

Crotalus durissus terrificus venom as a source of antitumoral agents

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Abstract: The basic knowledge on neoplasms is increasing quickly; however, few advances have been achieved in clinical therapy against tumors. For this reason, the development of alternative drugs is relevant in the attempt to improve prognosis and to increase patients' survival. Snake venoms are natural sources of bioactive substances with therapeutic potential. The objective of this work was to identify and characterize the antitumoral effect of *Crotalus durissus terrificus* venom (CV) and its polypeptide, crotoxin, on benign and malignant tumors, respectively, pituitary adenoma and glioblastoma. The results demonstrated that CV possess a powerful antitumoral effect on benign (pituitary adenoma) and malignant (glioblastoma multiforme) tumors with IC₅₀ values of $0.96 \pm 0.11 \mu\text{g/mL}$ and $2.15 \pm 0.2 \mu\text{g/mL}$, respectively. This antitumoral effect is cell-cycle-specific and dependent on extracellular calcium, an important factor for crotoxin phospholipase A₂ activity. The CV antitumoral effect can be ascribed, at least partially, to the polypeptide crotoxin that also induced brain tumor cell death. In spite of the known CV nephrotoxicity and neurotoxicity, acute treatment with its antitumoral dose established *in vitro* was not found to be toxic to the analyzed animals. These results indicate the biotechnological potential of CV as a source of pharmaceutical templates for cancer therapy.

Key words: *Crotalus durissus terrificus* venom, crotoxin, antitumoral effect, pituitary adenoma, glioblastoma multiforme.

INTRODUCTION

According to the World Health Organization (1), cancer, which accounts for 7 million deaths annually, ranks second, behind only cardiovascular disease, among causes of death by disease in most countries. Although brain tumors constitute only 1 to 2% of the tumors in adults, they have a poor prognosis and the patients' chance of survival generally is very low. Moreover, brain tumors represent one of the most common solid tumor in children, being responsible for 20% of childhood neoplasms (2).

Glioblastoma multiforme (GBM) is the most common malignant brain tumor. It can infiltrate diffusely into normal brain regions rendering

total surgical extirpation impossible and effective local radiotherapy difficult and thus requiring other therapy forms (3). Another type of brain tumor is the pituitary adenoma, a benign tumor, which represents about 7% of primary brain tumors (4). Patients with pituitary adenoma can have hypersecretion of growth hormone that may induce the development of acromegaly (5). Although surgery remains the treatment of choice for most patients, only 20 to 50% of patients can achieve control of hormone activity. Currently, gamma knife radiosurgery is the predominant method of treating failed pituitary surgery. However, radiosurgery poses the risk of radiation damage to the visual pathways. Somatostatin analogues are used for acromegaly treatment also,

although lifelong therapy is inconvenient and very expensive (6). For this reason, the development of alternative drugs is relevant in the attempt to improve prognosis and to increase the patients' chance of survival.

Snake venoms are natural sources of bioactive substances with therapeutic potential. The search for new drugs has indicated toxins from *Crotalus durissus terrificus* venom as inhibitors of cell adhesion, cell migration, epidermal tumor growth factor and metastases induced in experimental mice models (7).

Crotoxin (Crtx) is the main polypeptide isolated from *C. durissus terrificus* snake venom (CV) corresponding to 68% of CV total weight (8). Crotoxin (24 kDa) is a β -neurotoxin consisting of a heterodimeric non-toxic non-enzymatic acidic protein (CA) and a basic protein (CB) with phospholipase A₂ activity (9).

Previous reports have shown antitumoral effect of CV against tumor cells such as ovary carcinoma (CHO-K1) cells *in vitro* and Ehrlich tumor cells *in vivo* (10, 11). The antitumoral effect of CV has been mainly ascribed to Crtx.

Some authors have demonstrated that Crtx was cytotoxic to murine and human tumor cell lines *in vitro* including erythroleukemia, glioblastoma (U-87), mammary ductal (Hs 578T), lung (SK-LU-1) and Lewis lung carcinoma and breast (MCF-7) tumor cells (12-15).

It has been shown that apoptosis and autophagy make distinct and differentiable contributions to the Crtx-induced cell death involved in the antitumoral effect (15, 16). Donato *et al.* (17) reported that the Crtx-induced cytotoxicity appears to be highly selective toward cell lines expressing a high density of epidermal growth factor receptors, but the mechanisms of this antitumoral effect are still unknown.

All these literature data strongly corroborate the finding of Plata *et al.* (18) that crotoxin complex is a cytotoxic agent. Although Crtx has been used with some success in the treatment of several patients with squamous skin and breast cancer, to the best of our knowledge, no product derived from CV or its components, such as Crtx, has achieved worldwide application in clinics indicating that further studies should be performed (7, 19).

The aim of this work was to identify the antitumoral effect of *C. durissus terrificus* venom and its polypeptide, crotoxin, on benign and

malignant tumors such as pituitary adenoma and glioblastoma as well as to characterize the mechanisms of this antitumoral effect.

MATERIALS AND METHODS

Reagents

All chemicals used were of analytical grade.

Crotalus Venom and Crotoxin

C. durissus terrificus venom was obtained from the Ezequiel Dias Foundation (FUNED, Brazil) and crotoxin was purified according to Seki *et al.* (20).

Cell Culture

Rat glioma RT2 cells derived from Fischer 344 rats (kindly provided by Dr. William C. Broaddus, Department of Neurosurgery, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA, USA) and GH3 cells (benign pituitary adenoma) obtained from the American Type Culture Collection (ATCC, USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA), supplemented with 10% fetal bovine serum (FBS) (Cultilab, Brazil) and 50 U/mL penicillin, in a humidified atmosphere air/CO₂ (5%/95%) at 37°C.

