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Research Article

The Anti-Apoptotic Effect of *Lonomia obliqua* Hemolymph is Associated with the Mitochondria Pathway

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ABSTRACT

The apoptosis death is a very important factor in production processes that limited the industrial production of some proteins of economic interest. However, one of the forms to increase the cellular productivity would be inhibit or attenuate the cellular death. Recently we have demonstrated the presence of a potent anti-apoptotic protein in *Lonomia obliqua* hemolymph which extends the cell culture viability through apoptosis prevention. By the other side, has been reported that mitochondria have one important action in the apoptosis control process, being that Mitochondria Membrane Permeabilization (MMP) can be an important stage in this process. MMP associated or not with the loss of the electrochemical potential of the mitochondria and alteration of the matrix is responsible for the inter membranes protein release (e.g. cytochrome c, the AIF, etc) of cytosol. The result obtained showed that the addition of a protein from *Lonomia obliqua* hemolymph in the culture lead to a prolongation of the cellular life (3-4 days) and the cells leading a high electrochemical potential of the mitochondria. This protein can has its action in mitochondria membrane, avoiding the loss of the membrane permeability and the Cytochrome-C release. As positive control, apoptosis death in these cultures was induced by 50 µm of t-BHP or 600 µm of H202. The presence of apoptosis was characterized by flow citometry, microscopy electronic and agarose gel electrophoresis. The potential electrochemical of the mitochondria was determined by JC-1, Hoechst 33324 and DIOC6. Cytochrome C was identified in cytosol by an anti-cytochrome antibody.

Keywords: Hemolymph; Lonomia oblique; Bio-active peptide; Mitochondrial membrane permeabilization; Apoptosis; Bio-prospection

Abbreviations: HB: Hemolymph; T-BHP: Tert-Butylhydroperoxide; PI: Propidium Iodide DiOC6 (3), 3,3'Dihexyloxacarbocyanine Iodide; JC-1: 3,3'E-Tetraethylbenzimidazolylcar-bocyanine Iodide; HPI: Hours Post Infection; MOI: Multiplicity of Infection; MMP: Mitochondrial Membrane Permeabilization.

INTRODUCTION

Optimization of animal cell culture processes is essential for economical production of biopharmaceutical products, such as recombinant proteins, viruses and cells. Cell death in bioreactors represents a major problem in cell culture technology by decreasing the global productivity yield. Programmed cell death is directly related to the cells death in bioreactor, being 2 mechanisms of death can be observed; apoptosis and autophagy. The programed cell death (apoptosis) mechanism is activated by various external and internal factors as heat, irradiation nutrient depletion, shear stress, hypoxia, toxin accumulation, as well as a cell response to viral infections, [1-15]. These factor acts directly on the membrane of cells or on mitochondria, being acting on a caspase activation cascade [16-18]. Since these factors implicated on the loss of cell viability, due to cell death by apoptosis, the decreasing apoptosis in cell culture is an attractive strategy to improve global productivity yields [19]. For that, two main strategies can be followed: manipulation of the external environment or genetic manipulation of the internal cellular biochemistry. The medium culture can be supplemented with various components for prolongation of the viability cell. These components can be anti-apoptotic agents, nutrients or selected agents from serum. Caspases inhibitors also can enhance cell culture viabilities and protein titer [20-22]. Anti-oxidants were also described as factors to improve cell viability, N-acetylcysteine increases mammalian cell lifetimes upon Sindbis virus vector infection and catalase can improve recombinant protein production in baculovirus-insect cell system by preventing cell death [23,24]. We have showed that supplementation of insect

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cell culture with *Lonomia obliqua* hemolymph could extend culture longevity, avoiding apoptosis death [25]. Earlier we have identified, isolated and characterized this protein from *Lonomia obliqua* hemolymph (with about 51 kDa), presenting a potent anti-apoptotic effect. Addition of this purified protein to Sf-9 cell cultures allowed the prevention of apoptosis induced by nutrient depletion, as well as by actinomycin D. This protein showed action as in mammalian cell as well in insect cel showing a broad action range [26].

After this, when this protein was added to insect infected cell culture, was observed an improvement of recombinant rabies virus glycoprotein (rRVGP), expressed by Drosophila melanogaster Schneider 2 (S2) and improvement of recombinante bovine rotavirus glycoprotein Vp2, Vp6 and Vp7, expressed by baculovirus [27, 28]. Recently, we have observed that the addition of this protein to cell culture also leads to an increase in baculovirus production. This increase in viral production could be related to the increase in the production of recombinant proteins by baculovirus [29]. So, in this work, we seek to identify the place of action of this protein. Mitochondrial Membrane Permeabilization (MMP) can be a rate-limiting step of apoptotic just as of necrotic cell death. MMP associated or not to the loss of mitochondrial electrochemical potential ($\otimes\Psi m$) and matrix swelling, is responsible for the release of inter membrane proteins (e.g. cytochrome Cc, AIF, etc) into the cytosol. As a result of this release, apoptogenic proteases (caspases) and DNases are activated and the death process becomes irreversible. In addition, MMP can cause depletion of antioxidant enzymes, reactive oxygen species generation and cessation of ATP synthesis leading to an oxido-reductive and bioenergetic catastrophe [9,10,30]. Therefore studying the implication of mitochondria on the apoptosis inhibition by *Lonomia obliqua*, hemolymph factors is highly important to acquire a better knowledge about their mechanisms of action.

