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Apamin-induced alterations in J774 1.6 macrophage metabolism

[*Alterações induzidas por apamina no metabolismo de macrófagos J774 1.6*]

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ABSTRACT

Among the immune system cells, macrophages have an important role. Apamin, a bee venom constituent, is important in the defense of these insects. Thus, we aimed to evaluate the metabolism of J774 1.6 macrophage cell line when exposed to isolated and purified apamin, using cytotoxicity tests by MTT reduction and analysis by flow cytometry (apoptosis / necrosis, production of reactive oxygen species (ROS), membranous lipoperoxidation (LPO), electrical potential of the mitochondrial membrane (mMP) and DNA fragmentation). None of the tested concentrations (10 to 100µg/mL) were cytotoxic according to MTT reductions. Apoptosis rates decreased at concentrations of 2.5, 5.0, and 10.0µg/mL ($P<0.05$), while necrosis rates increased ($P<0.05$). However, rates of healthy cells at the highest tested concentration (10µg/mL) did not differ from control ($P>0.05$). Apamin did not alter ROS, LPO, or DNA fragmentation. Therefore, all analyzed concentrations (1.25 to 10µg/mL) decreased mMP. Such decrease in apoptosis might be due to a suppression of mitochondrial pro-apoptotic messengers, as this peptide causes no oxidative stress, lipid peroxidation, and DNA damage. Highly sensitive techniques are majorly important for proper interpretation of cellular toxicity mechanisms, combined with routine laboratory methods.

Keywords: cytotoxicity, mitochondrial function, ROS, flow cytometry, macrophage

RESUMO

Das células do sistema imunológico, macrófagos desempenham um papel fundamental. Apamina, constituinte do veneno de abelhas, é importante na defesa destas. Objetivou-se avaliar o metabolismo da linhagem de macrófagos J774 1.6 expostos à apamina isolada e purificada, avaliando-se citotoxicidade por redução de MTT e análise por citometria de fluxo (apoptose / necrose, produção de espécies reativas de oxigênio (EROs), lipoperoxidação membranosa (LPO), potencial elétrico da membrana mitocondrial (MMP) e fragmentação do DNA). Nenhuma concentração testada (10 a 100µg / mL) foi citotóxica. As taxas de apoptose diminuíram nas concentrações 2,5, 5,0 e 10,0µg / mL ($P<0,05$), enquanto as de necrose aumentaram ($P<0,05$). Entretanto, as taxas de células saudáveis na maior concentração testada (10µg / mL) não diferiram do controle ($P>0,05$). A apamina não alterou as ERO, a LPO nem a fragmentação do DNA. Portanto, todas as concentrações analisadas (1,25 a 10µg / mL) diminuíram a mMP. Tal diminuição na apoptose pode ser por uma supressão de mensageiros pró-apoptóticos mitocondriais, já que este peptídeo não causa estresse oxidativo, peroxidação lipídica nem dano ao DNA. Técnicas altamente sensíveis são importantes para adequada interpretação dos mecanismos de citotoxicidade.

Palavras-chave: citotoxicidade, função mitocondrial, ROS, citometria de fluxo, macrófago

INTRODUCTION

Macrophages are important immune system cells that participate in innate immunity and help to regulate adaptive immune responses through phagocytosis and antigen-presentation to lymphocytes, and their production of mediators interfere with a wide range of cytokines

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(Geissmann *et al.*, 2010). These cells originate from monocytes that migrate from blood vessels in response to antigenic stimulation (Van Ginderachter *et al.*, 2006). Macrophages, when in tissues, mature and adapt to the environment, differentiating into several types of cells with specific function such as Kupffer cells in liver and microglia in the central nervous system (Gordon and Taylor, 2005). Failures in macrophage differentiation can lead to diseases, including autoimmune diseases and cancer (Mantovani *et al.*, 2008).

Bee venom has been described as a potential therapy for arthritis, multiple sclerosis, and many types of cancerous tumors and also as a pain releaver (Son *et al.*, 2007). Part of the bee venom, an 18-amino acid peptide, apamin is a bioactive peptide constituting nearly 2-3% of its dry weight (Ovcharov *et al.*, 1976). Apamin is a less known neurotoxin which acts as a calcium channel blocker in postsynaptic membranes of the central and peripheral nervous system, responsible for blocking the transmission of certain inhibitory impulses (Banks *et al.*, 1979; Cruz-Landim and Abdalla, 2002).