Cytotoxicity Assay

Cytotoxicity was calculated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay which measures the cellular metabolic viability (21). The cells were cultured in 96-well plates and, 12 hours after the incubation, they were treated with different concentrations of CV (0.1 to 30 μ g/mL). Another group of cells were treated with increasing concentrations (0.5 to 200 μ g/mL) of cisplatin (positive control), a metallic complex commonly used as an antineoplastic in the clinic. After each treatment period, the cells were washed with phosphate buffer saline (PBS), incubated with MTT (0.5 mg/mL), and formazan crystals were solubilized in dimethyl sulfoxide (DMSO). Absorbance was measured in a microplate reader at 570 nm. IC₅₀ values were calculated as the concentration of antitumoral compound evoking 50% cytotoxicity.

Morphological Analysis

The cells were placed on 96-well plates and treated with different concentrations of

CV. Morphological changes were analyzed 48 hours before the treatment by contrast-phase microscopy (Nikon Eclipse TS100â, Japan).

DAPI Staining Assay

DNA alterations characteristic of apoptotic cells were detected by 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) staining. Cells were cultured on 96-well plates (Techno Plastic Products AG, Switzerland) and treated with CV for 48 hours. After the treatment, cells were washed with PBS and fixed with 70% methanol at room temperature for 30 minutes. Cells were incubated in 0.4 µg/mL DAPI (Sigma, USA) for one hour in the dark after washing with PBS. DNA alterations such as condensation and fragmentation were observed by fluorescence microscopy at 385-410 nm (excitation-emission wavelength).

DNA Cell Cycle Analysis

After 48 hours of treatment with different concentrations of CV, cells were washed twice with cold PBS and fixed in ethanol 75% (v/v). Cells were washed once with PBS and resuspended in cold propidium iodide solution (PI – 50 µg/mL) containing RNase A (100 µg/mL) in PBS for 30 minutes in the dark. Flow cytometry analysis was performed on a flow cytometry system (Becton Dickinson, USA). Population of cells containing sub-G1 DNA quantity was calculated as an estimation of the apoptotic cell population.

Calcium and Growth Factor Influence on CV Antitumoral Effect

C. durissus terrificus venom has proteins that secrete phospholipases (PLA₂s), enzymes that are activated by micromolar calcium concentrations (22). In order to verify whether extracellular Ca²⁺ and growth factor from serum play a role in the antitumoral activity of CV, the GH3 and RT2 cells were treated with CV (10 µg/mL) in the absence of Ca²⁺ (PBS + FBS) or in the absence of FBS (DMEM without serum). Twenty-four hours after the treatment, cell survival was measured by the MTT test. The results were compared with the treatment effect at this same CV concentration in complete DMEM containing Ca²⁺ and FBS.

Antitumoral Effect of the Main CV Polypeptide: Crotoxin

The antitumoral effect of increasing crotoxin concentrations (0.1 to 100 µg/mL) was evaluated

by the cytotoxicity assay, morphological analysis and DAPI staining assay.

Evaluation of the Acute Toxicity Produced by CV Antitumoral Dose Established *In Vitro*

The antitumoral CV dose, observed *in vitro*, was calculated in micrograms of CV per square millimeter of treated area from the IC₅₀ of MTT, after 24 hours of treatment (3 µg/mL). Given that 200 µL of drug had been used in each well of a 96-well plate (38.48 mm² area), the concentration 3 µg/mL represents a dose of 0.6 µg of CV per well, equivalent to a final concentration of 0.016 µg/mm².

Once the *in vitro* CV antitumoral dose was calculated, a group of healthy animals were obtained in which a local treatment comprising a fake tumor of 18 mm² was simulated for the toxicological analysis of the therapeutic CV dose. Twenty-four hours after treatment, blood samples were collected for the hematological analysis, and serum was separated for biochemical analysis (determination of blood urea, total proteins, alkaline phosphatase, albumin and creatine kinase). After this, the animals were sacrificed and the organs were taken for morphological analysis. All animal experimentation was performed according to the ethical protocol number 152/2007 approved by the Ethics Committee on Animal Experimentation (CETEA).

Statistical Analysis

Data were expressed as means ± S.D. Statistical significance of differences between means was determined by Student's t-test. Differences were considered significant at $p < 0.05$.

RESULTS

Cytotoxicity Assay

The results showed that CV was cytotoxic in a dose- and time-dependent manner to both glioblastoma and pituitary tumor lineages. However, GH3 cells were about twice as sensitive to the CV cytotoxic effect than were RT2 cells, as indicated by the concentration values that inhibit 50% of cell survival (IC₅₀): 48 hours after treatment, the IC₅₀ was 0.96 ± 0.11 µg/mL for GH3 cells and 2.15 ± 0.20 µg/mL for RT2 cells (Table 1).

The highest CV concentrations used (5 to 30 µg/mL) reduced the survival of GH3 cells significantly

Table 1. Cytotoxic effect of 48-hour treatment with CV and its main polypeptide, crotoxin, on brain tumor cells

Compounds	IC ₅₀	($\mu\text{g}/\text{mL}$)
	GH3	RT2
CV	0.96 \pm 0.11 $\mu\text{g}/\text{mL}$	2.15 \pm 0.20 $\mu\text{g}/\text{mL}$
Crotoxin	> 100.00 $\mu\text{g}/\text{mL}$	> 100.00 $\mu\text{g}/\text{mL}$
Cisplatin	5.01 \pm 0.50 $\mu\text{g}/\text{mL}$	4.96 \pm 0.40 $\mu\text{g}/\text{mL}$

in the first six hours of treatment (± 60 to 75% reduction, $p < 0.01$) (not shown). The highest CV cytotoxic effect on these tumor cells occurred 144 hours after treatment with 1 $\mu\text{g}/\text{mL}$ which was lethal for about 95% of the cells (Figure 1). For RT2 cells the maximum CV cytotoxic effect occurred 144 hours after the treatment with 5 $\mu\text{g}/\text{mL}$, when no cells were found alive (data not shown).

Morphological Analysis

As shown in Figure 2 (A and B), GH3 and RT2 cells treated with CV for 48 hours presented visible morphological alterations, namely: irregularities in cellular shape, cell shrinkage and bleb formation, all characteristics of apoptosis. These results are indicative that the reduction of cell survival after the treatment with CV, observed

in Figure 1 (A and B), occurred through apoptosis induction.

