

MANY experimental studies have been carried out using snake venoms for the treatment of animal tumors, with controversial results. While some authors have reported an antitumor effect of treatment with specific snake venom fractions, others have reported no effects after this treatment. The aim of this study was to evaluate the effect of *Bothrops jararaca* venom (*BjV*) on Ehrlich ascites tumor (EAT) cells *in vivo* and *in vitro*. In the *in vivo* study, Swiss mice were inoculated with EAT cells by the intraperitoneal (i.p.) route and treated with *BjV* venom (0.4 mg/kg, i.p.), on the 1st, 4th, 7th, 10th, and 13th days. Mice were evaluated for total and differential cells number on the 2nd, 5th, 8th, 11th and 14th days. The survival time was also evaluated after 60 days of tumor growth. In the *in vitro* study, EAT and normal peritoneal cells were cultivated in the presence of different *BjV* concentrations (2.5, 5.0, 10.0, 20.0, 40.0, and 80 µg) and viability was verified after 3, 6, 12 and 24 h of cultivation. Results were analyzed statistically by the Kruskal–Wallis and Tukey tests at the 5% level of significance. It was observed that *in vivo* treatment with *BjV* induced tumor growth inhibition, increased animal survival time, decreased mortality, increased the influx of polymorphonuclear leukocytes on the early stages of tumor growth, and did not affect the mononuclear cells number. *In vitro* treatment with *BjV* produced a dose-dependent toxic effect on EAT and peritoneal cells, with higher effects against peritoneal cells. Taken together, our results demonstrate that *BjV* has an important antitumor effect. This is the first report showing this *in vivo* effect for this venom.

Key words: Antitumor effect, Ehrlich ascites tumor, *Bothrops jararaca*, Venom

Antitumor effect of *Bothrops jararaca* venom

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Introduction

Many experimental studies have been carried out using snake venoms for the treatment of animal tumors. However, we have seen many controversies on this subject. Some authors have reported that tumor treatment with specific snake venom fractions has an important toxic effect on tumor cells, while others have reported no antitumor effects after inoculation with snake venoms.¹

The exact mechanisms that cause tumor regression in experimental animals after treatment with crude snake venom and/or any fraction of this venom still are unknown.¹ According to Lipps,² certain fractions isolated from snake venoms revealed a direct cytolytic activity on tumor cells. Markland,³ on conducting research on crotalase, proposed that malignant cells would produce a microenvironment around them, using substances of the host itself that protected them against defensive responses of the immune system. This microenvironment would be composed of fibrin

deposits from the nearby blood vessels. Crotalase apparently destroyed the microenvironment produced by the tumor cells attacking fibrinogen directly, which led to the formation of soluble fibrin monomers, or abnormal fibrin microclots, that were rapidly removed by secondary activation of the fibrinolytic system. Thus, immunological masking would not have occurred, which would leave tumor cells unprotected. The literature demonstrates also that snake venoms may induce apoptosis in both normal and tumor cells.^{4–12} In most of these works, the proposed apoptosis induction mechanism was H₂O₂ production induced by the venom enzyme L-amino acid oxidase. This enzyme was purified from *Crotalus adamanteus* venom and its structure showed a high degree of homology with murine interleukin-4.¹³

However, it has been demonstrated that continuous contact of venom and tumor cells was necessary to obtain an antitumor effect. Yoshikura *et al.*¹⁴ evaluated the effects of *Trimeresurus flavoviridis* crude venom and the hemorrhagic principles in different

cell strains *in vitro*. They demonstrated that treatment caused morphological and physiological alterations in tumor cells without causing cellular death. However, when these altered cells were removed from the venom-containing medium and placed in a venom-free culture medium, they showed normal morphology and physiology and grew in the medium. Leung *et al.*¹⁵ corroborated this and reported that the cardiotoxin from *Naja* sp. venom shows a cytolytic effect on Ehrlich ascites tumors cells and that cytolysis progression required continuous venom presence. This cytolytic activity completely stopped if the toxin was removed from the medium.