The present work aims to demonstrate the anti-apoptotic ability of hemolymph factors in anchorage-dependent HEK-293 cells cultures. The involvement of *Lonomia obliqua* hemolymph in the mitochondrial control of apoptosis is disclosed.

MATERIALS AND METHODS

Cells and media

Anchorage-dependent HEK-293 cells, purchased from ATCC (ATCC-CRL-1573), were routinely cultured in Minimum Essential Medium (MEM) supplemented with 5% (v/v) heat-inactivated (56°C, 30 min) Fetal Bovine Serum (FBS), 2 mM of glutamine, using an humidified atmosphere of 5% CO2 in air at 37°C.

Total soluble protein quantification

The total soluble protein content of the whole hemolymph and purified protein fraction were quantified using the Bradford method using bovine serum albumin as a standard [5].

Hemolymph

Hemolymph of *Lonomia obliqua* was collected from sixth-instar larvae after setae cut off. The collected hemolymph was centrifuged by 1.000 g/10 min; the supernatant was filtered with 0.2 µm membrane filter, inactivated by heat (60°C/30 min) and storage at 4°C. This material was used for supplementation the medium (1%) at start of the culture.

Hemolymph purification by chromatography

After centrifuged and filtered, 1 ml of hemolymph was loaded on a SuperdexTM 75 10/300 GL (Amersham Pharmacia Biotech) column at rate of 0.5 ml/min and eluted with sodium phosphate buffer. The eluated was harvested in fractions of 0.5 ml and monitored at 280 nm. Active fractions from SuperdexTM 75 10/300 GL column were loaded at an ion change column (Resource q). The chromatography was performed at an AKTA purifier chromatograph (Amersham Pharmacia Biotech). The purified fractions were applied at SDS-PAGE electrophoresis for analysis.

Chemical inductor of apoptosis

Apoptosis was trigged by oxidative stress induced with 50, 75 or 100 µM of T-BHP (Tert-butylhydroperoxide) (Sigma) or H202, in the concentrations of 400, 600, 800 or 1000 µM.

Fluorochromes used to determination of cellular death

Propidium iodide (1 µg/ml) and Hoechst 33342 (2 µM) was used as indicator of cellular death. The propidium was used as a fluorocrome that penetrates in all of the cells that lost the permeability emitting in red. Hoechst is a fluorochrome that stain cell nucleus allowing the visualization of morphologic changes of nuclei in apoptosis cell death process.

Fluorescent microscopy

HEK-293 cells were cultured in 13 mm-diameter cover slips. Eighteen hours later, cells were treated with tert-butylhydroperoxide (Sigma) and 4 hours after cells were stained with Hoechst 33342 (2 µM, Sigma), followed by fluorescence microscopic assessment of apoptotic nuclei. Cells were observed on a Leica DMRB microscope using a filter cube presenting UV excitation range with a band pass of 340-380 nm of wave length.

Flow cytometry

Samples of 0.5 ml were collected from the cell culture at different times. Apoptosis-associated changes were assessed by cytofluorometry on a BD FACSCalibur™ 4 colors (Becton Dickinson), while gating the forward and the side scatters on viable cells, using several fluorochromes: 3,3' dihexyloxacarbocyanine iodide (DiOC6(3), 20 µM) for $\Delta\Psi m$ quantification, propidium iodide (PI, 1 µg/ml) for the determination of cell viability. The acquisition and analysis of the results was performed with the software Cell Quest (Becton Dickinson).

Mitochondrial membrane potential measure

JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-Carbocyanine Iodide) (2 µm) and DIOC6 (3) (3,3´Dihexyloxacarbocyanine iodide), (20 µM) were used as mitochondrial membrane potential indicator.

Immunofluorescence for cytochrome C detection

For identification of cytochrome C was used a monoclonal antibody anti-cytochrome C and a second IgG anti-mouse antibody conjugated with alkaline phosphatase.

Measurement of cell viability

Culture samples were obtained daily and cell concentration was measured using a hemocytometer. Cell viability was determined by trypan blue exclusion test under light microscopy.

Participation of the mitochondria in the apoptosis death process

The participation of the mitochondria during the cellular death by induced apoptosis and the protector effect of the hemolymph was determined by the potential of the mitochondrial membrane. To this, apoptosis death in HeK-293 cells was induced by t-BHP or H202. The identification of the potential of the mitochondrial membrane in the cells cultured was accomplished by FACs (suspension cells) after cells stains with DIOC6 (3) or in fluorescence microscope (adherent cells) after cell stain with JC-1/Hoechst 33324. The pro-apoptotic factors (cytochrome C) determination liberated in the cytosol of the HEK-293 cell mitochondrial, after apoptosis induction, was performed by an anti-cytochrome C antibody, as described below.

Determination of the potential of the mitochondrial membrane in HEK-293 cell after JC-1 stain

For the determination of the protector effect of hemolymph against apoptosis induced (t-BHP or H202), cells HEK-293 were cultivated in suspension or adherent to micro plates. After semi confluence, cells were treated with 1% (v/v) of total Hemolymph (Hb) or with Purified Fraction (Frp) showing anti-apoptotic activity. After one hour of contact cells were treated with 50, 75 or 100 µM of t-BHP or with 400, 600, 800 or 1.000 µM of H202. The cells cultures were maintained at CO2 incubation by 4 hours and after this period cells were washed with PBS and treated with Hoechst 33324 and the fluorochrome JC-1 (2 µM).