DAPI Staining Assay

Brain tumor cells treated with CV presented nuclear condensation, DNA fragmentation and perinuclear apoptotic body formation, all characteristics of apoptosis (Figure 3 – A and B).

DNA Cell Cycle Analysis

The treatment with 5 $\mu\text{g}/\text{mL}$ of CV (48 hours) evoked significant increase (± 17 -fold increase, $p < 0.01$) in the number of subG1 GH3 cells, which is indicative of apoptosis (Figure 4 – A). In RT2 cells the CV treatment also evoked an increase (± 4 fold increase, $p < 0.01$) of subG1 cells (Figure 4 – B). Cells that did not suffer apoptosis accumulated in G1/G0 (Figure 4 – B), suggesting cell cycle arrest, which prevented their progression in the cycle from the S, G2 and M phases. This arrest was probably due to an attempt to repair the damage evoked by CV treatment. However, this attempt was not successful since all RT2 cells had died 144 hours after the treatment with 3 to 10 $\mu\text{g}/\text{mL}$ of CV (data not shown).

Calcium and Growth Factors Affect CV Antitumoral Activity

The CV antitumoral effect on GH3 and RT2 cells was dependent on the presence of growth factors (FBS) (Figure 5 – A and B). The CV effect was about 45% less potent in the absence of serum than in its presence ($p < 0.05$). This result suggests that the CV action is cell-cycle-dependent (more efficient in cells that are actively proliferating). However, the possibility that CV acted in the quiescent cells (G0 phase) cannot be ruled out, since CV did not lose its antitumoral activity.

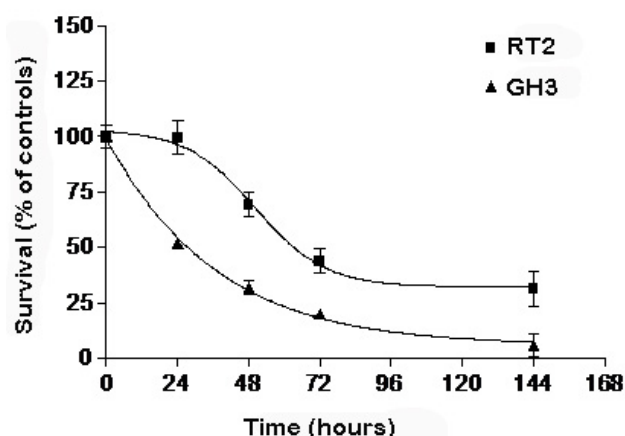


Figure 1. Kinetics of CV antitumoral effect on brain tumor cell lines. Brain tumor cells were treated with CV (1 $\mu\text{g}/\text{mL}$) for 24, 48, 72 and 144 hours. Cell viability was determined by MTT assay. Results are presented as survival ratio compared with the control group (untreated cells). GH3 and RT2 cells were sensitive to CV in a time-dependent manner.