It can be considered that antitumor effects of snake venoms depend on the venom biodistribution time. Tanigawa *et al.*¹⁶ demonstrated that jararafibrase-I and jararafibrase-II, two fibrinolytic/hemorrhagic enzymes of *Bothrops jararaca* venom (*BjV*), show rapid biodistribution in the body. Five minutes after intravenous inoculation in mice, its concentration in blood decreases by 60%. Similarly, Thwin *et al.*¹⁷ showed that *Vipera russeli* venom inoculated intravenously in mice was rapidly eliminated in urine.

The literature shows that mononuclear (MN) leukocytes may effectively participate in tumor cell elimination.^{18–26} A previous study²⁷ evaluated the percentage of macrophage spreading and found that, after treatment with *Crotalus durissus terrificus* venom, they were quite spread out with increases in size and presence of large cytoplasmatic vacuoles, suggesting high cellular activity. This increase was not observed in non-venom groups. However, Rabinovitch and Destefano²⁸ demonstrated that proteolytic enzymes may induce macrophage spreading as they are capable of removing macromolecular components from the cell surface and affecting macrophage surface receptors, resulting in a higher affinity of the cell membrane to the substrate. This suggests that although an increase in macrophage spreading percentage has been observed,²⁷ this may not lead to an increase in the functional activity of these cells.

Most studies report on different snake venoms, mainly *Crotalus* spp. and *Naja* spp.¹ There was only one study with *BjV*, which reports a cytophatic effect on normal and tumor cells *in vitro*.²⁹ The *in vivo* antitumor effect of this venom was not studied. The objective of the present paper is to evaluate the antitumor effect of *BjV* on tumor cells *in vivo* and *in vitro*.

Materials and methods

Animals

Swiss strain male mice, 4–6 weeks old, from our own animal facilities were used throughout the experiment.

Venoms

BjV was obtained from snakes maintained in captivity at The Center for the Study of Venoms and Venomous Animals of São Paulo State University, Brazil. Newly extracted venom was centrifuged for 10 min at 1500 rpm, filtered using a GSWP00250 Millipore filter and then lyophilized. The lethal dose 50 (LD₅₀) for this venom has been previously determined as 2.4 mg/kg of animal weight. The venom was stored at 4°C during the experiment. Preliminary protocols shown that 0.4 mg/kg *BjV* was efficient for tumor growth inhibition.

Ehrlich ascites tumor

The tumor was maintained in Swiss mice in the ascitic form. Tumor cells were collected by aspiration with a Pasteur pipette, centrifuged for 10 min at 200 × g, and washed twice with phosphate-buffered saline (PBS) (pH 7.2). Cell viability was evaluated by the trypan blue exclusion test, and only cell suspensions that presented more than 95% viability were used.

Peritoneal cells

Cells were harvested by peritoneal washing using 3 ml of sterile PBS. The number of cells was determined using a hemacytometer. Differential counts were performed on fixed and stained cell suspensions (0.5% crystal violet dissolved in 30% acetic acid).

In vivo experiment

Mice were inoculated by the intraperitoneal route (i.p.) with 1×10^3 Ehrlich ascites tumor (EAT) cells and treated with *BjV* (0.4 mg/kg) or saline (0.1 ml, i.p.). The first *BjV* dose was administered 24 h after tumor implantation and repeated five times in each 72-h interval. Additional control groups with no tumor bearing were treated with *BjV* or saline, in the same protocol of experimental groups. After 2, 5, 8, 11, and 14 days of EAT implantation, 10 animals from each group were sacrificed in a sulfur ether chamber, and peritoneal cells were analyzed for tumor, polymorphonuclear (PMN), and MN cell numbers. The remaining 10 animals from each group were kept in the laboratory for 60 days for survival analysis. These procedures were repeated twice and data were grouped for statistical analysis.

