comparado ao grupo TC (61,23 ± 9,503 mg/mL) não houve diferença significativa (p=0,7242). No inventário da cavidade abdominal dos grupos TU e TT, observou-se presença macroscópica de tumor em todos os rins direitos; não foram evidenciadas efusões ascíticas, formação de bridas ou metástases tumorais em outros órgãos ou tecidos adjacentes aos rins direitos. **Conclusão:** O tacrolimus exerceu efeito nefrotóxico e induziu exacerbação do crescimento do tumor de Walker 256, quando implantado no rim de ratos Wistar.

Descritores: Carcinoma 256 de Walker. Tacrolimo. Proteínas de Ligação a Tacrolimo. Ratos.

¹Research performed at Research Medical Institute, University Evangelic Hospital/ Evangelic Parana Faculty, Curitiba-PR, Brazil.

Introduction

Malignancies are the third cause of death in renal transplant recipients and account for 12% of deaths, surpassed only by cardiovascular and infectious causes¹. Those malignancies are related to the recipient's more advanced age, use of immunosuppressants and viral infections.

During the Second World War, it was found that transplants involving genetically similar tissues or organs could lead to rejection, especially when skin grafts were used.

The number of renal transplants in Brazil went from 3,332 cases in 2004 to 3,362 procedures in 2005. Today, Brazil is the third country in the world in absolute numbers, behind the United States and China only. The rise in immunosuppressive drug use to prevent rejection from the recipient organism is currently a fact. The most frequently used drugs for this purpose are tacrolimus – also known as FK 506 – and mycophenolate mofetil.

The occurrence of malignancies in transplant recipients is high and often implies a somber prognosis. In transplanted patients, kidney tumors occur more frequently than the expected rate for this type of tumor². Surgical treatment is restricted due to the risk of graft loss. The recommendation of immunosuppressive drug withdrawal in the event of kidney tumor in the transplanted graft is not always feasible, since the progression to kidney failure is rapid with the withdrawal of the drugs^{1,3,4}.

New lines of research involving drugs that act on the immune system have been proposed in the literature for tumor control in a situation of immunosuppression. The drug tacrolimus has been targeted in numerous clinical and experimental trials due

to its immunosuppressive properties. However, its action in the presence of tumors is scarcely known⁴.

Kidney implantation of a Walker tumor (carcinosarcoma 256) has a rapid and aggressive course, and is the experimental model of choice to simulate tumors and in the evaluation of the effect of immunomodulating drugs. The use of Walker tumors in studies related to tumor biology is extensive, and the tumor can be inoculated in several types of tissue⁵. Kidney implantation is used in research involving immunosuppression and kidney transplants. Such procedure carries a high level of accuracy for the evaluation of immunomodulating drugs and their action on tumor development⁶.

The objective of this study was to evaluate, through histological and renal function assessment, the course of Walker 256 carcinosarcomas inoculated in the right kidney of Wistar rats under immunosuppressive treatment with tacrolimus.

Methods

This study was conducted at the Institute for Medical Research of the University Evangelic Hospital/ Evangelic Parana Faculty as part of the *stricto sensu* activities of the Graduate Program in Principles of Surgery, and was approved by the Research Ethics Committee of the Sociedade Evangélica Beneficente of Curitiba, Brazil.

Forty Wistar rats aged between 120 and 140 days, weighing 265.34 ± 23.73 g, were used. The rats were divided into four groups of five animals each and identified with picric acid (Chart 1).

CHART 1 - Allocation of the study groups

GROUPS	N	GROUP OBJECTIVES
Tumor (TU)	10	Evaluate the natural course of a Walker tumor in the renal parenchyma for 15 days
Tumor-Tacrolimus (TT)	10	Evaluate the course of a Walker tumor in the renal parenchyma under tacrolimus for 15 days
Tacrolimus (TC)	10	Evaluate the action of tacrolimus for 15 days
Control (C)	10	Establish reference values for biochemical levels and a histological normality standard for the renal parenchyma

The rats were kept in a specific environment with controlled temperature and humidity and automatically regulated 12-hour light/dark cycles, and were fed a specific ration for the species (Nuvilab®, Nuvital) as well as water *ad libitum*.

Experimental design

a) Assessments

The following assessments were undertaken: 1) serum creatinine and urea in all rats of groups TC, TT, TU and C on day 15; 2) tacrolimus levels in group TT; 3) histological evaluation of both kidneys of each rat in the experimental groups, and one kidney of each control group rat on the 15th day of evolution.

b) Implantation of Walker 256 tumor cells and maintenance of the tumor line

Rats intraperitoneally injected with the Walker 256 tumor type 2 were provided by Professor Rui Curi, ICB – USP, São Paulo, Brazil.

