VIABILITY OF VERO E6 CELLS EXPOSED TO THE *Bothrops leucurus* SNAKE CRUDE VENOM

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ABSTRACT

Primary and continuous cell culture systems are a good *in vitro* alternative for the quantitative assessment of snake venom toxicity. There are still few studies evaluating the cellular response against *Bothrops leucurus* venom (BlV). This study aimed to evaluate the cytotoxic effect of crude BlV on culture of VERO E6 cells. Neutral Red incorporation (NR) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) tests were performed for viable cells, and Trypan Blue (TB) dye exclusion test was performed for non-viable cells. The differences between the groups were evaluated by ANOVA-One-Way and Dunnet through the program Graph Pad 5.0 with significance of 5%. The 50% cytotoxic concentration (CC_{50}) of BlV was 7.86 µg/mL. There was a trend of dose-dependent reduction on cell viability in all assays evaluated. However, MTT and NR tests were more sensitive for the evaluation of BlV cytotoxicity on the VERO E6 cell line.

Keywords: CC_{50}. Cell culture. Whitetail lancehead.

INTRODUCTION

According to data from the Convention on Biological Diversity (CBD), Brazil hosts between 15 and 20% of all world biodiversity, being the country with the largest number of endemic species (BARREIRO; BOLZANI, 2009). Regarding ophidian fauna, Brazil has representatives of nine families, 17 genera and 265 species of snakes, in which only two families, *Viperidae* and *Elapidae*, are considered true venomous and responsible for 20,000 accidents per year in the country (BRASIL, 2001).

Bothrops and *Bothropoides*, commonly known as jararacas, are members of the *Viperidae* family, and account for 70% of all snakebites in Brazil (ALBUQUERQUE et al., 2013; CHIPPAUX, 2015). These snakes have crepuscular or nocturnal habits, and aggressive behavior. *Bothrops leucurus* Wagler, 1824, popularly known as whitetail lancehead, is a venomous snake of medical importance, endemic to the Rainforest biome, which inhabits the Northeast region of Brazil, being common in forested areas. However, whitetail lancehead adapts well to domiciliary environments, consequently increasing its geographic distribution and invasion of different biota, probably due to the effects of deforestation and its great ecological plasticity (DA SILVA et al., 2014; LIRA-DA-SILVA, 2009).

Some studies have shown that the biological effects of *B. leucurus* envenomation have a similar profile to those presented by other snakes of the genus *Bothrops*, such as edema, tissue necrosis, coagulant activity, hemorrhagic activity, and renal failure *in vivo* (AKEF, 2018; NAUMANN et al., 2011). *B. leucurus* is characterized by producing venom of variable toxicity, but the venom composition reveals less presence of compounds responsible for the toxic effects when compared to the other species of South America (LIRA-DA-SILVA, 2009).

The protein fraction of the *B. leucurus* venom (BlV) consists mostly of serine proteinases, phospholipases A2 (PLA2), metalloproteinases, L-amino acid oxidases (LAAOs), cysteine-rich secretory proteins (CRISP), C-type lectins and pharmacologically active compounds that have possible effects on the homeostatic system, besides cytotoxic, antimicrobial and inflammatory action (WILSON, 2013).

There are actually variations within the venom of the same species of snakes, which can be evidenced by the differences of molecular masses and proteolytic activity of the purified

fractions, probably caused by different levels of glycosylation of these enzymes (WILSON, 2013). All the components of *B. leucurus* venom present great variability within the *Viperidae* family, both inter and intraspecific (QUEIROZ et al., 2008), due to the age, sex, seasonality, geographic variation (CHIPPAUX et al., 1991) and animal feeding (BARLOW et al., 2009).

Reassessment of the use of animals in experiments is world trend and aims to develop and validate new alternative methods. All these actions resulted in an internationally recognized program 3Rs (Reduction, Refinement, Replacement), which aims to reduce the number of animals used in the research, to minimize pain and discomfort, in addition to seek alternatives for replacing *in vivo* tests (CAZARIN et al., 2004).

Since the 1960s, several studies have been carried out searching for methodologies that use mammalian tissues and cells as an alternative form of research. The *in vitro* experimental methods demonstrate, among the advantages in relation to the *in vivo* tests, the limitation of the number of experimental variables and the easy and fast obtaining of significant data (ROGERO et al., 2003).