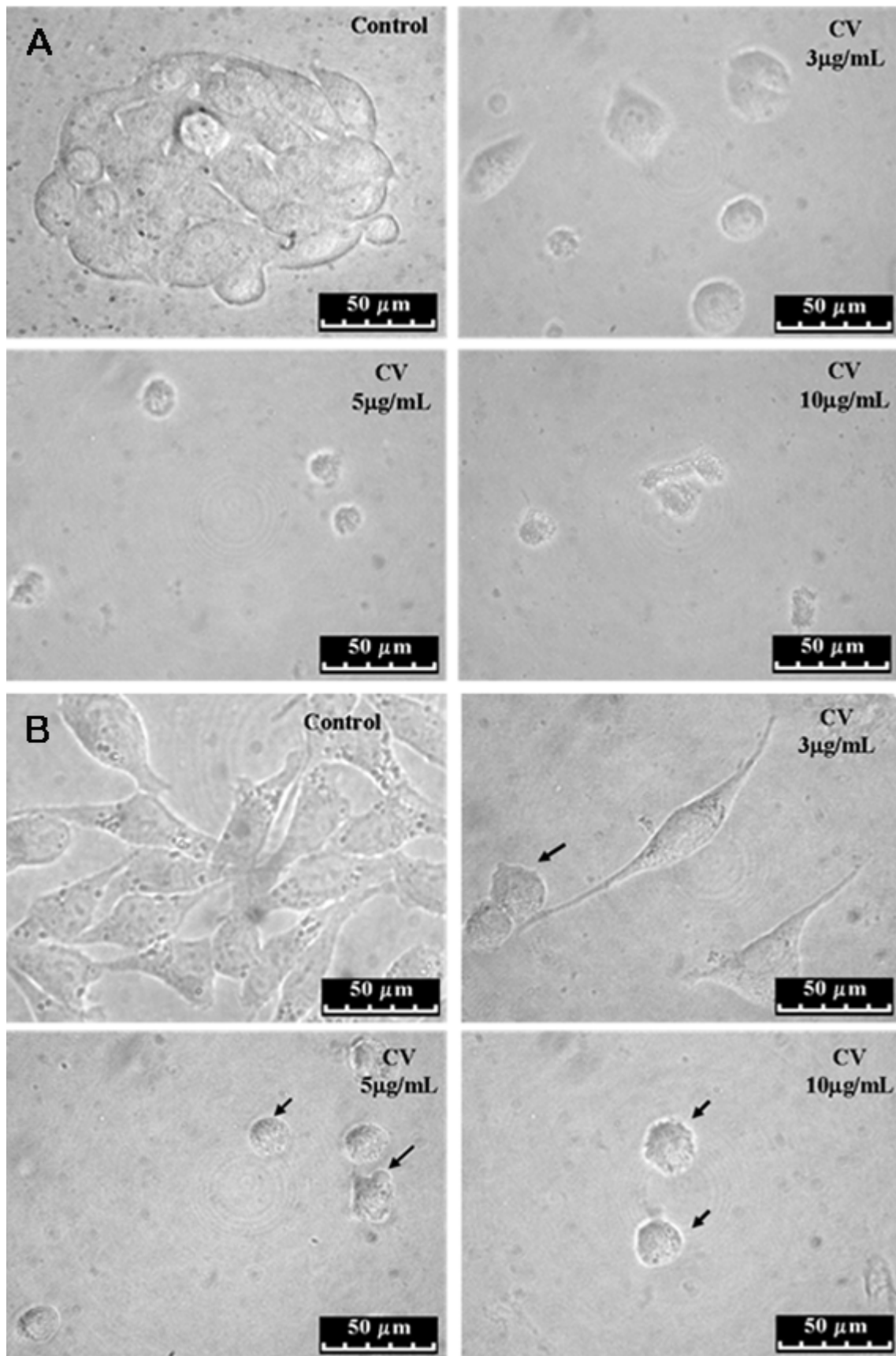


Figure 2. CV induces morphological changes in brain tumor cell lines. Photomicrographs from phase-contrast microscopy: (A) GH3 cells and (B) RT2 cells. Brain tumor cells were treated with different CV concentrations for 48 hours. Cell shrinkage and irregularity in cellular shape (indicated by arrows) were seen in the CV-treated cells.

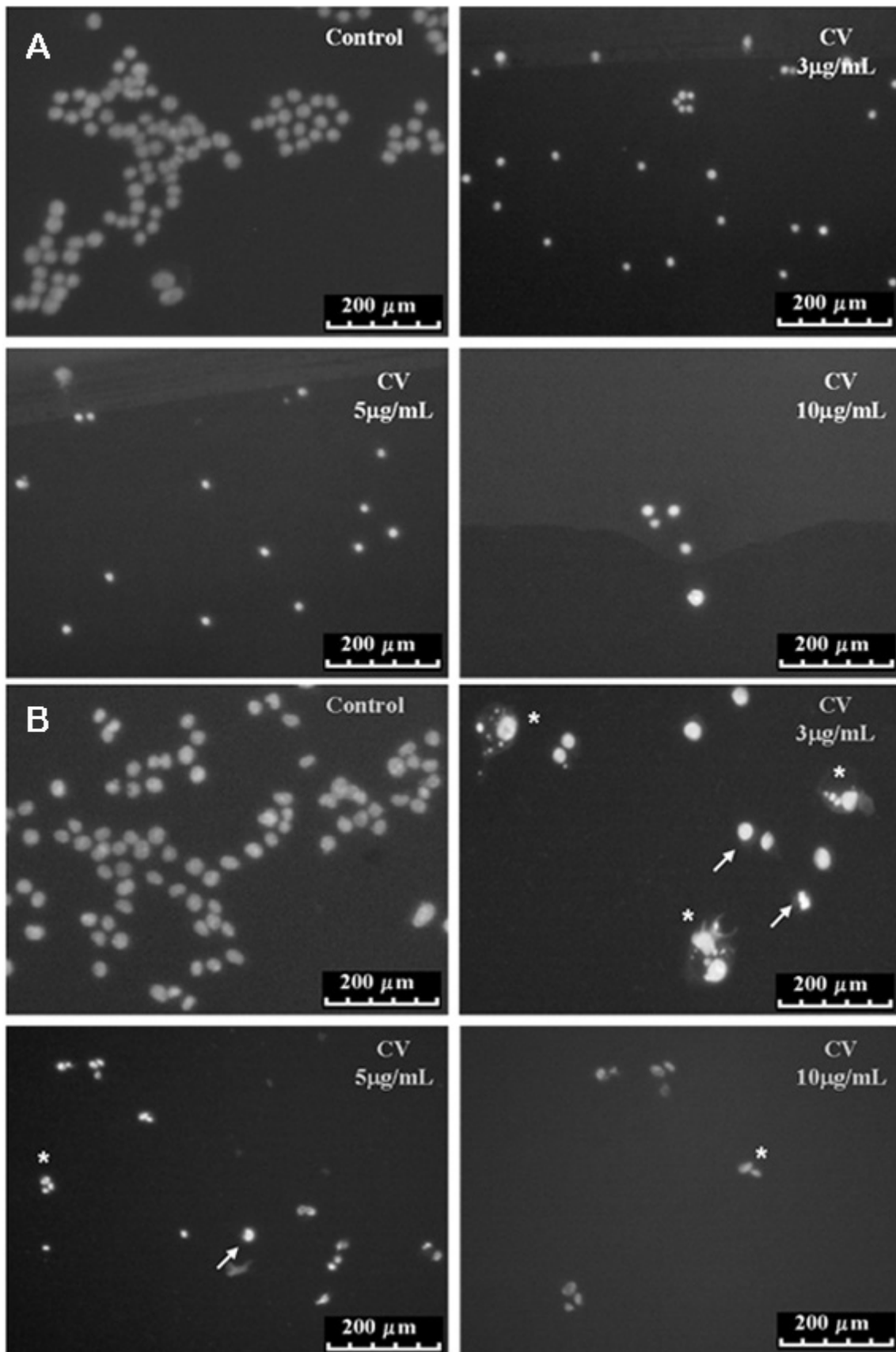


Figure 3. Cell death induced by CV: (A) GH3 cells and (B) RT2 cells. Brain tumor cells were treated with different CV concentrations for 48 hours. After the treatment the cells were stained with DAPI stain. Nuclear condensation (indicated by arrows) and apoptotic body formation (indicated by asterisks) were seen in the CV-treated cells.