The determination of the cells potential of the mitochondrial membrane was performed by FACs (FACSort Becton Dickinson (Air laser, to 488 nm (excitement) and 620 nm (emission), for cells in suspension or in fluorescence microscope for adherent cells). Cells showing mitochondria with low and high potential of the mitochondrial membrane are stained in green and red respectively. Normal cells present homogeneous nucleus stained in blue (Hoechst) while apoptotic cells present the nucleus fragmented.

Identification of Cytochrome C in the cytosol of the mitochondria by immunofluorescence

To the determination of the protector effect of the hemolymph in the apoptosis induced by t-BHP, cells HEK-293 were cultivated in micro plates of 12 wells. After semi confluence, the cells were treated with 1% (v/v) of total hemolymph (Hb) or it´s purified fraction (Frp). After contact of one hour the cells were treated with 50 or 75 µM of t-BHP. The cultures were incubated by 4 hours and after this period, cells were washed with PBS and fastened with paraformaldeide (4%) and picric acid (0,18%) and incubated by 45 minutes. The cells were washed with PBS, treated with BSA (3%) and incubated by 30 minutes.

The cells were then washed with PBS and marked with the cytosol monoclonal antibody. The reaction was revealed with an anti-mouse IgG antibody conjugated with alkaline fosfatase by 75 minutes being after this added 2 µM of hoechst. The material was observed in a fluorescence microscope (microscope Leica DMRB).

RESULTS

Hemolymph purification and apoptosis identification

The protein antiapoptotic was purified in two steps of chromatography. The results are shown in Figure 1 [26].

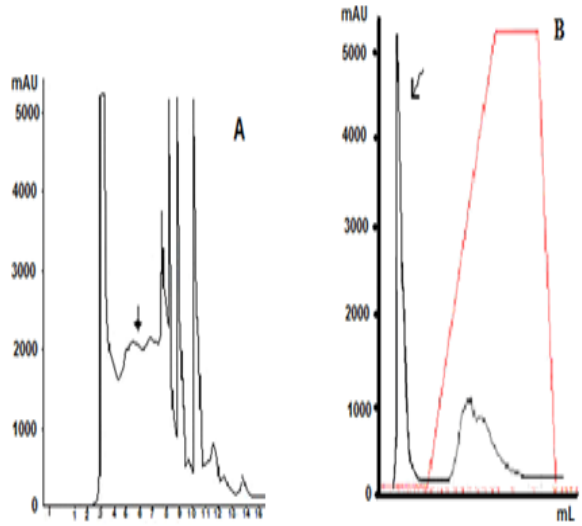

Figure 1: Purification of total hemolymph of *Lonomia obliqua* and its subfractions. Total hemolymph was initially purified by gel filtration in a Superdex G-75 column (a). Fractions were tested for antiapoptotic activity. The chromatography fraction with activity (arrows in Figure 1a) was applied on an ion exchange column (Resource Q). The chromatography fraction showing antiapoptotic effect is presented with an arrow in figure (b).

The whole and purified fraction was then applied to SDS-PAGE gel electrophoresis. The purified protein against death effect appeared as one band with molecular weights of approximately 51 kDa (Figure 2).

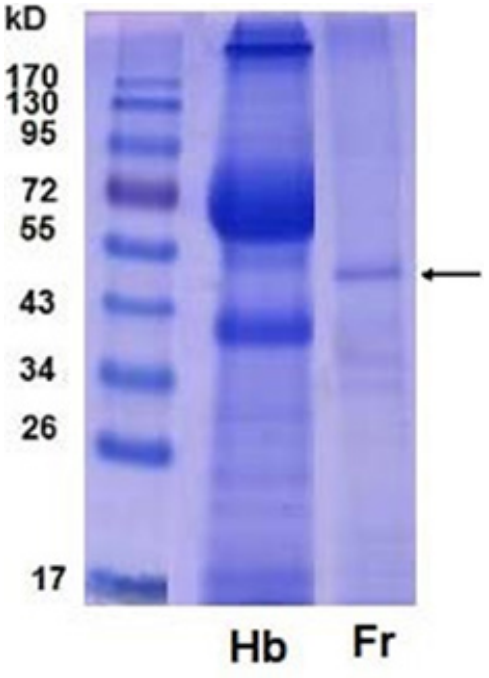

Figure 2: SDS-PAGE of total hemolymph (Hb) and the purified antiapoptotic protein (Fr) analyzed on a 12% gel.

In order to evaluate the effect of hemolymph supplementation in cell death by apoptosis process, cultures were incubated with 1% v/v of whole hemolymph (30.0 mg/ml) or purified protein fraction (1.0 mg/ml). The final concentration in culture was 300 µg/ml and 20 µg/ml respectively. The results obtained in hemolymph supplemented cultures were compared with those cultures performed without supplement addition. The results showed that hemolymph supplementation (whole and purified fractions) had a strong positive protection effect in cell culture, suggesting the presence of a potential anti-apoptotic in hemolymph of *Lonomia obliqua* (Figure 3).