Toxicity tests are among the first *in vitro* bioassays used to check the action of toxic substances on a particular tissue by assessing the number of viable surviving cells. There are several methods and models of cytotoxicity testing. Its use depends on the category of cytotoxicity to be evaluated and the substance in question. Thus, cell viability analyzes are shown as tools that can qualify or quantify cells that are metabolically active in a culture (WHITE; MEIER, 2017).

There are still few studies available that evaluate the cellular response against animal toxins, especially those involving the *B. leucurus*, due to their restricted geographic distribution. The biological mechanisms of *Bothrops* snake venom, although widely studied, demonstrate divergent aspects in some unexplored places, especially with the endemic species of the country. For this reason, regionalized studies of the effects of BlV are important to understand their action on the affected cells. The present study investigated the cytotoxic effect of BlV on cultures of VERO E6 cells, with a view to its use in animal venoms cytotoxicity protocols.

MATERIAL AND METHODS

Bothrops leucurus venom (BlV)

A pool of *B. leucurus* adult snake venoms kept in the scientific breeding ground of the Laboratory of Venomous Animals and Herpetology of the State University of Feira de Santana (UEFS), Bahia - Brazil was used. BlV was sterilized, lyophilized and stored at -20 ^{o}C until use.

Cell culture

African green monkey kidney cells (VERO) of the E6 lineage (ATCC® CRL-1586) were seeded in 96-well plates in triplicate at a concentration of 1.8 x 10^5 cells/well in Dulbecco's Modified Eagle Medium (DMEM- Thermo Fischer Scientific) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin (200 µg/mL) and streptomycin (200 µg/mL). The plates were incubated at 37 ^{o}C with 5% CO_2 and controlled humidity. For the detachment of the monolayers, a solution of 0.25% Trypsin and 0.02% EDTA was used. Treated cells were observed under an inverted microscope (Olympus CK40).

Neutral Red (NR) Test

For each assay, VERO E6 cells (1.8 x 10^5 cells/mL) were seeded in seven replicates and maintained in DMEM medium for 24 h at 37 ^{o}C and 5% CO_2. After this time, the medium was changed, and 20 µL of BlV was added at different concentrations (50 µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL) for 24 and 48 h. After incubation and removal of the supernatant, cell viability was determined using the dye-uptake technique with modifications. Two hundred microliters of 0.01% NR solution (Sigma, St. Louis, Missouri, USA) diluted in DMEM medium was added and incubated for 3 h. After removing the supernatant, the cells were washed in saline solution, and 200 µL of formaldehyde solution (0.5%) in $CaCl_2$ (1%) were added for 5 min. 200 µL of acid alcohol solution (1% acetic acid in 50% ethanol) were added, under stirring for 10 min, and analyzed in a spectrophotometer at 540 nm. To obtain the cytotoxic concentration of 50% of the cell cultures (CC_{50}), the Reed–Muench method was used.

Trypan Blue (TB) dye exclusion test

Two assays were performed; in the first the cells were incubated with 50 µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL of BlV for 24 and 48 h. In the second assay, with 24 h of incubation, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL and 0.125 mg/mL of BlV was used. Saline solution was used as negative control (0.9%). Assays were performed in eight replicates and the visualization of the 96 well plate occurred after the incubation times of each assay. After these periods, 10 µL of TB dye (4%) (Thermo Fisher Scientific) were added to 10 µL of the cell suspension. For the viability analysis, quantification of cell death was performed in a Neubauer chamber, using inverted light microscopy (Leica – DM-IL-LED).

3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl-tetrazolium bromide (MTT) Test

The cellular viability of the BlV treated cultures was evaluated by the MTT colorimetric assay performed, with 1×10^5 cells/well seeded in triplicate in a 96-well plate for adhesion. After 24 h, the cells were incubated with 200 µL of DMEM medium without FBS, with saline (PBS 0.9%) (control) or with different concentrations of BlV (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL and 3.125 µg/mL). After 24 h, the cells were incubated with MTT (Termo Fischer Scientific) (5 mg/mL, 20 µL/well) for 3 h and the formazan crystals were dissolved in 100 µL of saline solution (PBS) containing SDS (10%) and HCl (0.01 N). After 18 hours, the optical density was determined at 540 nm on a plate reader (Multiskan GO Thermo Scientific, USA).