In vitro experiment

EAT and peritoneal cells were cultivated with 0 (control), 2.5, 5, 10, 20, 40, and 80 µg of *BjV*. Peritoneal washing was performed on mice inoculated with EAT cells and on normal mice. Cellular suspensions adjusted to 1×10^3 EAT cells and 5×10^3

peritoneal cells were cultivated in RPMI medium supplemented with 5% fetal bovine serum for 24 h at 37°C and 5% CO₂ atmosphere. The cultures were then washed and the cells placed in RPMI medium containing *BjV* at different doses. After 3, 6, 12, and 24 h, cell viabilities were analyzed. These procedures were repeated twice and data were grouped for statistical analysis.

Statistical analysis

Data were analyzed by the Kruskal–Wallis or analysis of variance tests for independent sets, and by the Tukey or Student–Newman–Keuls tests for differences between the groups. The significance level was 5%.

Results

In vivo experiment

Animals inoculated with EAT cells and treated with saline (G3) presented exponential growth of tumor cells after the 8th day, with a total number of 70×10^6 cells/ml at the 14th day. In the animals of the group inoculated with EAT cells and treated with *BjV* (G4), a significant reduction in tumor cells was observed on the 11th and 14th days; the final number was 0.7×10^6 cells/ml (Fig. 1).

PMN leukocytes influx in animals inoculated with EAT (G3) showed significant influx of these cells on the 8th, 11th, and 14th days. Animals inoculated with EAT cells and treated with *BjV* (G4) showed a significant increase throughout the study. PMN leukocyte influx was also observed in non-tumor-bearing animals treated with *BjV* (G2), the *BjV* only group (Fig. 2).

In addition, animals inoculated with EAT cells and/or *BjV* (G2, G3, and G4) showed a significant influx in MN leukocytes throughout the study (Fig. 3).

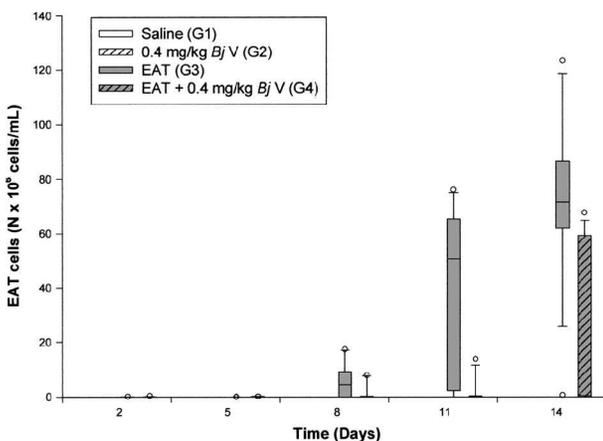


FIG. 1. Distribution of the median of the number of EAT cells on the 2nd, 5th, 8th, 11th, and 14th days of tumor growth. Each box represents 25–75% values, with the median as an internal line; the error bar represents the percentiles 10 and 90, and the circle represents the outliers. Statistics: 11th and 14th days, G3 > G4; $p < 0.05$.

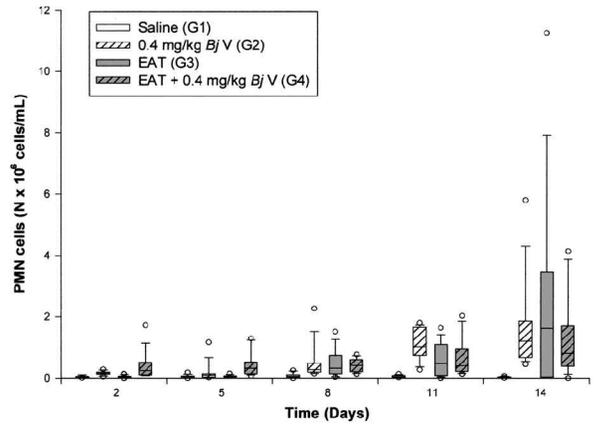


FIG. 2. Distribution of the median of the number of PMN cells on the 2nd, 5th, 8th, 11th, and 14th days of tumor growth. Each box represents 25–75% values, with the median as an internal line; the error bar represents the percentiles 10 and 90, and the circle represents the outliers. Statistics: 2nd day, G2 = G4 > G1 = G3; 5th day, G4 > G1 = G3; 8th, 11th, and 14th days, (G2 = G3 = G4) > G1; $p < 0.05$.