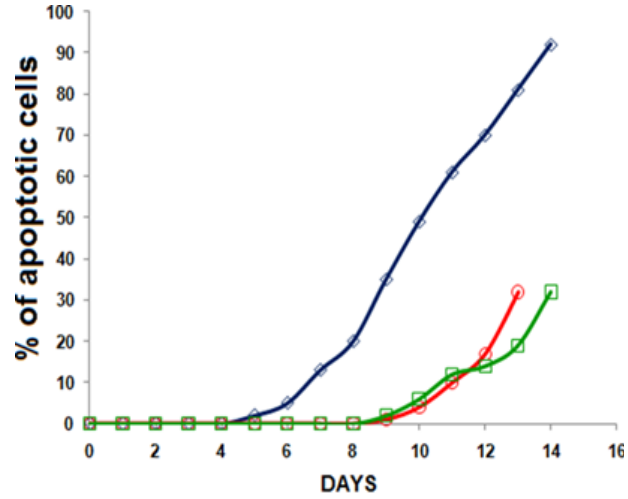

Figure 3: Percentage of apoptotic cells in HEK-293 cultures treated with 1% v/v of total (red) or purified fraction of hemolymph (green). Culture samples were obtained daily and cellular death was determined after propidium iodide (1µg/ml) and Hoechst 33342 (2 µM) stain. **Note:** (—◆—) Control; (—●—) Hb; (—□—) Fr

Determination of the potential of the mitochondrial membrane of HEK-293 cell after induction by t-BHP and H_20_2.

Effect of different amounts of H202 in the mortality and in the potential of the mitochondrial membrane of HEK-293 cells: Aiming to verify the action of the anti-apoptotic protein in potential of the mitochondrial membrane of HEK-293 cells after induction by t-BHP and H202, HEK-293 cells were previously treated with 1% (v/v) whole or a purified fraction of hemolymph. After 1 hour, cell death was induced with H202 (600, 800 or 1.000 µM) overnight. After this period the cultures were labelled with 1 µg/ml PI and 20 µM of DIOC6(3). After 20 minutes, the samples were applied to a flow cytometer and the potential of the mitochondrial membrane was determined. As can be seen in Figure 4, there is an increase in the loss of potential of the mitochondrial membrane after H202 addition. By the other hand, both, total (Hb) and purified hemolymph fraction (Frp) were able to inhibit cell death, keeping potential of the mitochondrial membrane higher than that observed in control culture. The loss of potential of the mitochondrial membrane (and consequently cell death) was proportional to the increase in the inductor amount. The total hemolymph was able to inhibit virtually almost all cell death, even with 1.000 µm H202. In this condition, the number of dead cells in control was 62%, while that, in the treated hemolymph culture, the amount of cell death did not exceed 4.75%. Even the fraction was able to inhibit the cell death but in lower amount (37%). HEK-293 cells were very sensitive to incubation under the experimental conditions (overnight). In all experiments it was observed a loss of cell viability in control cultures in this period (26.88%), but loss was not observed in cultures incubated with total hemolymph (2.65%). These data suggest that hemolymph also protect the cells of control against initial loss of viability.

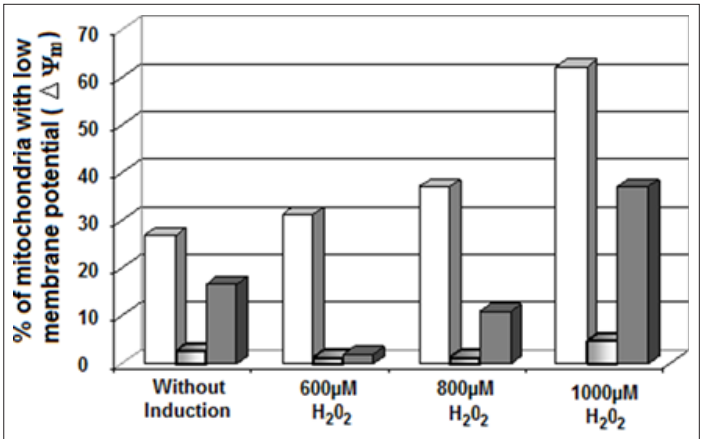

Figure 4: Effect of hemolymph in the protection of the cellular death by H202. HEK-293 cells were previously treated or not with 1% (v/v) of total or purified hemolymph. After this period the cellular death was induced with 600, 800 or 1000µM of H202 overnight. After this period the cultures were stained with 1µg/ml of PI and 20µM of Dioc(6)3. After 20 minutes of contact the samples were applied at FACS and the death number cells were determined. **Note:** (□) Control; (■) Hemolymph; (■) Fraction

To verify the protective effect in the potential of the mitochondrial membrane of cells, HEK-293 cells treated with 1% (v /v) of total or purified fractions of hemolymph. After 1 hour, cell's death induced with 400 or 800 µM of H202, for 4 hours. After this period the cultures were labelled with DIOC6 (3) and the fluorescence was determined. In Figure 5 is observed the % of fluorescent cells showing high mitochondrial membrane potential. As can be observed, the hemolymph and it purified fraction were able to remain the membrane potential even after treatment of cells with 800 µM of H202. Moreover, the control of the culture incubated with total hemolymph or it fraction, shown higher viability when compared with the control of the culture without this treatment.

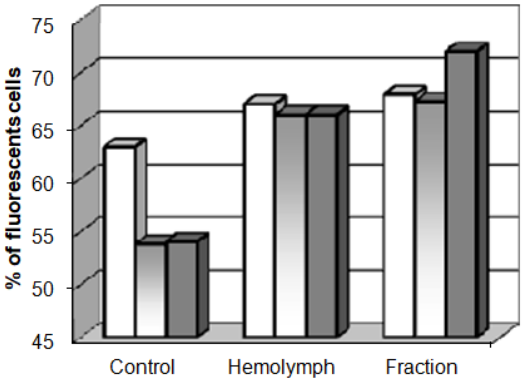

Figure 5: Hemolymph effect in the membrane potential of cells HEK-293. The HEK-293 cells were previously treated or not with 1% (v/v) of total or purified hemolymph. After this period the cellular death of the cells was induced by 4 hours with 400 or 800µ M of H202. After this period the cultures were stained with 1µg/ml of PI and 20µM of DIOC6(3). After 20 minutes of contact, the number of cells showing high fluorescence was determined. **Note:** (■) Without H202; (■) 400µM H202; ■) 800µM H202