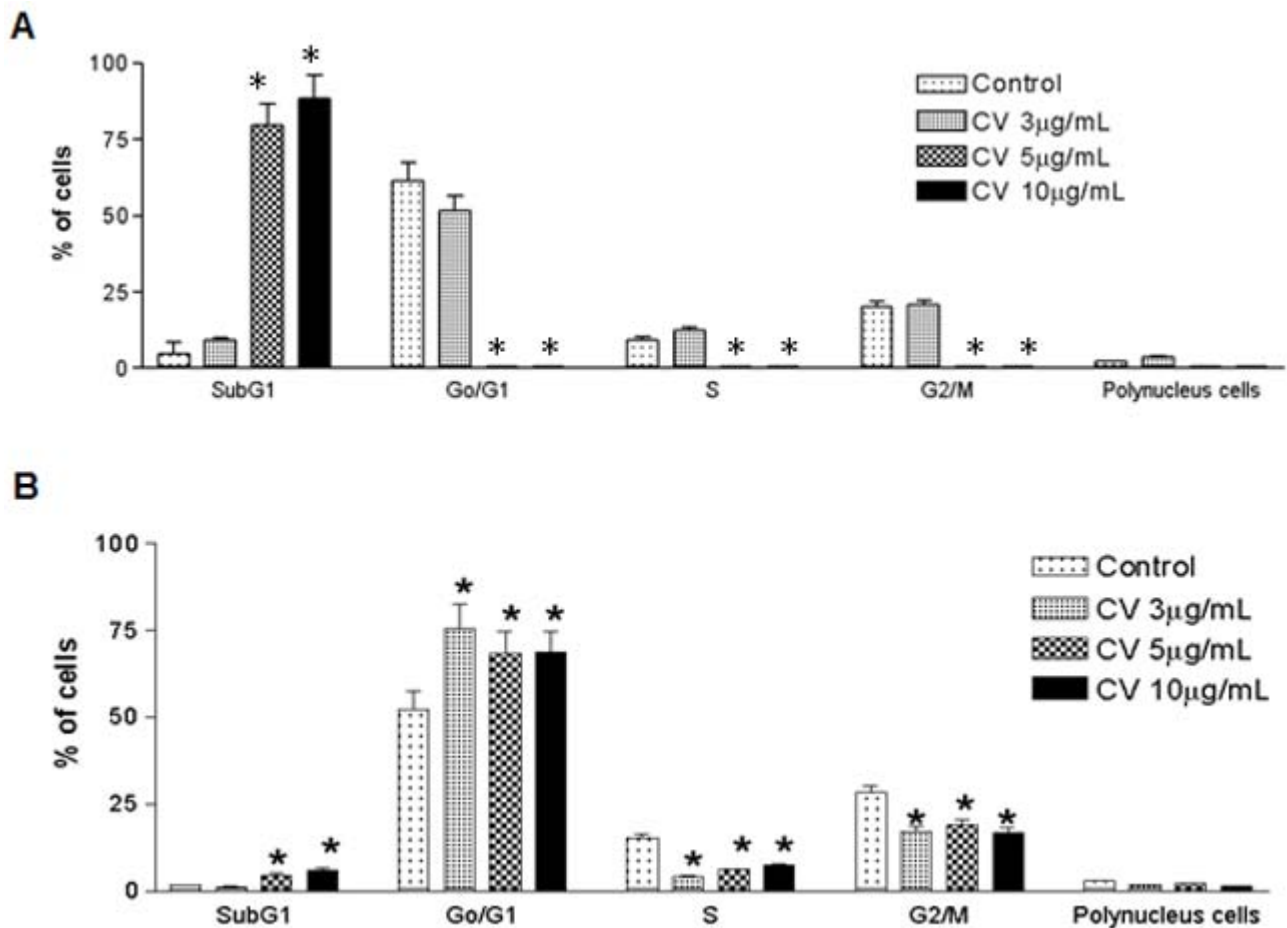


Figure 4. Flow cytometry analysis of DNA content in CV-treated cells: (A) GH3 cells and (B) RT2 cells. Brain tumor cells were treated with different CV concentrations for 48 hours. Cell cycle was analyzed by flow cytometry using propidium iodide. CV induced death and cell cycle arrest in brain tumor cells (* $p < 0.01$).

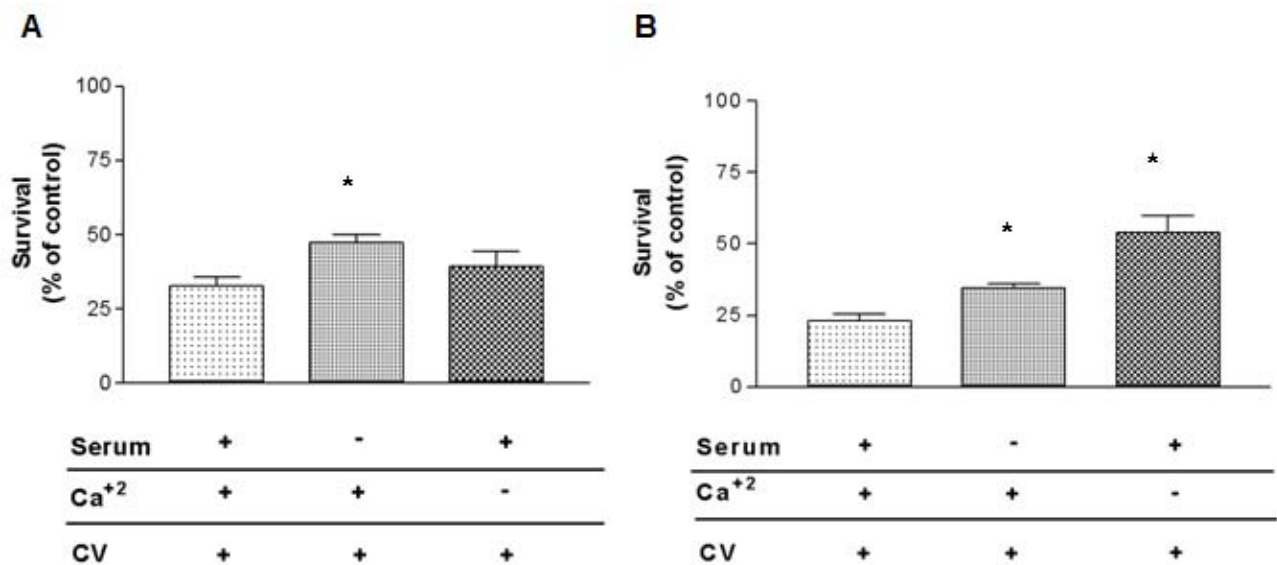


Figure 5. Calcium and growth factors influence on CV antitumoral effect: (A) GH3 cells and (B) RT2 cells. Brain tumor cells were treated with 10 µg/mL of CV for 48 hours in the presence and absence of calcium and FBS. In GH3 cells, the CV antitumoral effect was reduced only in the absence of serum. In RT2 cells, the CV antitumoral activity was reduced as in the absence of either serum or calcium (* $p < 0.01$).