Survival analysis revealed that the animals inoculated with EAT cells and treated with *BjV* have increased survival times and decreased mortality, compared with untreated animals (Fig. 4).

In vitro experiment

All tested venom concentrations showed a toxic effect on tumor cells. We observed higher toxicity when the higher doses of *BjV* were used, and *vice versa*. We have also observed that, for concentrations of 2.5, 5, and 10 µg, there was a mean increase in cell viability at 6 h in comparison with 3 h. This increase was not seen with concentrations of 20, 40, and 80 µg (Fig. 5).

Similar to the tumor cells, all the tested venom concentrations showed a toxic effect on mice peritoneal cells, with higher toxicity associated with treatment with higher doses of venom (Fig. 6).

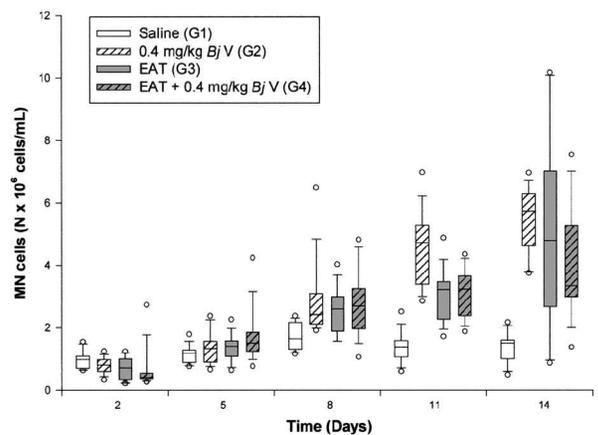


FIG. 3. Distribution of the median of the number of MN cells on the 2nd, 5th, 8th, 11th, and 14th days of tumor growth. Each box represents 25–75% values, with the median as an internal line; the error bar represents the percentiles 10 and 90, and the circle represents the outliers. Statistics: 11th and 14th days, (G2 = G3 = G4) > G1; $p < 0.05$.

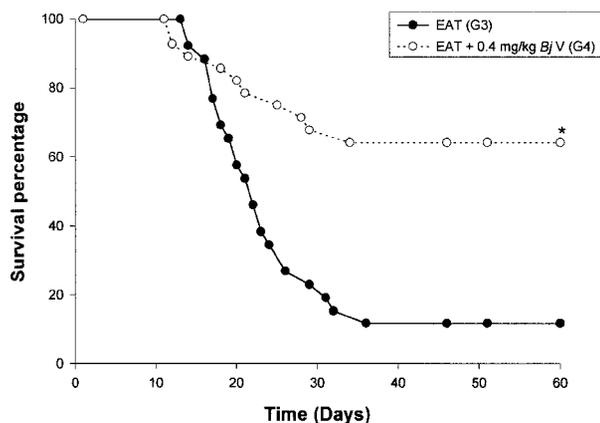


FIG. 4. Survival percentage of animals inoculated with EAT cells and treated with *BjV* or saline solution (* $p < 0.05$).

The comparative *in vitro* evaluation showed a peritoneal cell viability rate significantly higher than tumor cells after 3 h culture at 2.5, 5, and 10 μg venom. At 20 μg , although the tumor cell mean viability rate was higher than peritoneal cells, it was not statistically significant. At concentrations of 40 and 80 μg , the tumor cells showed significantly higher viability than the peritoneal cells. After 6 h culture at 2.5 and 5 μg , we observed no significant difference between tumor and peritoneal cells. At 10, 20, 40, and 80 μg , however, tumor cell viability was significantly higher than peritoneal cells. At 12 h, only the 2.5 μg concentration showed no significant difference. For all the other concentrations (5, 10, 20, 40, and 80 μg), tumor cell viability was higher than the normal cells. At the end of the experiment (24 h), viability of tumor cells was higher than normal cells at all venom concentrations (Figs 5 and 6).