Effect of different amounts of t-BHP in the potential of the mitochondrial membrane of HEK-293: In Figure 6 is showed the effect of different concentrations of t-BHP on membrane potential of mitochondria in HEK-293 cells (four hours of induction). As can be seen there is a progressive increase in the loss of membrane potential of mitochondria according to increasing concentrations of t-BHP. To verify the protective hemolymph effect in the potential of the mitochondrial membrane, HEK-293 cells treated with 1% (v /v) of total hemolymph or it purified fraction. After 1 hour, cell death induced with 50, 75 or 100 µM of t-BHP, for 4 hours. After this period, the cultures were labelled with DIOC6 (3) and fluorescence determined by flow cytometry. In Figure 7 is observed the percentage of fluorescent cells showing high mitochondrial membrane potential. As can be observed, total and hemolymph fraction were able to inhibit cell death, maintaining the potential of the mitochondrial membrane higher than that observed in control culture.

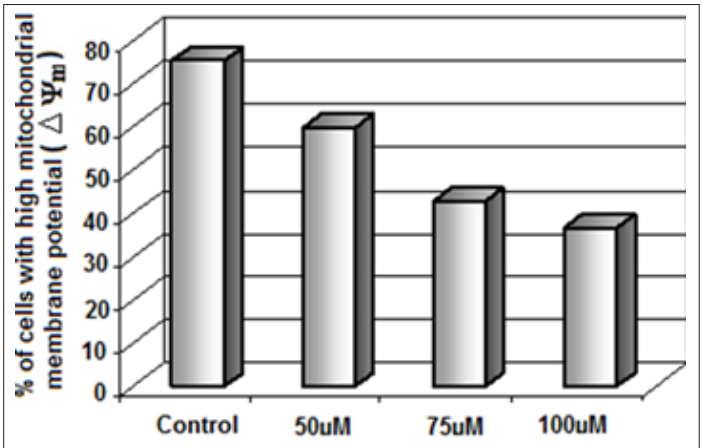

Figure 6: Hemolymph effect in the membrane potential of cells HEK-293. The cellular death was induced by 4 hours with 50, 75 or 100µM of t-BHP. After this period the cultures were stained with 1µg/ml of PI and 20µM of DIOC6 (3). After 20 minutes of contact the samples were applied to a FACs and the membrane potential was determined. **Note:** (■) t-BHP

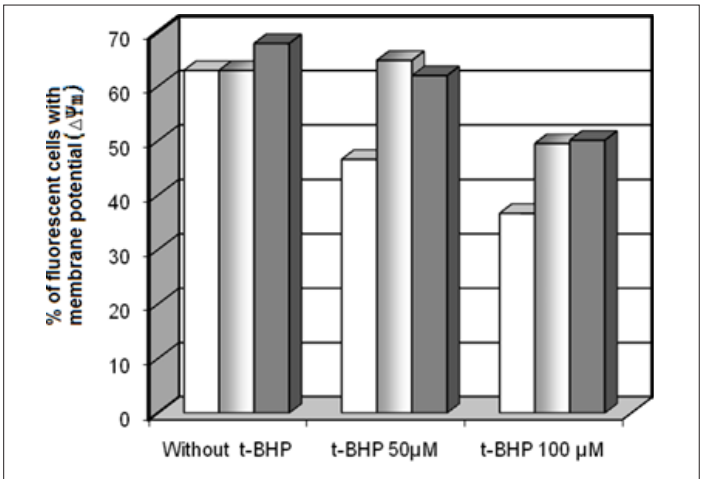

Figure 7: Hemolymph effect in the membrane potential of cells HEK-293. The cells previously treated or not with 1% (v/v) of total or purified hemolymph. After this period the cellular death of the cells was induced by 4 hours with 50 or 100µM of t-BHP. After this period the cultures were stained with 1µg/ml of PI and 20µM of DIOC6(3). After 20 minutes of contact the number of cells showing high fluorescence was determined. Note: (□) Control; (■) Hemolymph; (■) Purified Fraction

Determination of potential of the mitochondrial membrane in the HEK-293 using JC-1/Hoechst 33324 staining after cell death induction by t-BHP and H202

In the Figure 8 is showed a comparison between the protector effect of hemolymph and the purified fraction in the HEK-293 cells culture treated with 50 µM of t-BHP or 600 µM of H202. To this, HEK-293 cells grew adhered on the glass slides and treated with total hemolymph or purified fraction 1% (v/v). After 1 hour, cell's death was induced with 50 µM of t-BHP or with 600 µM of H202, and maintained overnight. Then, the cells were marked with 1 µg/ml of PI and 20 µM of JC-1 and Hoechst. The potential of the mitochondrial membrane (A) and the number of nucleous (B) with apoptotic morphology respectively were determined by fluorescent microscope. As can be observed, there is a good correspondence between the potential of the mitochondrial membrane and the number of nuclei with apoptotic morphology.