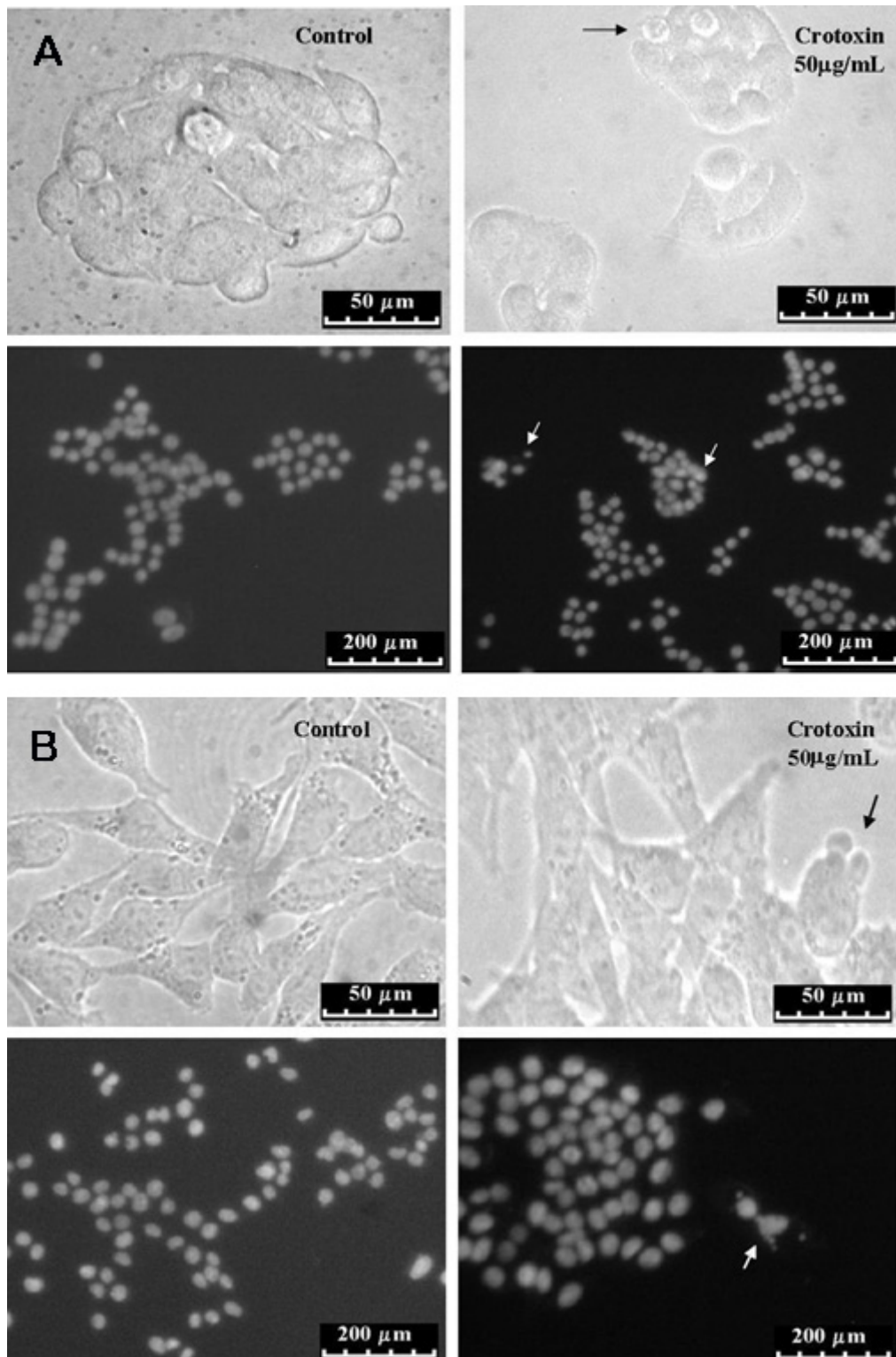


Figure 6. Crotoxin induces morphological changes and death in brain tumor cell lines: (A) GH3 cells and (B) RT2 cells. Brain tumor cells were treated with different crotoxin concentrations for 48 hours. Cell shrinkage, irregularity in cellular shape (indicated by black arrows), nuclear condensation and apoptotic body formation (indicated by white arrows) were seen in the crotoxin-treated cells.