Statistical analysis

Cell viability percentages were calculated in relation to controls using the formula: [(Optical Density (O.D.) of treated cells / median O.D. of control) x 100]. The CC_{50} value was estimated by linear regression analysis of concentration–response curve. The results were presented as mean ± standard deviation, independently analyzed, and the differences between the groups were evaluated by ANOVA-One-Way and Dunnet, using the Graph Pad 5.0 program with significance of 5%.

RESULTS AND DISCUSSION

The effect of BlV on the loss of cell morphology can be seen in Figure 1B, which shows rounded cells and reduced confluence when compared to control (Figure 1A). Figure 1 also shows that in the highest concentration of BlV incubated for 24 h (1 mg/mL), the detachment of the VERO E6 cell monolayer occurred, making it impossible to count individual cells. This same effect was observed in a study with endothelial cells exposed to BlV crude and Leucurolysin-a (Leuc-a) (GREMSKI et al., 2007). This result was also verified with *Elapidae* venom, in a study that used the TB test to evaluate the extent of cell death.

In Bustillo et al.'s (2009) study, it was verified that after incubation of C2C12 murine cells with 10 µg/mL of *B. alternatus* venom (BaV), several morphological alterations occurred, as reduction of nuclear sizes, chromatin condensation, cell rounding, detachment cells, cell shrinkage and the formation of blebs on cell surface.

Venoms contain many toxic components and can target different cell types, resulting in a multiplicity of pathological lesions. The detachment activity of the monolayer can be explained by the presence of desintegrins derived from snake venom, whose effect would be to block the integrins, membrane surface proteins responsible for cell differentiation, proliferation and activation (DA SILVA et al., 2009; ROJNUCKARIN, 2008).