Preview studies suggest that the apamin itself may induce a mast cell stabilizing effect, reducing allergic airway inflammation, and also can inhibit the destruction of myelin, being used as a facilitator of neurotransmission in a variety of nerve disorders (Son *et al.*, 2007). The cytotoxicity of a compound can be assessed by the mitochondrial activity of cells it is in contact with it, by apoptosis or induced necrosis, as well as by ROS, LPO, mMP and DNA fragmentation. In this study we aimed to describe the metabolic changes in J774 1.6 macrophages when exposed to apamin.

MATERIALS AND METHODS

Cells were cultured in Eagle minimum essential medium (E-MEM; Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with antibiotics [penicillin (Sigma-Aldrich®, USA), streptomycin (Vetec®, Brazil), and enrofloxacin (Bayer®, Brazil)] as well as amphotericin B (Cristália®, Brazil), using polystyrene 96-well flat bottom microplates (KASVI®, Brazil). Fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) was added when the cell multiplication was required. Apamin was commercially purchased (Sigma-Aldrich Corp., St. Louis, MO, USA) at 99% purity and dissolved in sterile E-MEM, then stored at a concentration of 1mg/mL at -70°C. Murine macrophages of the J774 1.6 cell line were obtained from Rio de Janeiro's cell bank. Dimethyl-sulfoxide (DMSO), MTT [3- (4,5-dimethylthiazol-2yl) -2-5-diphenyl-2H-tetrazole reagent], and reagents used in flow cytometry (Hoechst 33342, Annexin V, propidium iodide, rhodamine 123, 2', 7' dichlorofluorescein, C11-BODIPY581/591, and acridine orange) were purchased commercially (Sigma-Aldrich Corp., St. Louis, MO, USA).

The J774 1.6 cells were kept in cell-culture bottles with E-MEM supplemented with FBS, inside a laboratory oven at 37°C and 5% CO_2. When the cell monolayer reached approximately 80% confluence, the cells were transferred to 96-well plates (100µL/well) at a concentration of $3x10^4$ cells/mL. After 24 hours in the oven, the medium was carefully aspirated and transferred to the wells (100µL /well) at different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100µg /mL). Tests were carried out in six-fold for 72 hours, under the same conditions, until the moment of the analyses. Cells in E-MEM without exposure to apamin were used as a negative control.

The MTT reduction assay was used to estimate cell viability through mitochondrial functionality, as proposed by Mosmann (1983), which consists of a quantitative assessment of living cells, therefore, mitochondrial activity. It's expected that functional mitochondria, through the action of dehydrogenases, reduce the MTT into formazan, changing the compost color and then it can be measured using a spectrophotometer. After exposure to apamin, an aliquot of 50µL of MTT solution (1mg / mL) was transferred to each well, with plates being incubated for 4 hours in the laboratory oven at 37°C and 5% CO_2. Then, the supernatant was removed, and 100µL of DMSO was poured on to the cell monolayers for solubilization of generated formazan salts. After 15min stirring, the plates were subjected to spectrophotometry for absorbance measurements at 540nm. Cytotoxic concentrations (CC) 50% and 90% were calculated by the formula: CC = (TA / CA) x100; where in: TA is the mean absorbance of wells whose cells received treatment, and CA the mean of control wells.

The J774 1.6 cells cultured in 96-well plates and exposed to four apamin concentrations (1.25, 2.50, 5.00 and 10.00µg/ mL) for 72 hours were subjected to specific reagent treatment for each analysis and then removed from wells to be resuspended in 100µL E-MEM and preserved (refrigerated). An Attune® acoustic focusing cytometer (Applied Biosystems, Foster City, CA, USA) was used for flow-cytometry analyses, and the results were analyzed using Attune Cytometric software v 2.1 (Life Technologies). A fluorescence probe (2mM Hoechst 33342) was used to separate cell populations (Hoechst positive) from debris (Hoechst negative). They were detected by a VL1 photomultiplier (450/40 filter). Twenty thousand events were analyzed per sample at a flow rate of 50µL per second. FSC and SSC scatter plot was used to select cell populations.