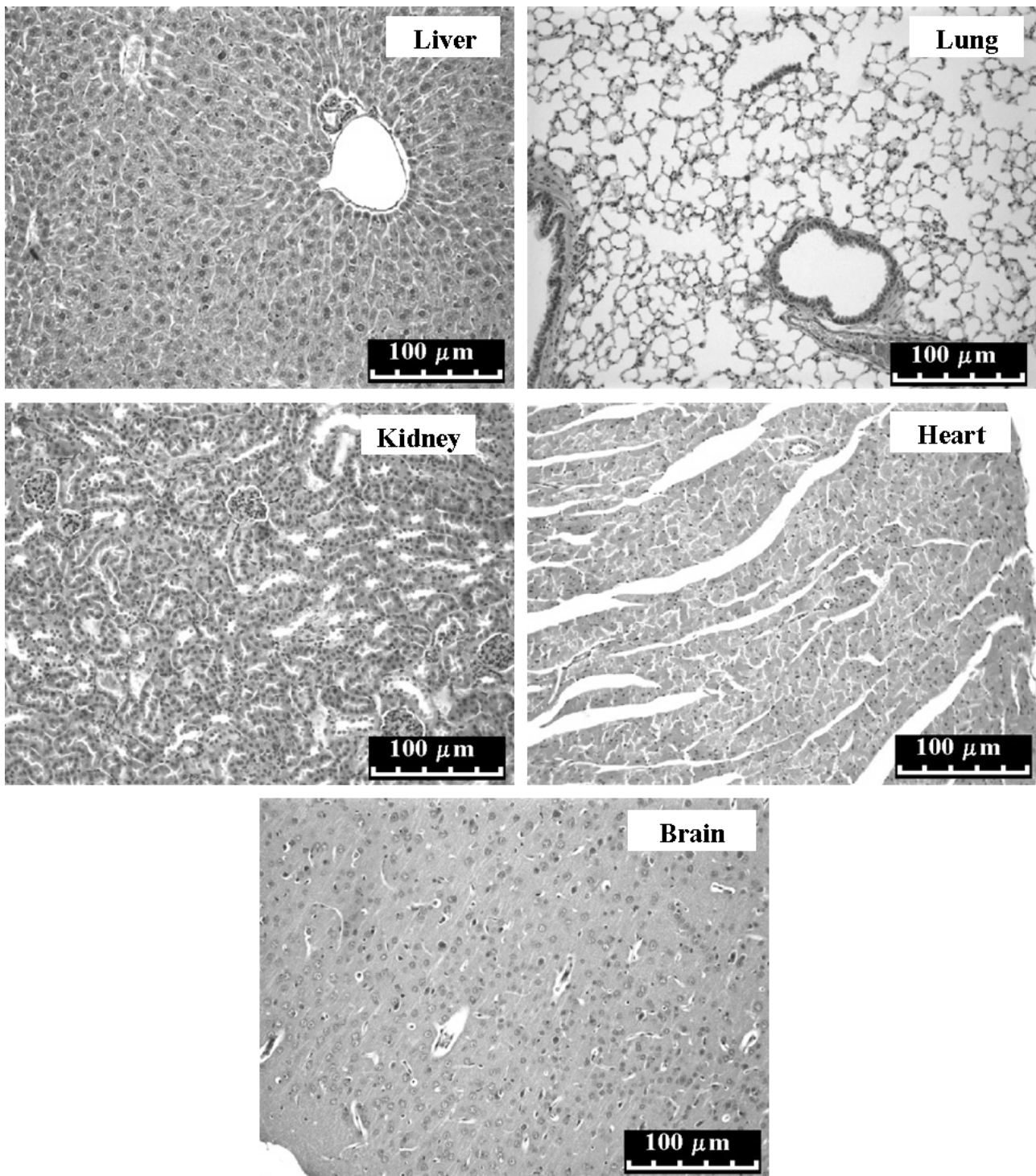


Figure 7. Liver, lung, kidney, heart and brain photomicrographs from a representative animal treated with the antitumoral CV dose established *in vitro*. No apparent morphological alteration was observed in the organs of the CV-treated animals.

Table 2. Biochemical and hematological analysis of mice treated with the therapeutic CV dose established *in vitro*

	Control group	Treated group
Blood urea	60.46 ± 4.02 mg/dL	59.25 ± 6.85 mg/dL
Total proteins	7.18 ± 0.39 mg/dL	7.86 ± 0.27 mg/dL
Alkaline phosphatase	209.10 ± 30.86 U/mL	219.70 ± 40.50 U/mL
Albumin	2.17 ± 0.13 g/dL	2.13 ± 0.19 g/d
Creatine kinase	23.00 ± 2.00 U/L	26.00 ± 2.00 U/L
Erythrocytes	100.00 ± 10.00%	93.80 ± 9.30%
Lymphocytes	67.70 ± 3.06%	69.60 ± 14.00%
Monocytes	3.62 ± 5.58%	5.40 ± 5.60%
Granulocytes	28.44 ± 6.29%	24.69 ± 10.6%

Unlike GH3 cells, the CV antitumoral effect was partially calcium-dependent in RT2 cells. The effect of CV on these cells was about 135% lower in the absence of calcium than in its presence ($p < 0.05$).

Antitumoral Effect of the Main CV Peptide: Crotoxin

Crotoxin was cytotoxic to both GH3 and RT2 cells. After treatment with crotoxin, GH3 and RT2 presented irregularities in cellular shape, cell shrinkage, nuclear condensation and DNA fragmentation (Figure 6). These morphological alterations observed in crotoxin-treated cells indicate that, as in the case of CV, this polypeptide induces cell death in brain tumors. However, treatment with the maximum concentration of 100 µg/mL was cytotoxic only to 14 ± 0.7% of GH3 and 11 ± 0.57% of RT2 cells ($IC_{50} > 100$ µg/mL).

Evaluation of the Acute Toxicity Produced by Antitumoral CV Dose Established *In Vitro*

The results showed that the blood cell number in treated animals remained equivalent to the one in controls. Moreover, plasmatic concentrations of blood urea, total proteins, alkaline phosphatase, albumin and creatine kinase were not significantly modified by CV treatment (Table 2) and no apparent histopathological alteration was found in the organs of treated animals (Figure 7).

DISCUSSION

An efficient antitumoral drug must be cytotoxic to the tumor cells without causing extreme damage to normal tissue (23). The results obtained in this work showed that CV was cytotoxic to two brain tumors lineages: a benign tumor of pituitary adenoma (GH3) and a malignant glioblastoma multiforme (RT2). Moreover, the CV cytotoxic effect was significantly higher than the effect evoked by cisplatin, a drug used clinically for tumor treatment (CV: $IC_{50} = 0.96 \pm 0.11$ µg/mL against GH3 and 2.15 ± 0.20 µg/mL against RT2, cisplatin: $IC_{50} = 5.01 \pm 0.50$ µg/mL against GH3 and 4.96 ± 0.40 µg/mL against RT2).