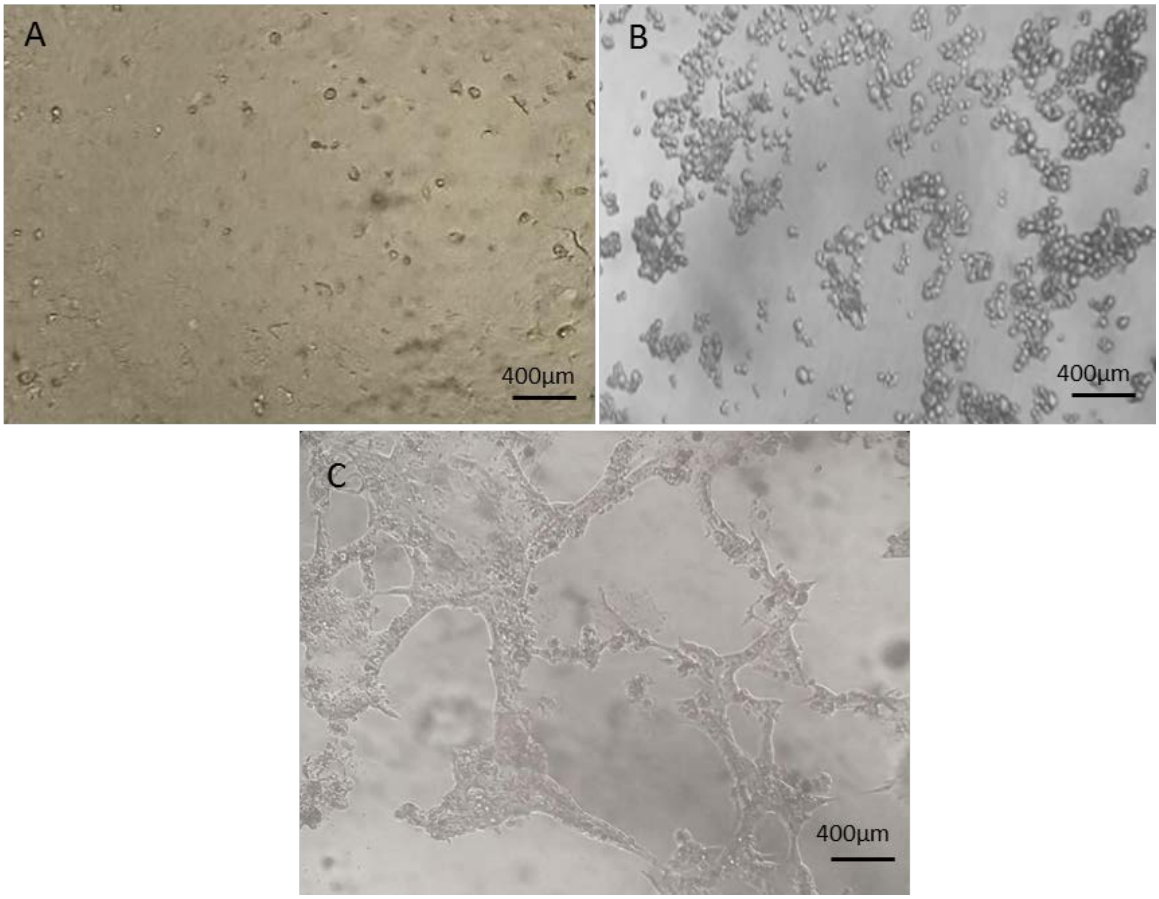

Figure 1 - Micrographs. A - VERO E6 cells from the control group, without BlV. B - VERO E6 cells morphologically altered (50 µg/mL BlV). C - detachment of the VERO E6 cell monolayer (1 mg/mL BlV), incubated for 24 h.

The NR test is based on the ability of viable cells to incorporate and capture this vital dye at the level of lysosomes. Changes in the lysosomal membrane result in a reduction of NR incorporation, thus distinguishing between viable (incorporate NR) and nonviable cells (do not incorporate NR) (REPETTO et al., 2008).

In the NR test, dose-dependent cell death was induced by BlV, relative to the untreated control (Figure 2). The CC_{50} obtained was 7.86 µg/mL, in the assay using BlV concentration of 6-50 µg/mL after incubation for 48 h. No cytotoxic effect was observed at these concentrations after incubation for 24 h. The cultivation of LLC-MK2 and HK-2 cells with *B. alternatus* venom (BaltV) decreased cell viability at high concentrations (50, 100 and 200 µg/mL). Differently, in LCC-MK2 the cell viability decreased, at low concentrations (0.19,

0.39, 0.78, 1.56, 3.12, 6.25, 12.5 and 25 µg/mL). Therefore, an increase of cell viability was observed, with an IC50 of 221.3 µg/mL for LCC -MK2 (NOGUEIRA JÚNIOR, 2017).

An assay using endothelial cells incubated with BlV, found that there was no reduction in cell viability (NAUMANN et al., 2011). However, evaluating the cytotoxicity of the cell lines (MGSO3, VERO and HeLa), against venom of Peruvian *Bothrops* snakes, it was observed that all the venoms were able to reduce viability cell culture in all strains, and Bl-LAAO (L-amino acid oxidase of *B. leucurus*) is responsible for this cytotoxic activity (GUERRA-DUARTE et al., 2015). NR test also presents some problems in the evaluation of the cytotoxicity, considering that the cytotoxicity is obtained from the decrease in neutral red uptake by cells remaining in the monolayer, disregarding the cells of the supernatant during the washing step. This procedure may be misleading, since the cells that are detached from the substrate, however viable, would be withdrawn and therefore the absorption of neutral red would be reduced, without this reduction necessarily reflecting cytotoxicity (GUTIÉRREZ; RUCAVADO, 2000).