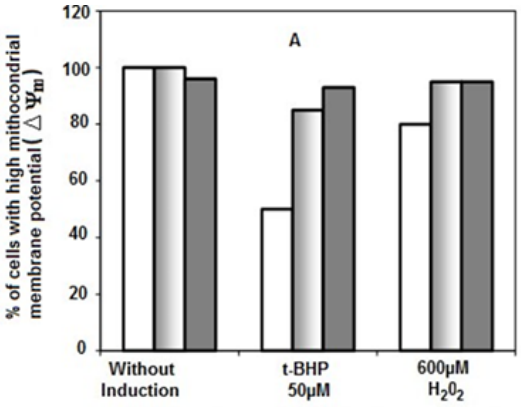

Figure 8: Effect of the hemolymph in the protection of the cellular death by apoptosis chemical inductor. HEK-293 cells previously treated or not with 1% (v/v) of total or purified hemolymph. After this period the cellular death was induced with 50µM of t-BHP or 600µM of H202 overnight. After this period the cultures were marked with 1µg/ml of PI and 20µM of JC-1 and Hoechst. After 20 minutes of contact the samples were applied to a FACs and the membrane potential was determine A). The number of cells with fragmented nucleus was determined by fluorescence microscope. Note: (□) Control; (■) Hemolymph; (■) Fraction

As showed in Figure 9, also there is a good correspondence between the results obtained from both dyes (t-BHP (1st column) and H202 (2nd column) after JC-1 and Hoechst stain. In Figures 9A, 9B and 9E, F are shown viable HEK-293 cells showing high potential of the mitochondrial membrane. After t-BHP or H202 induction, almost all cells were stained green (Figures 9A-9F). It was correlated with percentage of fragmented cell nucleus after Hoechst stain showing that the loss of potential of the mitochondrial membrane is related with the cell death and DNA fragmentation (Figures 9G and 9H). On the other hand, when total hemolymph or its fraction was added, this effect was not observed (Figure 9K). The Figures 9I and 9J correspond to the overlap of staining with Hoechst and JC-1. In the Figures 9K and 9L are presented two pictures with overlap of the two markers, the first cell were apoptosis induced and treated with hemolymph and the second the cells were apoptosis induced but without treatment with hemolymph (Figures 9K and 9L). These figures are representative of all experiment performed with the cultures were death were inducted by t-BHP or H202 and treated or not with hemolymph. The results obtained in cultures treated with total or fraction hemolymph were identical to the normal controls without induction.

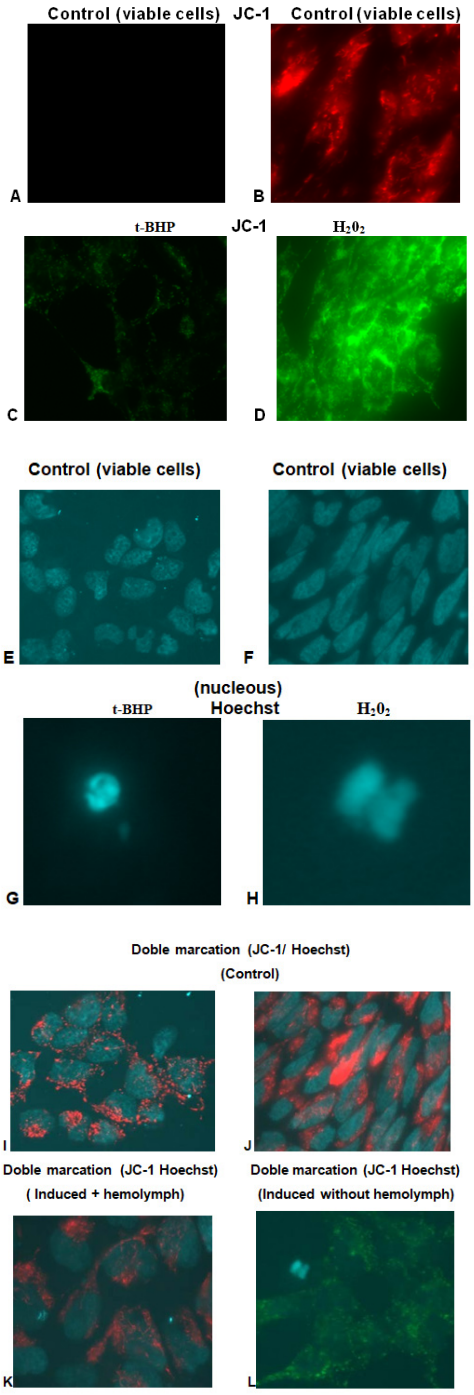

Figure 9: Photomicrography of HEK-293 cells without induction of death cell (A-B-E-F), or after induction (C-D-G-H) by chemical agents (t-BHP, 1st column or H202, 2nd column). After eighteen hour, cells were stained with JC-1(2μM) and Hoechst 33324 (2μM) for 20 minutes. The samples were observed in fluorescence microscope with an increase of 40x. In I-J is showed the sobreposicion of the two colors in normal culture (without induction). In Figure K, the culture were apoptosis induced and after this treated with hemolymph (sobreposicion of the two colors). In Figure L, the culture were apoptosis induced but without hemolymph treatment (sobreposicion of the two colors).

Identification of cytochrome C in HEK 293 cells after induction of apoptosis and the protective action of hemolymph

Cytochrome C is the first mitochondrial intermembrane protein released into the cytosol, with apoptogenic action. The literature relates that, together with dATP, cytochrome C is required for proteolytic activation of procaspase-3. The no release of cytochrome C in the cytosol in cultures treated with hemolymph is an indicator of the hemolymph action as an inhibitor of apoptosis cascade activation.