After exposed to apamin, cells were analyzed by adding fluorescent compounds to plate wells, as described by Masango *et al.*, 92015. An aliquot of 2µL of fluorescein isothiocyanate-conjugated Annexin V antibody (FITC) was added to each well for one hour, and propidium iodide (PI, 50µg / mL) for 10 minutes. The cells were removed from plates and kept under refrigeration until the reading. Viable cells were not labeled (FITC, PI-). Apoptotic cells externalized phosphatidylserine were recognized by Annexin-V (FITC +, PI); yet, necrotic cells were recognized by nuclear membrane rupture and IP bond to genetic material (FITC-/ PI+ and FITC+/PI +). Fluorescence readings were carried out through a photomultiplier BL3 (640LP filter). The results were expressed as a percentage of cellular populations calculated by the formula: percentage of cells = (number of positive events / total number of events) x 100.

Rhodamine 123 fluorescent dye was used to label active mitochondrial membranes when electrons were donated to respiratory chain complexes. After exposure to apamin, rhodamine 123 (100nm) was added to the J774 1.6 cells, remaining for one hour. Fluorescence intensities emitted by mitochondria, more active (higher concentration of fluorescence, greater accumulation of rhodamine) and less active (less fluorescence, less accumulation of rhodamine), were analyzed through a photomultiplier BL1 (filter 530/30). The data were expressed in percentages of cells with low mMP and calculated by the formula: percentage of cells with low mMP= (number of cells with low mMP / total cell number) X 100.

Intracellular production of ROS was measured using fluorescent dye 2', 7'-dichlorofluorescein diacetate (H2DCF-DA). Fluorescence was read in a photomultiplier BL1 (530/30 filter). The technique was adapted from Domínguez-Rebolledo *et al.* (2011) and is based on H2DCF-DA oxidation by intracellular ROS, producing fluorescence. H2DCF-DA dye (1mM) was added to the cells after exposure to apamin and remained for one hour prior to other reading procedures. Results were expressed as mean green fluorescence intensity, with an added standard error. Cell membrane lipid peroxidation was evaluated by a lipophilic fluorophore probe (C11-BODIPY$^{581/591}$), which was incorporated into lipid membranes for its similarity to unsaturated fatty acids. When intercalated with intact membranes, the reddish fluorescence properties are altered with lipid peroxidation, emitting orange-green fluorescence (Aitken *et al.*, 2007).

By the time cell monolayer was established, they were supplemented with C11-BODIPY$^{581/591}$ for 2 hours; after aspiration, the apamin concentrations were added. Then, after 72-h incubation, the cells were removed from plates and subjected to the procedures for reading. Results were expressed by a percentage of cells containing lipids in peroxidized membranes; for this, the following formula was used: percentage of cells with peroxidized membranes = (number of events with red fluorescence / total number of events) X 100. The analysis of DNA Fragmentation was performed using an acridine orange fluorescence technique (AO) in the carpet of cells, after exposure to apamin for 5 minutes, as proposed by Ojeda *et al.* (1992).

The AO probe was inserted into DNA double-strand structure as a monomer bound to a single-stranded DNA. The monomeric AO bound to intact DNA emitted green fluorescence, while AO attached to the fragmented DNA emitted orange to red fluorescence (Hoshi *et al.*, 1996). The results were expressed as percentages of cells with DNA fragmentation and calculated by the formula: percentage of cells with DNA fragmentation = (number of cells with orange fluorescence/total number of cells) X 100.

The data from MTT test were represented by mean ± standard deviation (SD). The difference between treatment groups and control were assessed by analysis of variance (ANOVA, Tukey test), as were flow cytometry analyses (ANOVA, LSD test). Pearson correlations were performed to evaluate associations between the analyzed variables. In all cases, STATISTIX statistical software version 10.0® was used and significant results were considered when P<0.05.

RESULTS

None of the apamin concentrations tested here differed from the control cells, for the MTT reduction test, which remained in E-MEM medium throughout the entire experiment. Therefore, the 50% cytotoxic concentration of apamin for J774 1.6 macrophage cell cultures could not be determined. Figure 1 shows the apoptosis and necrosis rates of J774 1.6 macrophages exposed to apamin. A decrease in apoptosis rates with respective healthy cell rate reduction was observed when cells were exposed to the concentrations of 1.25, 2.5, and 5µg/mL apamin (P<0.05), besides the increase in necrosis rates (P<0.05). Conversely, healthy cell rates in macrophages treated with the highest concentration (10µg/mL of apamin) match those of control (P>0.05), while apoptosis remained lower than the control. Table 1 displays the results of the other functional parameters of J774 1.6 macrophages when exposed to apamin.