Every four days, the ascitic effusion produced by the tumor was collected through an extensive laparotomy and aspiration by syringe and needle. Subsequently, the animal underwent lethal ether inhalation. The rat's waste and all the material used in the procedure were disposed of as laboratory waste of high biological hazard and immediately destroyed by incineration.

The sample obtained was submitted to tumor cell count and diluted so as to contain 2×10^7 cells/mL of the Walker 256 tu-

mor, and 1 mL was injected into another animal. After four days, this procedure was repeated, thus maintaining the cell line identified as a “tumor seed” (Figure 1).



FIGURE 1 - Aspect of the ascitic tumor collection on the 4th day of evolution

c) Obtaining the inoculum for the experiment

On the day of tumor implantation, the rat bearing the “tumor seed” was anesthetized as previously described and 10 mL isotonic saline, pH 7.2, were injected into the abdominal cavity. Subsequently, the abdomen was opened and the fluid extracted through a Pasteur pipette. A 2-mL aliquot was taken for bacterial and fungal sterility testing.

Subsequently, cell count was carried out in a Neubauer counting chamber (Figure 2), and 0.1 mL of that suspension, containing 3×10^5 cells/mL, was injected into the right kidney of the rats which were the subjects of this study⁶.

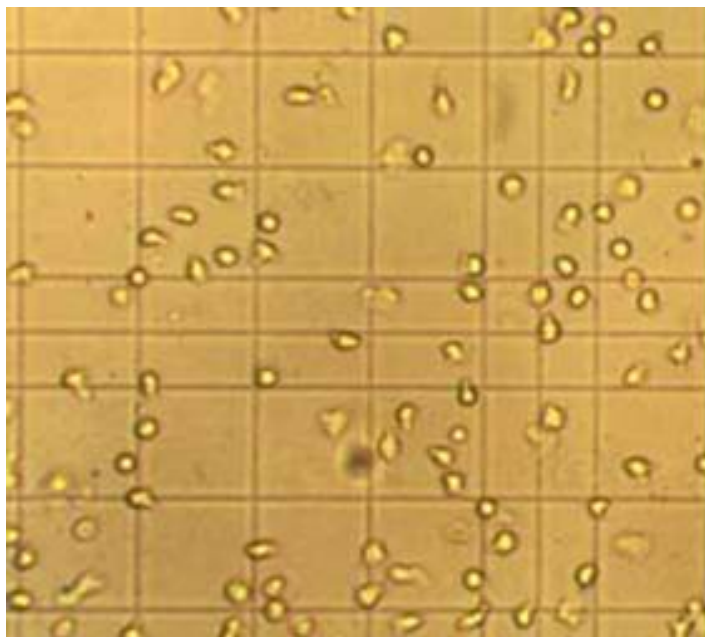


FIGURE 2 - Aspect of the Walker 256 tumor cell count in a Neubauer counting chamber

d) Inoculation of Walker tumor 256 in the kidney of rats

The inoculated rats (groups TU, TT) were separated on the previous day and fur-marked with picric acid. They were weighed and submitted to extensive abdominal shaving. On the following day, they were anesthetized with 10% chloral hydrate at the dose of 300 mg/kg intraperitoneally.

Subsequently, sterile drapes were placed and the abdominal cavity was opened (a median laparotomy of approximately 3 cm). The bowel loops were pushed aside, and thus the right kidney was located – where 0.1 mL of the tumor suspension described above was inoculated.

Inoculation was standardized with a 1-mL sterilized syringe and hypodermic needle with a piece of latex in order to restrain the trajectory and direction of inoculation so as to be done always in the medullary region of the lower pole of the kidney. The inoculation of 2×10^6 tumor cells was then carried out, in a slow fashion to prevent intra-abdominal extravasation of the inoculum (Figure 3). The contralateral kidney, by the same procedure, was injected with only 0.1 mL of the medium of the tumor cell suspension and isotonic saline, pH 7.2.

The procedure was similar for group TC, yet no inoculation of tumor suspension was conducted; both kidneys were inoculated with 0.1 mL isotonic saline, pH 7.2.

After the inoculations, the bowel loops were replaced and the surgical wound was closed on two planes with continuous 3-0 mononylon suture.