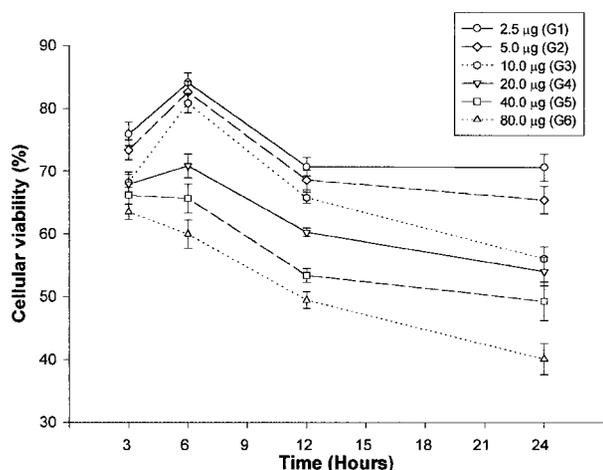


FIG. 5. Cellular viability of tumor cells after 3, 6, 12, and 24 h of cultivation in RPMI medium at 37°C and 5% CO_2 , and incubation with 2.5, 5, 10, 20, 40, and 80 μg of *BjV*. Each point represents the mean and the standard error values ($n = 48$). Statistics: 3 h, (G1 = G2) > (G3 = G4 = G5 = G6); (G1 = G2 = G3) > (G4 = G5) > G6; 12 h, (G1 = G2) > G3 > G4 > G5 > G6; (G1 = G2) > (G3 = G4 = G5) > G6; $p < 0.05$.

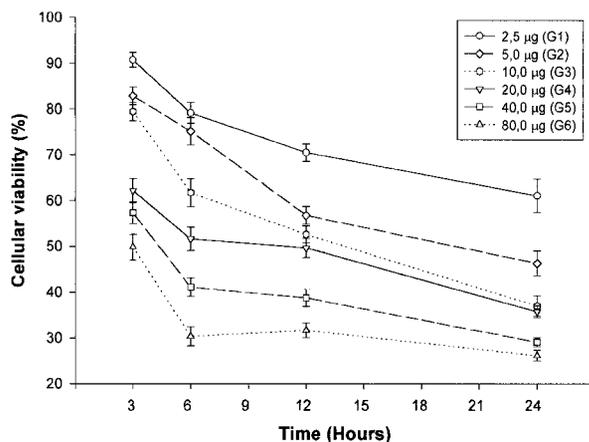


FIG. 6. Cellular viability of peritoneal cells after 3, 6, 12, and 24 h of cultivation in RPMI medium at 37°C and 5% CO_2 , and incubation with 2.5, 5, 10, 20, 40, and 80 μg of *BjV*. Each point represents the mean and the standard error values ($n = 48$). Statistics: 3 h, G1 > (G2 = G3) > (G4 = G5) > G6; 6 h, (G1 = G2) > G3 > G4 > G5 > G6; 12 h, G1 > (G2 = G3 = G4) > G5 > G6; 24 h, G1 > G2 > (G3 = G4) > (G5 = G6); $p < 0.05$.

Discussion

This study evaluated the effect of *BjV* treatment on the kinetics of tumor growth, inflammatory influx, and animal survival *in vivo*, and on its toxic and/or mitotic effect *in vitro*.

Analysis of total number of cells in the peritoneal cavity shows that the number of cells in the group that received tumor cells and venom treatment was around 10% of that of the group with tumor cells only. This suggests that although there was tumor growth in the treated group, this was of lower magnitude than in the non-treated group.

With regard to number of tumor cells, treatment with *BjV* promoted 99% tumor growth inhibition. These data show that treatment of EAT mice with 0.4 mg/kg of *BjV* induces major tumor growth inhibition. These results are in agreement with previous studies evaluating the effects of *BjV* on tumor cells²⁹ and others showing an antineoplastic effect associated with snake venom treatment.³⁰⁻⁴¹

Tumor growth inhibition caused by *BjV* was accompanied by inflammatory influx alterations. Treatment of EAT animals with *BjV* induced a significant increase of PMN leukocytes in the initial stages (2nd and 5th days) in comparison with the non-treated EAT group. This suggests that tumor growth inhibition may be related to PMN leukocyte influx at the beginning of the experiment - the beginning of tumor growth. At this stage, the presence of PMN leukocytes, attracted to the tumor growth site and activated by the venom either by oxygen free radical generation⁴² or modulation of the inflammatory response via tumor necrosis factor- α ⁴³, might play a role in modifying the environment necessary for tumor development.