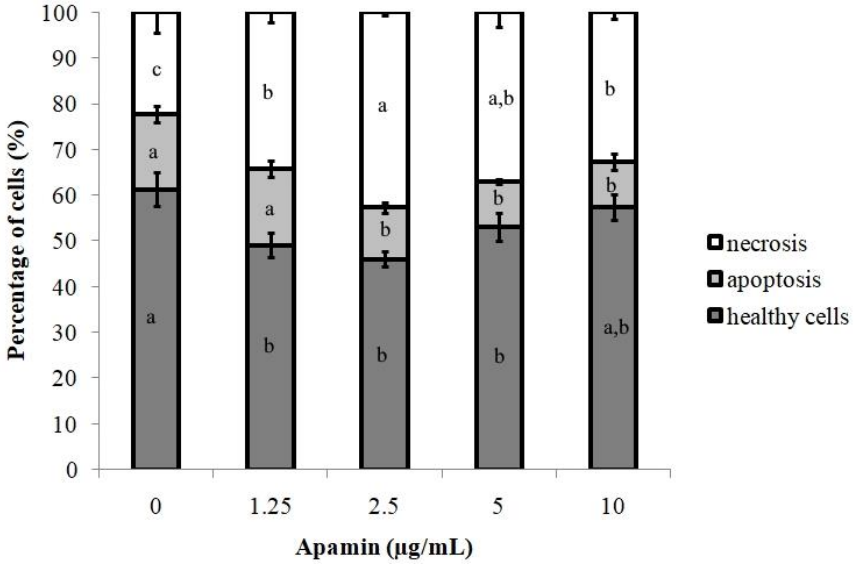

Figure 1 Rates of normal, apoptotic, and necrotic cells of J774 1.6 macrophages analyzed by flow cytometry after 72 hours of exposure to apamin. Different letters within the same cell populations indicate statistical difference by the LSD test (P<0.05).

Table 1 Parameters evaluated by flow cytometry in J774 1.6 macrophages exposed to apamin for 72 hours

Apamin	Parameters			
	ROS (IL)	LPO (%)	DNA fragmentation (%)	MMP (%)
0µg/mL	69810 ± 6800.7 a	62.7 ± 0.6 a	0.4± 0.3 a	17.8 ± 0.2 a
1.25µg/mL	58913 ± 1674.9 a	66.8 ± 0.1 a	0.6 ± 0.1 a	15.6 ± 0.6 b
2.5µg/mL	63043 ± 2031.6 a	64.9 ± 0.5 a	0.9 ± 0.1 a	16.2 ± 0.0 b
5µg/mL	64568 ± 1291.9 a	66.6 ± 0.3 a	0.7 ± 0.2 a	15.1 ± 0.2 b
10µg/mL	62653 ± 2686.9 a	65.7 ± 0.2 a	1.0 ± 0.2 a	15.4 ± 0.6 b

Different letters in the same column indicate statistical differences by the LSD test. ROS (intracellular production of oxygen reactive species), IL (luminescence intensity), LPO (membrane lipoperoxidation), mMP (mitochondrial membrane electrical potential).

Significant changes were not observed for apamin-induced DNA fragmentation (P>0.05). Hence, J774 1.6 macrophage apoptosis can be inhibited via mitochondrial pathway when exposed to apamin since DNA fragmentation is one of the changes caused by caspase-3, which is activated by cytochrome c released from mitochondria when apoptosis is stimulated by this same metabolic pathway. Table 2 shows the Pearson's correlations for all evaluated parameters.

Table 2 Pearson's correlations for cytometry parameters of J774 1.6 macrophages exposed to apamin for 72 hours

	Apoptosis	Necrosis	LPO	ROS	DNA Frag	MMP
Apoptosis	-					
Necrosis	-0.4072**	-				
LPO	-0.1580	0.0046	-			
ROS	0.3410*	0.2095	-0.040	-		
DNA Frag	-0.1062	0.0058	-0.3630*	-0.0055	-	
MMP	-0.0353	0.2927	-0.1594	0.5575**	0.1275	-

* (P<0.05), ** (P<0.01). LPO (membrane lipoperoxidation), ROS (intracellular production of oxygen-reactive species), DNA Frag (DNA Fragmentation), mMP (mitochondrial membrane electrical potential).