FIGURE 3 - Inoculation of the Walker tumor in the right kidney

The rats were then caged in a controlled environment as previously described and maintained there for 15 days. The following clinical parameters were recorded daily on a protocol: piloerection, occurrence of blepharitis, the animal's deambulation pattern in its box, presence of feces and weighing on an electronic scale every five days.

e) Tacrolimus treatment

The product was used as oral FK 506 (Tacrolimus). The dose administered was 5 mg/kg of body weight via orogastric gavage^{7,8}.

Specimen collection and laboratory tests

On the 15th day of evolution, all rats in the study underwent anesthesia with 10% chloral hydrate and intracardiac puncture was performed for blood sampling and euthanasia.

Following confirmation of death, extensive opening of the abdominal cavity was undertaken, as well as an inventory and resection of the kidneys, which were subsequently immersed in bottles with formalin and identified.

In the inventory, the aim was to note the tumor take rate, i.e., the percentage of tumors induced, the presence of ascitic effusion, adhesion formation or tumor metastases. For the control group, blood and kidney sampling followed the same methodology.

Serum urea and creatinine levels were measured in a Cobas Mira automated biochemical equipment – COBAS System – MIRA “S”, with specific reagents and standards.

Monitoring tacrolimus levels

The IMx tacrolimus II assay (Abbot csc 0800-11-90-99)⁹, based on the microparticle enzyme immunoassay methodology (MEIA), was used in this study.

Prior to the initiation of the automated IMx sequence, a manual pretreatment step was performed, in which the whole blood specimen was extracted with a precipitation reagent and centrifuged. The supernatant was decanted to the sample well and IMx tacrolimus II reagents, along with the sample, were added to the reaction vessel (RV). The probe/electrode assembly dispensed the microparticles coated with anti-tacrolimus murine monoclonal antibodies and the tacrolimus-alkaline phosphatase conjugate into the incubation well of the reaction vessel. Tacrolimus and the conjugate competed to bind to the antibody-coated microparticles, composing an “antibody-antigen” complex and an “antibody-antigen-alkaline phosphatase” complex. An aliquot containing the “antibody-antigen” and “antibody-antigen-alkaline phosphatase” complexes bound to the microparticles was transferred to the glass fiber matrix. The microparticles bound irreversibly to the matrix,

which was washed in order to remove unbound materials. The 4-methylumbelliferyl phosphate substrate was added to the matrix, and the fluorescent product was measured by the optical assembly of the equipment. Positive and negative controls were employed, and photolorimetric readings interpolated according to the IMx software assay implant module/TDM, version 4.0. The results were expressed in ng/mL.

Histopathology

After fixation in 10% formalin, the samples were referred for routine staining and mounting by the hematoxylin-eosin technique.

The histological slides were examined under light microscopy according to the criteria by Hard¹⁰, who considered the description of the neoplasia infiltration as medullary and/or cortical, the characterization of the neoplastic cells, the determination of the mitotic index and the characterization of the inflammatory infiltration.

Statistical analysis

The statistical method ANOVA was applied and the significance level of *P* was set at 5%. For comparisons between results of animals within a group at different time points, the non-parametric Wilcoxon method was used, and between different groups, the Tukey-Kramer test. The 95% confidence intervals were also calculated.

Results

Maximum reference values of ≤ 48.16 mg/mL for urea and ≤ 0.60 mg/mL for creatinine were established.

The biochemical test levels of the Control group for urea and creatinine were, respectively: means, 44.8 and 0.576; standard deviations, 6.058 and 0.03627, confidence intervals (95%), 40.48 to 48.16 and 0.55 to 0.6019 (Table 1).

TABLE 1 - Values from the biochemical tests of the animals in the Control group

Rats	Urea(mg/mL)	Creatinine(mg/mL)
1	42.2	0.53
2	45.1	0.57
3	34.4	0.60
4	48.1	0.59
5	55.5	0.64
6	44.3	0.55
7	44.0	0.60
8	42.6	0.57
9	37.5	0.52
10	53.8	0.59
MEAN	44.8	0.576
STANDARD DEVIATION	6.058	0.03627
CONFIDENCE INTERVAL (95%)	40.48 to 48.16	0.55 to 0.6019

Gross examination, biochemistry of the inoculated groups and tacrolimus dosing

During the 15 days of the experiment, no deaths occurred of any rat; however, signs of prostration and piloerection were observed in the animals of groups TU and TT. In the abdominal cavity inventory, the presence of the tumor was detected in all right kidneys; ascitic effusions, adhesion formation or tumor metastases were not found in other organs or tissues adjacent to the right kidneys.