It is reported that the action of Bcl-2, a protein witch known for anti-apoptotic action, is exactly at mitochondrial membrane level. To determine the hemolymph effect in the protection of apoptosis induced by t-BHP, HEK 293 cells were grown in 12 wells microplate. After semi confluence, cells were treated with 1% (v/v) of Purified Hemolymph Fraction (PRF) showing anti-apoptotic activity. After contact by one hour the cells were treated with 25, 50 or 75 μM t-BHP. The cultures were kept in CO_2 incubator for 4 hours and after this period washed with PBS and fixed in paraformaldehyde (4%) and picric acid (0.18%) and incubated for 45 minutes. The cells were then washed with PBS and treated with 3% BSA and incubated for 30 minutes. The cells were again washed with PBS and labelled with monoclonal anti cytocromo C for 2 hours. The reaction was revealed with an anti-mouse IgG alkaline phosphatase conjugate for 75 minutes. After this, was added 20 μM of Hoechst and the cell culture was observed in a fluorescence microscope (Leica DMRB microscope). The results are shown in Figure 10. Was observed that, as in control cultures (without death's induction) as well in induced cultures, but treated with hemolymph, a high fluorescence in the mitochondria membrane. However, in cultures were the induction was performed, but without hemolymph treatment, this fluorescence was not observed. With the increase of the membrane porosity, and with consequent release of cytochrome C in citosol, the cytochrome C is distributed in a diffuse mode and cannot be clearly identified.

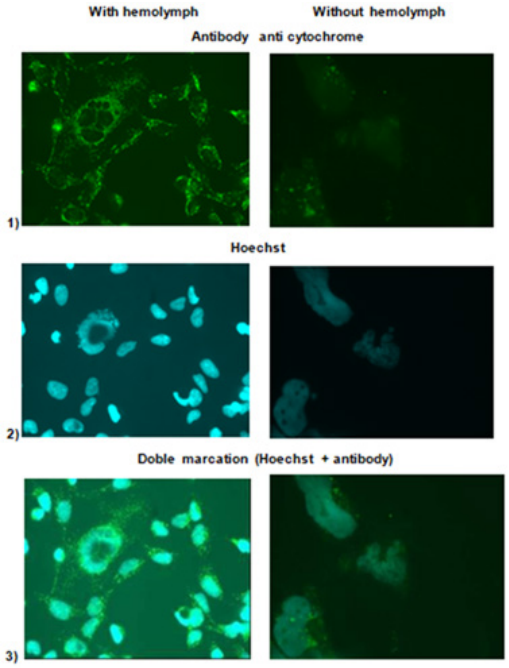

Figure 10: Photomicrography of HEK-293 cells cultured in microplate of 12 wells. After semi confluence the cells were treated with 1% (v/v) of the purified fraction of the hemolymph (Frp) showing antiapoptotic action. After one hour of contact the cells were treated with 25, 50 or 75μM of t-BPH. The cultures were maintained with CO2 by 4 hours and after this period washed with PBS and fastened with paraformaldeid 4% and picric acid 0, 18% and incubated by 45 minutes. The cells were then stained with the antibody monoclonal anti cytochrome C 2. The reaction was revealed with an antibody anti mouse IgG conjugated with alkaline fosfatase by 75 minutes. Was added then 2μM of Hoechst and the cultures were observe in a fluorescence microscope with an increase of 40x. (A) Cells treated with Frp (B) cells no treated with hemolymph. Both were induced with t-BHP.

DISCUSSION

Mitochondria are involved in many essential processes for cell survival, including energy production, redox control, calcium homeostasis and certain metabolic pathways and biosynthesis. In addition, the mitochondria have an essential role in the process of cell's death known as apoptosis. This mitochondrial pathway of apoptosis may suffer dysfunctions causing various diseases such as cancer, diabetes, ischemia and neurodegenerative disorders like Parkinson's and Alzheimer's [4]. In one of the classic routes of apoptosis induction, the activation of caspases is directly related to Mitochondrial Outer Membrane Permeabilization (MOMP). Many signals that generate pro-apoptotic molecules and pathological stimulus converge on mitochondria and induce MOMP. The local regulation and for the execution of MOMP involve several types of proteins such as Bcl-2 family, mitochondrial lipids, proteins that regulate the metabolic flow and other components that act on the membranes pores. The MOMP is a lethal process because results in the release of capsize activating molecules and effectors of death that are caspase-independent. Some drugs that suppress the MOMP can prevent cells death or, when necessary, as in the case of cancer, restore the apoptosis process [11].

During the transduction of apoptosis signal, there is a change in the mitochondria membrane permeability, which causes the translocation of cytochrome C and apoptogenic proteins in the cytoplasm activatingb proteolytic proteins known as caspases. Shimizu et al. has demonstrated that some pro-apoptotic proteins (Bax, Bak, etc.) would act on some Voltage Dependent of the Anion Channels (VDAC) accelerating the opening of these channels and increasing the permeability of the membrane, allowing that pro apoptotic activators factors of caspases proceed to the cytosol [25]. On the other side, they also demonstrate that anti-apoptotic proteins family, known as Bcl-x (L) closes these channels by binding directly to them. While the proteins Bax and Bak allow cytochrome C to pass through these channels VDAC, keeping them open, the protein Bcl-x (L) prevents this occurring, regulating thus the mitochondrial membrane potential ($\Delta\psi m$), blocking the release of cytochrome C during apoptosis-inducing events. On the other hand, Shimizu et al. has shown that in some cases as the BH3 proteins (Bid and Bik) may have apoptogenic induction releasing cytochrome C by mechanisms other than those requiring the direct link to VDAC. Antibodies that inhibit VDAC prevent cytochrome release by the action of Bax avoiding the loss of mitochondrial membrane permeability [24]. However, this is not observed with induction of release of cytochrome C by Bid. These anti-VDAC antibodies also inhibit apoptosis induced by chemical agents, inducers of apoptosis such as etoposide, paclitaxel and staurosporine. However, Cartier et al. observed that, despite the fact that family of the proteins Bcl-2 are very efficient in blocking apoptosis in mammalian cells they fail to prevent death's induced by actinomycin-D or induced by baculovirus (AcMNPV) [8]. Unlike what happens with the Bcl-2, p35, a potent inhibitor of apoptosis present in the genome of baculovirus, prevents apoptosis induced by this class of virus suggesting that the mechanism of action of two regulators may be functionally distinct. Due numerous mechanisms of the death apoptosis activation, the determination of the action mechanism of various anti-apoptotic proteins, particularly those of a broad spectrum of action (which inhibit death by different inducers or preventing this death in different types of cells) may be of high medical and biotechnological interest.