Table 2 shows that apoptosis and necrosis rates are inversely proportional (r=-0.4072, P<0.01). Although no statistical difference was noted for ROS rates, this parameter had a positive correlation with apoptosis rates (r=0.3410, P<0.05). This result is quite typical when apoptosis is extrinsically induced by intracellular ROS increases with subsequent membrane peroxidation and phosphatidylserine loop, which characterizes apoptosis. However, in J774 1.6 macrophages, neither ROS nor LPO modified with the use of apamin.

Still, mitochondrial membrane potential had a correlation with ROS (r=0.5575, P<0.01). Even with no statistical difference (P>0.05), intracellular ROS tended to decrease with an increase in apamin concentrations. On the other hand, mMP decreased and presented statistical difference (P<0.05).

DISCUSSION

Zhou *et al.* (2013) evaluated apamin toxicity on two cell lines. After MTT reduction tests these authors observed that L02 cells (human hepatocytes) maintained 99.08% viability after exposure to 100μg/mL apamin for 72 hours, and for HepG2 cells (human hepatoma cells) exposed to 800μg / mL apamin for 48 hours viability was 66.15%. Both studies show that the 50% cytotoxic concentration for cell cultures could not be determined. Through MTT reduction tests, apamin presented low toxicity, but this test evaluates cell viability through a single parameter - mitochondrial functionality. However, active mitochondria do not always denote cellular health since these organelles increase activity in cases of injury.

The other flow cytometry analyses showed changes that there were non-perceptible by the MTT reduction test. Annexin V binding to external phospholipids indicates apoptotic cells, whereas propidium iodide interspersed with DNA sequences, which signals cell membrane integrity since its molecular weight prevents it from entering into cells with intact membranes. Necrosis rates increased with apamin exposure, except at the highest concentration, in which it decreased, but still higher than that of untreated cells.

The reductions in apoptosis rates observed in our study corroborate Kim *et al.* (Kim *et al.*, 2012a). These authors counted apoptotic macrophages stimulated to apoptosis using oxidized low-density lipopolysaccharides (oxLDL), treated or not with apamin. They observed that the apamin-treated group had lower apoptosis rates. Similarly, our studies show that apoptosis levels in J774 1.6 macrophages exposed to apamin were lower than the untreated group. As intracellular ROS and LPO of cell membranes did not increase, we suggest that the anti-apoptosis mechanism found here corroborates with the findings of Kim *et al.*

(2012a). They reported a suppression of pro-apoptotic messengers (cytochrome c, caspase 3 and PARP and Bax) together with an increase in anti-apoptosis messengers (Bcl-2 and Bcl-xL).

Pro-apoptotic processes might occur due to several factors, such as oxidative stress (Dickhout *et al.*, 2005) and oxLDL (Seimon and Tabas, 2009). Kim *et al.* (2012a) examined the intracellular accumulation of lipids in macrophages after exposure to oxLDL, with or without apamin; they claimed that apamin suppressed such deposition of lipids. This mechanism may be responsible for a decrease in mitochondrial pro-apoptotic messengers. Thus, apamin suppressed the expression of cytochrome c, caspase-3, PARP (poly ADP-ribose polymerase) and Bax, and pro-apoptotic proteins, in addition to raising levels of anti-apoptosis proteins - Bcl-2 and Bcl-xL. These results show that this peptide inhibited the pro-apoptotic effects on the macrophages evaluated in our study.

During apoptosis, mitochondrial functioning is controlled by Bcl-2 family proteins (Pradelli *et al.*, 2010). The pro-apoptotic Bax protein can be inserted into the outer mitochondria membrane, raising permeability and releasing apoptotic factors, such as cytochrome c (Hsu *et al.*, 1997). In contrast, the anti-apoptotic proteins, Bcl-2 and Bcl-x, hinders membrane permeability and disruption, blocking cytochrome c release from mitochondria (Kroemer, 2002).

Mitochondria play a crucial role by activating caspase in apoptosis processes of different cell types. It occurs by releasing cytochrome c to activate caspase (Danial and Korsmeyer, 2004). Caspase-3-mediated apoptosis is associated with PARP cleavage, which has been recognized as a sensitive marker of caspase-mediated apoptosis (Utz and Anderson, 2000). This mediator induces DNA fragmentation and other morphological changes corresponding to apoptotic cell death (Janicke *et al.*, 1998). Kim *et al.* (2012a) claimed that in their studies DNA fragmentation in treated macrophages decreased with exposure to apamin if compared to the group with apoptosis induced by oxLDL. Yet in our studies, DNA fragmentation was not different between control and apamin-exposed groups. This corroborates the already described action mechanisms of apamin as an apoptosis inhibitor.