With regard to MN cells, we observed an increase of tumor growth from day 11 in all groups inoculated

with EAT and/or venom. This indicates that both BJV and the presence of EAT may induce an increase in MN cell influx. Although no difference was detected in the quantity of MN cells in the peritoneal cavity, we do not rule out the possibility that different subpopulations may be involved in tumor growth inhibition.¹⁸⁻²⁶ The inflammatory capacity of BJV has also been demonstrated by the evaluation of PMN and MN leukocyte influxes into the venom only group. This group showed increased PMN on the 2nd, 8th, 11th and 14th days, and increased MN on the 11th and 14th days.

Our study also evaluated the *in vitro* antimetabolic and/or toxic effect of BJV on EAT cells and mice peritoneal cells. We observed that the venom showed a toxic effect both on tumor cells and peritoneal cells, with a higher toxicity for peritoneal cells. These results are not in agreement with studies showing the preferential effect of venom on tumor cells more than normal cells,^{2,33} but are in agreement with work by Rizzo and Tuchiya²⁹ that showed the *in vitro* toxic effect of BJV on HeLa, KB, and Hep-2 tumor cells, and on normal rabbit kidney and chicken embryo cells. These authors²⁹ have also demonstrated that the tumor cells were more resistant to venom than normal cells, as seen in this study.

BJV has different actions, such as cytotoxic action.^{44,45} It is possible, then, that this venom's proteolytic enzymes may be responsible for the death of tumor and peritoneal cells, as suggested by Lipps.²

However, in the *in vitro* cultivation conditions used in this study, the venom remained in direct contact with cells for 24 h. In the *in vivo* study, immediately after venom inoculation, the biodistribution process starts and it is soon eliminated from the peritoneal cavity, as previous demonstrated.^{16,17} Therefore, in contrast to the *in vitro* study, we can suggest that BJV venom does not act for a long period of time on tumor cells *in vivo*. Also, even *in vitro* at 10 µg concentration, corresponding to 0.4 mg/kg concentration in a 25 g animal, tumor cell viability after 24 h venom contact was 56.1%. Assuming that this toxic effect was repeated *in vivo*, the non-affected part (43.9%) could still continue to multiply, leading to tumor growth despite venom treatment. This can be seen in the *in vivo* study since, for the venom-treated EAT group, the reduction of tumor growth was not 100%. Even after venom treatment, some animals still showed tumor growth. A similar observation was reported by Yoshikura *et al.*¹⁴ and Leung *et al.*¹⁵

Although the antineoplastic effect of venom treatment has been observed *in vivo* and works on apoptosis induction have been performed only *in vitro*,⁴⁻¹² we can consider that the apoptotic processes may be involved in the growth inhibition of the EAT. In addition, L-amino acid oxidase, the main venom enzyme associated with apoptotic proces-

ses,¹³ has been described as one of the venom components from all snakes of the *Bothrops* genus,^{46,47} and therefore we cannot exclude the possible action of this enzyme on EAT cells. It is important to emphasize that, as already discussed for the venom effect on tumor cells, venom biodistribution in the body should also be considered for apoptosis; it would be important to demonstrate that the venom has enough contact time with tumor cells to induce apoptosis.

In the *in vitro* study, we also observed that tumor cells were more resistant to the venom toxic effect than peritoneal cells. We believe that *in vitro* induction of apoptosis may have a major role in cellular death. As we observed a higher sensitivity from peritoneal cells to venom, and as it is a characteristic of this venom to induce apoptosis, we can suggest that the peritoneal cells may be more susceptible to the apoptotic effect of the venom than the tumor cells.

Taken together, our results demonstrated that BJV has an important antitumor effect. This is the first report showing this *in vivo* effect for this venom. Future studies with protein fractions of BJV may reveal some biological product will contribute to improve the antitumor effect.

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