We have reported earlier the presence of anti-apoptotic protein present in the hemolymph of Lonomia oblique with his supplementation in cell's culture inducing high levels of cell growth and that the cells viability can be maintained for longer periods [16,18,19,26,30]. However, contrary that occurs with the anti-apoptotic Bcl-2 family proteins, has the ability to inhibit apoptosis in different types of cells, whether mammal, either insect, by different inducers [25,26,30]. The present data suggests that the site of action of this protein could be similar to Bcl (x) proteins, since that, in all experiments, as we observed a blockade of apoptosis, with clear protection of mitochondrial membrane potential. The action of hemolymph on the cells is clearly demonstrated in early experiments, whether in the cell viability increase, either in the increased of the recombinant proteins production [30]. This action, at least in part related to an anti-apoptotic protein, which is evident in with the cell's death blocking, the cell remains functional (Figures 4-8). In this case we have observed increasing the viral title or in the recombinant proteins production [19]. As demonstrated throughout the study, apparently the site of action of this protein could be on the membrane of mitochondria, maintaining so, the high membrane potential, avoiding the membrane permeability. This anti-apoptotic protein not only blocks death induced by the virus, but also death's that normally occurs by physiological stress culture, as observed in control culture (Figure 8). In this case, the cell's death observed in the control culture was reduced by half, indicating that this protein could also be used as a supplement to culture media. The protein responsible for this effect has been identified as a protein with a molecular weight of 51 kDa [26]. The protective effect of p51 protein is evident in Figures 4-8, where death was induced with a potent cell death inducer (t-BHP or H202). Under these conditions p51 was able to inhibit the death induced in insect cells (Sf-9) at concentrations above 50 μM t-BHP or 400 μM of hydrogen peroxide.

Flow cytometry proved an excellent tool to characterize different cell populations. This technique was used in all experiments used to determination of potential of the mitochondrial membrane as well apoptosis cell's death (Figures not showed). The definition of the different cells population (viable, necrotic and apoptotic cells) were determined by information obtained from literature. Apoptotic cells show small size (low FSC) and high granularity (high SSC) and intensely are stained by PI. These cells showed too low potential of the mitochondrial membrane. Necrotic cells are stained by PI. The differentiation between necrotic from apoptotic death is that in necrotic cells shown a normal size with a high potential of the mitochondrial membrane. Normal cells show a high viability, high potential of the mitochondrial membrane and are not labelled with PI. Was observed a material showing low size and low granularity and are not stained by any markers. These materials are cellular debris or apoptotic bodies. The characteristic of each of these populations has been confirmed by other techniques such as electron microscopy, and agarosis gel electrophoresis to determine DNA fragmentation [22, 28]. The determination of DIOC6 (3) was effective potential of the mitochondrial membrane, determination. However, to confirm that the hemolymph action site is really on the mitochondria, we used a second dye (JC-1). This stain been widely used by other researchers for this purpose. The results obtained with this marker were similar to those obtained with the DIOC6 (3). Also was observed, in all the experiments, cell death in the control culture (without induction) when the incubation is performed overnight.

Nevertheless, when hemolymph was added to cell culture it was able to inhibit virtually 100% of the harmful effect observed in culture.

The cytochome C is the first protein intermembrana mitochondrial liberated in the cytosol, with action apoptogenic. It is mentioned in the literature that, together with the dATP, the cytochrome C is necessary for the proteolitic activation of the procaspase-3. If the cytochrome C is not liberated in the cytosol in the cell's culture treated with hemolymph, it would be an indicator of the hemolymph actions in the inhibition of this cascade need to apoptosis activation. According Zou et al., the cytochrome C attached to Apaf-1, which in presence of ATP (or dATP), promote the activation of the procaspase-9, generating the activated caspase-9 [15,31-33].

CONCLUSION

Therefore, caspase-9 act on the procaspase-3 to begin the activation of the caspase cascade. If the hemolymph were acting about the liberation of the cytochrome C in the cytosol, as we suspected, there would be a suppression of the activation of the caspase-3, blocking this way the apoptosis induction. This blocked of the cytochrome C maintains the functionality of the mitochondria, avoiding the lack of energy for the cell, and therefore the cellular death by apoptosis. In this study, we demonstrate the action of hemolymph on the mitochondria and release of cytochrome C in cytosol. As expected, when cultures were treated with hemolymph, there was no release of cytochrome C, which remained fixed on the membrane of mitochondria, generating an intense fluorescence. Meanwhile, in cultures induced with t-BHP and non-treated hemolymph, this was not observed.

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