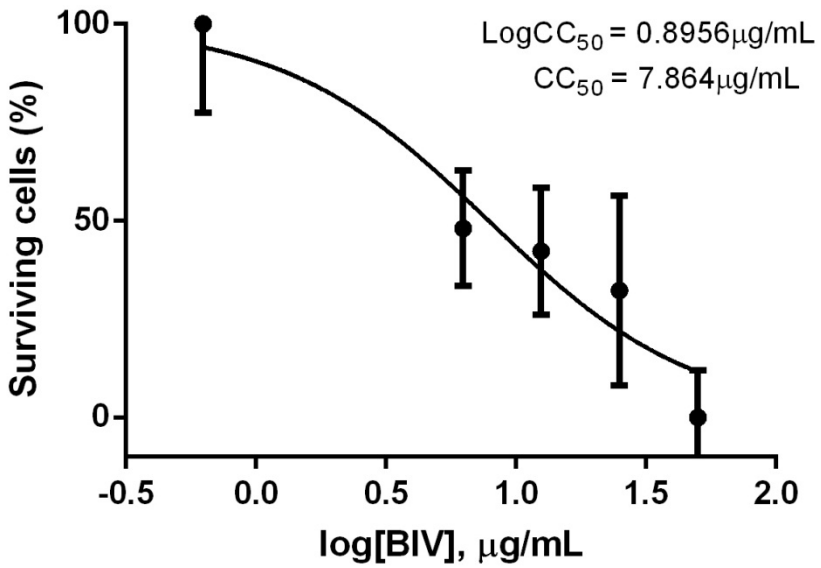

Figure 2 - Determination of cytotoxic concentration 50 (CC_{50}=7.86) and decrease in cell viability of VERO E6 cells for 48 h (B) after the inoculation with crude BlV venom (6.5 - 50 µg/mL); exposure assessed by dye-uptake assay of the neutral red dye. The mean of the control group, treated with DMEM medium alone, was taken as 100% of dye uptake value. Data (mean + SD) are representative of independent assays performed in seven replicates. For better viewing, the concentration data have been transformed into log10.

The dose-response curve obtained (Figure 2), reveals similarity with a study that used the quantitative survival method when using NR to detect cytotoxicity. After exposure of the cells to *Bothrops* venoms, it was observed that the amount of incorporated NR was directly proportional to the number of viable cells in the culture (OLIVEIRA et al., 2002). However, when the CC_{50} = 7.86 µg/mL, determined in VERO E6 cells, was compared with those found in closely *Bothrops* venoms (GUERRA-DUARTE et al., 2015), the BlV showed to be more toxic than the *B. atrox* (12.2 µg/mL), *B. neuwiedi* (27.14 µg/mL) and *B. jararaca* (18.78 µg/mL) venoms in this cell lineage.

In a study evaluating the venom of seven South American *Bothrops* species, BlV CC_{50} (4.95 µg/mL) showed larger cytotoxicity and closer to the *B. neuwieddi mattogrossensis* venom (4.74 µg/mL) on VERO cell line (OLIVEIRA et al., 2002). In a study of exposure of human glioma cells (U251 and U87) to BlV, CC_{50} of 3.34 and 30.9, respectively, were observed. Variations in CC_{50} can also be observed with proteins isolated from BlV. In a study performed with stomach cancer cells (MKN-45) and colorectal cancer (RKO), the BlV presented CC_{50} of 0.41 and 0.04 µM respectively (NAUMANN et al., 2011).

BlV exhibited cytotoxic activity on tumor cells and induced apoptosis in K562 cells, but BIL fails to induce apoptosis in cultured human nontransformed cells (NUNES et al., 2012). In a study of the Bl-LAAO fraction, an increase in reactive oxygen species was observed in MDCK cells, resulting in an important role in the induction of apoptosis in renal cells (MORAIS, 2015). The Bl-LAAO is a cytotoxin, the main component of the venom that would act primarily in the generation of high amounts of H_2O_2 (NAUMANN et al., 2011).

The study using a murine skeletal muscle cell line revealed that an apoptotic mechanism mediates the cellular destruction caused by *Bothrops* venoms (BUSTILLO et al., 2009). However, studies conducted with the galactose-binding lectin (BlL) have demonstrated that this component does not induce apoptosis in untransformed human cells, exhibiting selective cell death on B16-F10 melanoma cells by increasing the free $[Ca^{2+}]_{cyt}$ concentration and mitochondrial superoxide generation, resulting in the opening of mitochondrial permeability transition (MPT) pore (ARANDA-SOUZA et al., 2014), and cytotoxic and apoptotic activities on K562, NCI-H292 and Hep-2 cells (NUNES et al., 2012). In a study using

BlV on MDCK cells, cell death was induced predominantly by necrosis, with presence of pycnotic nuclei, increase in $[Ca^{2+}]_{cyt}$, and decrease of membrane potential in mitochondria (MORAIS, 2011).

During the cytotoxicity tests using Trypan Blue (TB) (Table 1), at low concentrations of BlV (25 µg/mL, 12.5 µg/mL, 6.25 µg/mL), dead cells were observed only after incubation for 48 hours, in concentrations equal or above 12.5 µg/mL. The tendency to increase cytotoxicity by increasing incubation time was observed with HepG2 cells against *Naja naja oxiana* venom (EBRAHIM et al., 2016).

Table 1 - Assays of viability of VERO E6 cells exposed to different concentrations of BlV using the Trypan Blue (TB) dye exclusion test. In assay 1, low BlV concentrations (6.25 - 25 µg/mL) were used with incubation times of 24 h and 48 h. In assay 2 larger BlV concentrations (0.125 – 1.0 mg/mL) were used with incubation time of 24 h.