Serum creatinine levels were higher in group TT (1.013 ± 0.3028 mg/mL), which differed significantly from groups TU (0.5670 ± 0.03536 mg/dL) with $P=0.00256$ and TC (0.711 ± 0.1653 mg/mL) with $P=0.02832$ (Figure 4).

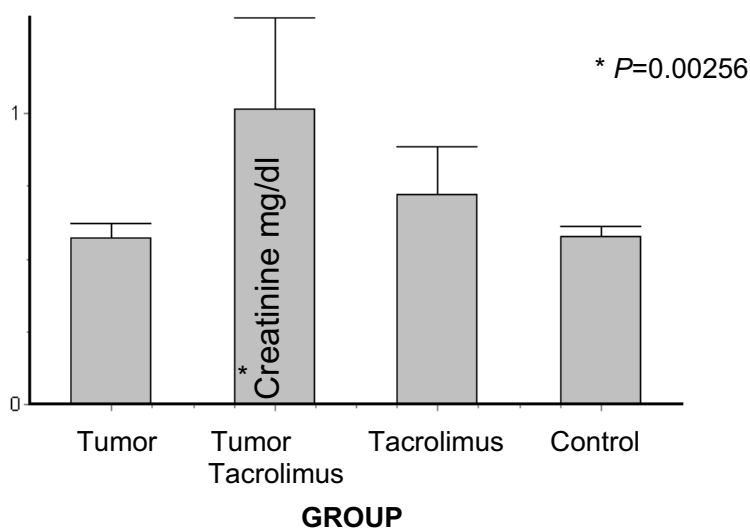


FIGURE 4 - Means and confidence intervals (95%) of creatinine serum levels in the study groups

Urea serum levels were higher in group TT (71.32 ± 17.14 mg/mL), which differed significantly from group T (45.83 ± 5.046 mg/dL) with $P=0.000318$, while no significant difference was found when compared with group TC (61.23 ± 9.503 mg/mL), with $P= 0.7242$ (Figure 5).

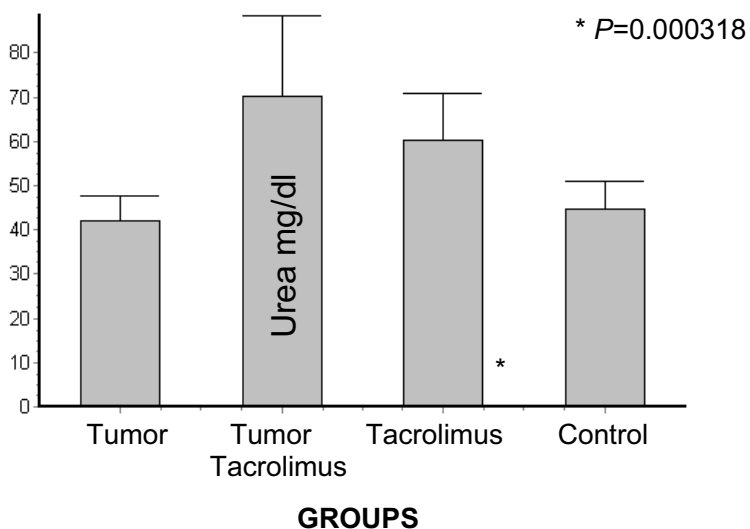


FIGURE 5 - Means and confidence intervals (95%) of urea serum levels in the study groups

Tacrolimus dosing was conducted only in the rats of groups TC and TT, which showed values of 11.29 ± 3.22 ng/mL and 10.58 ± 4.29 ng/mL ($P= 1.4462$), respectively.

Histopathology

Specimens from the animals in groups C and TC were histologically similar and showed no histological alterations.

Only blood vessel congestion was observed in the left kidney specimens of the animals in groups TU and TT, all inoculated with isotonic saline.

In the right kidneys of groups TU and TT, inoculated with the Walker tumor, the following histological differences were found: the neoplasms in the specimens of group TT were larger, affecting a more extensive area of the organ; the foci of tumor necrosis and areas of hemorrhage were also larger and inflammatory infiltration was a little more pronounced. Inflammation was similar for these two groups, as was the mitotic index of the neoplastic cells. The principal differences of the specimens between these groups are described in Chart 2.