When exposed to bee venom at 5 and 10μg/mL, activated RAW 264.7 macrophages decreased the expression of mRNA coding for cyclooxygenase 2 (COX-2), nitric oxide synthase (iNOS), and NF-kB (nuclear factor kappa B) (Jang *et al.*, 2005). In another study, Kim *et al.* (2012b) reported that apamins inhibited the expression of TNF-α and adhesion molecules (VCAM-1 and ICAM-1) in THP-1-derived macrophages (monocyte lineage), lipopolysaccharide activated, besides inhibiting NF-kB transcription factor activation in these cells. While LPS-treated macrophages (control) induced NK-kB activation, the apamin-treated ones inhibited it. NF-kB is a nuclear factor that, once activated by agents (e.g. lipopolysaccharides), binds to a gene promoter region and activates transcriptions (Sen and Baltimore, 1986). This transcriptional factor is a heterodimer consisting of two subunits - p65 and p50 (Siebenlist, 1997). On the other hand, other subunits have been reported in the literature, such as c-Rel, RelB, and p52; thus, varied combinations can activate different genes, or block p50/p65 transcription.

NF-kB action has been described for several cells, being even superior to the other transcription factors hitherto characterized. Such superiority is related to various stimuli and to many genes regulated by this factor. Among these stimuli are neurotransmitters, cytokines (interleukin-1 and tumor necrosis factor), glucocorticoids, virus and bacterial products, ultraviolet irradiation, enzyme reaction products (nitric oxide synthetase and cyclooxygenase type 2) (O'neill and Kaltschmidt, 1997). Regardless of the stimulus, reactive oxygen species (oxidative stress) and intracellular calcium increases are involved in NF-kB activation. Kim *et al.* (2012b) reported that intracellular calcium accumulation and serum TNF-α levels were lower in apamin-treated mice when compared to untreated animals.

If not stimulated, NF-kB factor remains bound to inhibitory protein (IkB) in the cytoplasm. This complex blocks NF-κB translocation to the nucleus. Thus, phosphorylation and IkB degradation are essential for occurring this translocation (Baldwin, 1996). Kim *et al.* (2012b) said that apamin-treated macrophages had IkB phosphorylation suppressed, hindering NF-kB translocation and inhibiting gene transcription accordingly.

Apoptosis reduction, assessed by phosphatidylserine externalization, and mMP decreases in macrophages exposed to apamin ($P<0.05$) may indicate a blockage in the release of mitochondrial pro-apoptotic messengers, or even an increase in the release of anti-apoptosis proteins. Phosphatidylserine loop, which characterizes apoptosis, may have been caused by membrane destabilization after lipid peroxidation; as there was an increase in intracellular production of oxygen reactive species. However, ROS and LPO rates did not change significantly ($P>0.05$) when compared to the control.

Jang *et al.* (2005) described the action of bee venom towards RAW 264.7 macrophages through Griess reaction; their results demonstrate a suppression in nitric oxide production, besides of an inhibition in iNOS expression, suggesting an anti-inflammatory effect. In our study, results showed that apamin contributes to this effect since ROS production did not change in J774 1.6 macrophages, decreasing apoptosis rates, i.e. acting as 'protector'. In general, ROS are considered to be harmful to cells but still play an important role in signal transmission (Finkel and Holbrook, 2000) and gene expression (Goldstone *et al.*, 1996). Some reports suggest that ROS may be involved in the differentiation of hematopoietic cells or macrophages. It is known that ROS are crucial for macrophages to perform their functions; in addition to being involved in monocyte differentiation, they are responsible for the way it happens, both by classic and alternative pathway (Zhang *et al.*, 2013).

CONCLUSIONS

Our results suggest that apamin causes neither oxidative stress, nor DNA fragmentation, nor membrane changes in J774 1.6 macrophages. However, in this study, this substance reduced mitochondrial membrane electrical potential. Thus, reductions in apoptosis rates could be a consequence of the low release of pro-apoptotic messengers from mitochondria. The different analyses performed in this study helped to clarify the action mechanisms of this venom on J774 1.6 macrophages, besides emphasizing the need to use the latest techniques along with conventional ones.

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