Assay	Venom concentration (µg/mL)	Incubation (h)	Death cells/mL (Mean ± SD)	Viability % (Mean ± SD)
1	6.25	24	-	100 ± 1.067
	12.5	24	-	100 ± 1.091
	25.0	24	-	100 ± 2.123
	6.25	48	$1.0 \times 10^8 \pm 0.135$	96 ± 2.034
	12.5	48	$2.25 \times 10^8 \pm 0.088$	84 ± 1.292
	25.0	48	$4.25 \times 10^8 \pm 0.092$*	41 ± 1.374*
2	125.0	24	$1.25 \times 10^8 \pm 0.022$	96 ± 2.036
	250.0	24	$2.0 \times 10^8 \pm 0.043$	91 ± 2.010
	500.0	24	$3.0 \times 10^8 \pm 0.091$	89 ± 1.098
	1000.0	24	$5.25 \times 10^8 \pm 0.097$*	36 ± 1.497*
	Negative control	24	-	100 ± 2.106

SD = standard deviation. * = Statistically significant values (ANOVA / Dunnet, $p<0.05$).

Our study found that, regardless of the technique chosen, incubation time would be an important factor in the cytotoxicity evaluation on VERO E6 cells. However, in a comparative study of two methods of assessing the viability of mammalian cells, it was found that fewer nonviable cells were detected using TB test, and as the culture aged, the TB test significantly overestimated viable cells and underestimating nonviable cells, resulting in an incorrect estimate (ALTMAN et al., 1993). Although the TB test result shows a trend of change in cell viability in a dose-dependent manner (Table 1), a trend appears to exist when smaller concentrations are used with longer incubation times, or large concentrations with shorter

incubation time. Thus, the TB test does not seem to us a good method to evaluate the cytotoxicity of snake venoms.

Since, for most cell populations, total mitochondrial activity is related to the number of viable cells, the MTT test is based on the conversion of MTT to formazan crystals by mitochondrial reductases of metabolically active (living) cells, yielding intracellular blue crystals. Figure 3 shows that BlV reduced the cell viability of the VERO E6 lineage in a dose-dependent manner.

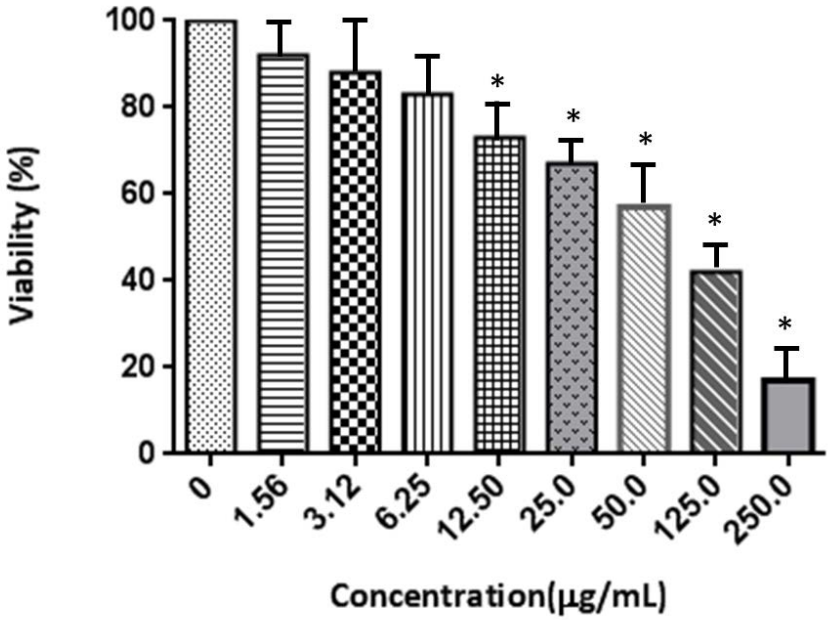

Figure 3 - Analysis of crude BlV cytotoxicity on VERO E6 cells by MTT assay. The cultures were treated with different concentrations (1.56, 3.12, 6.25, 12.5, 25.0, 50.0, 125.0 and 250.0 µg/mL) of BlV for 24 h. These results are representative of at least 3 independent experiments. Data show the mean ± standard deviation (S.D.). *Statistically significant difference ($p<0.05$) compared with control.