Discussion

Clinical¹¹ and experimental¹² studies with tacrolimus have demonstrated its advantages over other immunomodulating drugs such as cyclosporine concerning the rejection of transplanted organs. Many studies have reported on tacrolimus as a promising therapy in cases of failure with other immunomodulating drugs^{13,14,15}. After a few years of tacrolimus use, an increase was observed in the development of malignancies, pointing to its withdrawal or replacement¹⁶. Few experimental studies have evaluated its actual effect and impact on tumor progression.

In the present study, the dose of tacrolimus was 5 mg/kg of body weight, since that dose promotes greater lymphocyte inhibition and immunosuppression. Barten *et al.*⁸, in 2005, proved through flow cytometry, proliferating cell nuclear antigen (PCNA) and CD25 lymphocyte receptor expression that the maximal dose for lymphocyte inhibition in rats was 5 mg /kg.

Immunosuppressive therapy is a risk factor for increased tumor incidence or progression in a transplanted organ recipient¹⁷. Growth factor beta 1 (TGFbeta1) is associated with tumor invasion and aggressiveness.

Maluccio¹⁸, in a study of rats with renal cell carcinoma that were treated with tacrolimus at the dose of 2 and 4 mg/kg, noted an increase in pulmonary metastases in the treated animals, which showed a worsening evolution with higher doses of tacrolimus and greater TGFbeta1 production. In the present study, the implantation of carcinosarcomas in kidneys of rats under immunosuppression showed an aggravation in the clinical course and tumor aggressiveness comparable to that in the abovementioned study.

CHART 2 - Histological aspects of the kidney specimens inoculated with Walker tumor in the rats of groups TU and TT

CRITERIA	TUMOR GROUP		TUMOR-TACROLIMUS GROUP	
	%	Findings	%	Findings
Description of the tumor area	100%	Tumor area affecting the medullary region of kidneys, with infiltration by a neoplastic population of epithelial cells. Tumor growth was invasive and compact	100%	Tumor area affecting the cortical and medullary regions of kidneys, with infiltration by a neoplastic population of epithelial cells. Tumor growth was invasive and compact, with areas of tumor necrosis and multifocal hemorrhage
Characterization of the neoplastic cells	100%	Poorly differentiated, disorganized proliferation between renal tubules, showing a large round nucleus and indistinct cytoplasm, frequently vacuolar. Severe anisokaryosis and nuclear and nucleolar atypias were observed.	100%	Poorly differentiated, disorganized proliferation between renal tubules, showing a large round nucleus and indistinct cytoplasm, frequently vacuolar. Severe anisokaryosis and nuclear and nucleolar atypias were observed.
Determination of the tumor mitotic index	100%	High, an average of 5.4 mitotic figures / field (40x) and atypical mitotic figures were observed	100%	High, an average of 5.6 mitotic figures / field (40x) and atypical mitotic figures were observed
Inflammatory infiltration	100%	Diffuse mild, chiefly by neutrophils and lymphocytes	100%	Diffuse mild, chiefly by neutrophils and lymphocytes

Everett *et al.*¹⁹, in a study involving rats with the Walker tumor treated with hydrocortisone and cyclophosphamide, demonstrated a tendency to metastases when those drugs were used. In the present study, the action of tacrolimus was comparable, since it revealed a tendency to increased tumor aggressiveness.

In most studies described in the literature, tacrolimus accelerates tumor growth as found in the present study; there are, however, cases of tumor regression with tacrolimus. Taguchi *et al.*²⁰ reported total regression of pleural metastases with the use of tacrolimus in a thymoma patient.

A study conducted by Niwa²¹ with rats submitted to induced skin tumors found that topical application of tacrolimus accelerated carcinogenesis when compared with other drugs for topical application. Shinozuka²², in an experimental study of liver tumors,

found a worsening picture with the use of tacrolimus. In the present study, similar results were found with the implantation of Walker tumors, the same being true for other authors.

Rettori *et al.*²³ remarked that Walker tumors, when experimentally implanted, may trigger a number of water-electrolyte alterations such as sodium retention and reduced creatinine clearance. In the present study, those findings were observed in the groups inoculated with this tumor and in the tacrolimus-treated tumor groups.

The Walker tumor causes metabolic alterations in the lymphocytes and macrophages of rats; these changes could compromise the animal's immune response, metabolism and renal function. The present study confirmed those findings, since animals in groups TU and TT exhibited a decline in renal function indices.

Conclusion

Immunosuppressive treatment with tacrolimus in the presence of a Walker tumor in murine kidney induced exacerbated tumor growth and nephrotoxicity with elevation of serum urea and creatinine levels.

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