The CV-treated cells presented morphologic alterations including irregularities in cellular shape, cell shrinkage, nuclear condensation, DNA fragmentation and perinuclear apoptotic body formation, all characteristics of apoptosis. The analysis of the cell cycle also suggests that the treatment with CV evoked an increase of the apoptotic cells; moreover, this treatment induced a cell cycle arrest of RT2 cells in the G0/G1 phase. This fact is indicative that the sensory proteins (as ATM, ATR and DNA-PK) recognized the damage provoked by CV and tried to repair it. This repair attempt, however, seems not to be successful since, as demonstrated by the MTT test, all RT2 cells died 144 hours after CV treatment.

Apoptosis of tumor cells induced by CV also was recently demonstrated by Tamietti *et al.* (10)

in ovarian carcinoma cells (CHO-K1). These authors suggested that CV induced apoptosis in CHO-K1 cells through the mitochondrial pathway associated with the death promoter Bcl-2 (Bad). CHO-K1 cells possess the mutant P53 gene and, for this reason, do not possess the G1 checkpoint in the cell cycle (24). Probably, the Bad activation, in CHO-K1 cells treated with CV, was mediated by p53 protein in G2 phase. The glioblastoma multiforme cells used in our experiments (RT2) possess wild type p53, which enables the DNA damage repair at G1/G2 checkpoints (25-27). We observed that the treatment of these p53 wild type cells with CV induced cell damage that activated cell cycle arrest in the G1 phase and induced apoptosis.

Taken together, the literature and the present data indicate that CV is capable of inducing apoptosis after cell cycle arrest in the G1 and G2 phases in the cell lines investigated in this work. Despite this, the induction of autophagy cannot be ruled out when using breast cancer cells as described by Yan *et al.* (15).

Growth factors are essential for cell proliferation. In the absence of these factors, cells in G1 leave the cell cycle and stay in G0 (quiescent phase), where cell metabolism is reduced and cell growth is absent. The quiescent cells can return to the cycle since there is growth stimulation (28). Antineoplastic agents can be cell-cycle-specific, more efficient in cells that are proliferating actively, or cell-cycle-unspecific, that is, possess effectiveness independent of the cell proliferation rate. Our results indicate that CV is cell-cycle-specific.

In the absence of growth factors (FBS), the CV cytotoxicity was about 45% lower than when they were present ($p < 0.01$). However, the possibility of CV acting in the G0 phase cannot be ruled out since, although less powerful, CV was cytotoxic to tumor cells in serum's absence. In addition to its dependence on growth factors, the CV cytotoxic effect was also dependent on the extracellular Ca^{+2} in RT2 cells. The previously described importance of Ca^{+2} in triggering apoptosis signals and the presence of calcium channels in GH3 and RT2 cells were also corroborated in the present work (29-31). The reduced CV cytotoxic effect in the absence of extracellular calcium probably occurred because the bioactive components, whose activity is calcium-dependent (such as phospholipases and metalloproteases), were not activated.

The main CV polypeptide, crotoxin, also

induced cell death of brain tumor cells as demonstrated by the morphological alterations and DNA degradation. CV (10 $\mu\text{g/mL}$) was cytotoxic to all GH3 and RT2 cells, 144 hours after treatment. This CV concentration contains approximately 6.8 $\mu\text{g/mL}$ of Crtx (8). Some authors demonstrated that Crtx was cytotoxic to a variety of murine and human tumor cell lines *in vitro* including erythroleukemia, lung (SK-LU-1), mammary ductal (Hs 578T), glioblastoma (U-87 MG) and breast (MCF7) tumor cells (13, 15-17). In the present work, at the maximum concentration used, 100 $\mu\text{g/mL}$, Crtx caused only mild cytotoxic effect to the brain tumor cells, suggesting that Crtx probably is not the main antitumoral component of CV, at least in relation to these cell lines. Snake venoms are constituted by a mixture of biologically active substances, each bioactive component of which can exert its activity separately or with other components (32, 33). Crotoxin, for example, can activate proteases and phospholipases A_2 , indicating that it is capable of intensifying crotoxin activity (34). Studies using other CV polypeptides are necessary to identify whether they have some component more effective than Crtx in CV antitumoral action.

The identification and characterization of the antitumoral effect of CV indicated its therapeutic potential. In the development of new pharmaceuticals, toxicological studies are essential to evaluate possible adverse effects. The toxicological studies demonstrated that the administration of CV antitumoral dose, established *in vitro* (0.016 $\mu\text{g/mm}^2$ of tumoral mass), did not evoke acute toxic effects; no histopathological or functional alteration was observed in treated animals. Brigatte (35) also demonstrated that a five-day crotoxin treatment (18 μg) inhibited the Walker 256 tumor (mammary carcinosarcoma) growth with no histopathological alterations in the analyzed animals. These results are encouraging and support the safe use of some CV components as a platform for the development of therapeutic antitumor drugs. Novais *et al.* (36) has successfully labeled Crtx with $^{99\text{m}}\text{Tc}$, a radioisotope commonly used in diagnostic imaging, and the labeled molecule was proven useful for biodistribution and imaging studies. It remains to be demonstrated whether compounds derived from CV can also be useful as modeling tools for diagnostic imaging.

CONCLUSIONS

In this work, it was possible to identify and characterize the CV antitumoral effect on benign and malignant tumors. This antitumoral effect is probably mediated by apoptosis induction, is cell-cycle-specific and dependent on extracellular calcium, an important factor for venom components including metalloproteases and phospholipases A₂. The CV antitumoral effect can be ascribed, at least partially, to the polypeptide crotoxin, which also induced brain tumor cell death. Moreover, the CV antitumoral dose established *in vitro* evoked no significant toxicity *in vivo*. These results strongly indicate the use of CV components as template sources to develop pharmaceuticals for low-toxicity tumor therapy.

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CONFLICTS OF INTEREST

There is no conflict.

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ETHICS COMMITTEE APPROVAL

The present study was approved by the Ethics Committee on Animal Experimentation (CETEA) of the Federal University of Minas Gerais.

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