Study investigating the viability of LLC-MK2 renal cells against venom of *B. alternatus*, through the MTT assay, observed a significant reduction in viability only in the highest concentrations tested (50-200 µg/mL) (NOGUEIRA JÚNIOR, 2017). Similar results were obtained with other venoms of snakes of the *Bothrops* genus, as *B. jararacussu* (TEIXEIRA-CRUZ, 2014) and *B. alternatus* (NASCIMENTO et al., 2007). In a study conducted with

Montivipera xanthina, another snake of the *Viperidae* family, the authors did not find a cytotoxic effect on VERO cells, even at high venom concentrations (YALCIN et al., 2014). In a recent study with the action of *Bothriopsis bilineata smaragdina* venom (BbsV) on VERO cells, the authors verified cytotoxic effect only from high concentrations, suggesting that this venom appears to be less toxic for this cell line (RODRIGUES et al., 2018).

These studies indicate that there is a variation of the cytotoxic effect of the venom in each cell line tested, but there are also differences within the same lineage, with the same venom, which may suggest a variation, confirming the pattern of the dose-response curve seems to be species-specific. Considering that venom cytotoxicity is related to LAAO activity (SANTOS et al., 2008), perhaps these variations in LAAO concentration of BlV could result in different cytotoxicity patterns.

CONCLUSION

BlV reduced cell viability of VERO E6 cells in a concentration-dependent manner, being the MTT test the best method for this evaluation. The results of this study show the potential of using cell culture system to evaluate venom toxicity and to prevent the over use of animal models to study toxins. Considering that the cellular pathways triggered by this venom and its toxins are still poorly understood, research studies of their mechanisms of action on cellular models will produce relevant information that is close to what happens in real accidents.

VIABILIDADE DE CÉLULAS VERO E6 EXPOSTAS AO VENENO BRUTO DA SERPENTE *Bothrops leucurus*

RESUMO

Sistemas de cultura de células primários e contínuos são uma boa alternativa *in vitro* para a avaliação quantitativa da toxicidade de veneno de serpente. Ainda existem poucos estudos avaliando a resposta celular contra o veneno de *Bothrops leucurus* (BlV). Este estudo teve como objetivo avaliar o efeito citotóxico do BlV bruto em cultura de

células VERO E6. Testes de incorporação de vermelho neutro (VN) e de brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil-tetrazólio (MTT) foram realizados para células viáveis, e teste de exclusão de corante azul de Tripan (AT) foi realizado para células não viáveis. As diferenças entre os grupos foram avaliadas por ANOVA-One-Way e Dunnet, através do programa Graph Pad 5.0 com significância de 5%. A concentração citotóxica de 50% (CC_{50}) do BlV foi de 7,86 µg/mL. Houve uma tendência de redução dose-dependente na viabilidade celular em todos os ensaios avaliados. No entanto, os testes do MTT e do VN mostraram-se mais sensíveis para a avaliação da citotoxicidade do BlV na linhagem celular VERO E6.

Palavras-chave: CC_{50}. Cultura celular. Jararaca-do-rabo-branco.

VIABILIDAD DE CÉLULAS VERO E6 EXPUESTAS AL VENENO BRUTO DE LA SERPIENTE *Bothrops leucurus*

RESUMEN

Los sistemas de cultivo de células primarias y continuas son una buena alternativa *in vitro* para la evaluación cuantitativa de la toxicidad de veneno de serpiente. Todavía existen pocos estudios evaluando la respuesta celular contra el veneno de *Bothrops leucurus* (BlV). Este estudio tuvo como objetivo evaluar el efecto citotóxico del BlV bruto en cultivo de células VERO E6. Los ensayos de incorporación de rojo neutro (RN) y de bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil-tetrazolio (MTT) se realizaron con las células viables, y los ensayos de exclusión de colorante azul de Tripan (AT) se realizaron en las células no viables. Las diferencias entre los grupos fueron evaluadas por ANOVA-One-Way y Dunnet, a través del programa Graph Pad 5.0 con significancia del 5%. La concentración citotóxica del 50% (CC_{50}) del BlV fue de 7,86 µg/ml. Se observó una tendencia de reducción dosis-dependiente en la viabilidad celular en todos los ensayos evaluados. Sin embargo, las pruebas del MTT y del RN se mostraron más sensibles para la evaluación de la citotoxicidad del BlV en el linaje celular VERO E6.

Palabras clave: CC_{50}. Cultivo celular. Jararacá de la cola blanca.

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