Autophagy protects against neural cell death induced by piperidine alkaloids present in *Prosopis juliflora* (Mesquite)

VICTOR D.A. SILVA[1], CARLOS CUEVAS[2], PATRICIA MUÑOZ[2], MONICA VILLA[2], ULISES AHUMADA-CASTRO[2], SANDRO HUENCHUGUALA[2], CLEONICE C. DOS SANTOS[1], FILLIPE M. DE ARAUJO[1], RAFAEL S. FERREIRA[1], VANESSA B. DA SILVA[1], JULIANA H.C. E SILVA[1], ÉRICA N. SOARES[1], EUDES S. VELOZO[3], JUAN SEGURA-AGUILAR[2] and SILVIA L. COSTA[1]

[1] Laboratório de Neuroquímica e Biologia Celular, Avenida Reitor Miguel Calmon, s/n, Universidade Federal da Bahia, Instituto de Ciências da Saúde, Departamento de Bioquímica e Biofísica, 40110-100 Salvador, BA, Brazil
[2] Farmacologia Molecular e Clínica, Avenida Independência 1027, Universidade do Chile, Faculdade de Medicina, 8380453, Santiago, Chile
[3] Laboratório de Pesquisa em Matéria Médica, Rua Barão de Jeremoabo, 147, Universidade Federal da Bahia, Faculdade de Farmácia, 40170-115 Salvador, BA, Brazil

ABSTRACT

Prosopis juliflora is a shrub that has been used to feed animals and humans. However, a synergistic action of piperidine alkaloids has been suggested to be responsible for neurotoxic damage observed in animals. We investigated the involvement of programmed cell death (PCD) and autophagy on the mechanism of cell death induced by a total extract (TAE) of alkaloids and fraction (F32) from *P. juliflora* leaves composed majority of juliprosopine in a model of neuron/glial cell co-culture. We saw that TAE (30 μg/mL) and F32 (7.5 μg/mL) induced reduction in ATP levels and changes in mitochondrial membrane potential at 12 h exposure. Moreover, TAE and F32 induced caspase-9 activation, nuclear condensation and neuronal death at 16 h exposure. After 4 h, they induced autophagy characterized by decreases of P62 protein level, increase of LC3II expression and increase in number of GFP-LC3 cells. Interestingly, we demonstrated that inhibition of autophagy by bafilomycin and vinblastine increased the cell death induced by TAE and autophagy induced by serum deprivation and rapamycin reduced cell death induced by F32 at 24 h. These results indicate that the mechanism neural cell death induced by these alkaloids involves PCD via caspase-9 activation and autophagy, which seems to be an important protective mechanism.

Key words: programmed cell death, autophagy, glial cells, neurons, piperidine alkaloids, *Prosopis juliflora*.

INTRODUCTION

Prosopis juliflora (Swartz) DC (Algarrobo, Mesquite) is a shrub native to Central America, northern South America and the Caribbean islands

(Burkart 1976) that has become seriously invasive in other regions throughout the world such as India, Saharan and southern Africa, the Middle East, Pakistan and Hawaii (USA) (Pasiecznik et al. 2001). In Brazil, this plant was introduced in the 1940s and has been used to feed cattle, sheep, goats, chickens and equine (Moreno et al. 2005,

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Correspondence to: Silvia Lima Costa
E-mail: costasl@ufba.br

Rossi et al. 2009, Silva et al. 2002a, 2002b, Stein et al. 2005). It has also been used for human consumption in breads, biscuits, jellies, sweets, and coffee drink substitutes (Choge et al. 2007, Vieira et al. 1995).

This species contains steroids, alkaloids, coumarins, flavonoids, sesquiterpenes, and stearic acid (Almaraz-Abarca et al. 2007). Extracts of *P. juliflora* seeds and leaves have several *in vitro* pharmacological effects such as antibacterial (Ahmad et al. 1995, 1986, 1989b, Al-Shakh-Hamed and Al-Jammas 1999, Cá-Ceres et al. 1995, Kanthasamy et al. 1989, Satish et al. 1999), antifungal (Ahmad et al. 1989a, Kanthasamy et al. 1989, Kaushik et al. 2002), and anti-inflammatory properties (Ahmad et al. 1989b) and has been shown to stimulate the immune system (Ahmad et al. 1992) and function as an acetylcholinesterase inhibitor and butyrylcholinesterase inhibitor with Ca^{2+} channel blocking activity (Choudhary et al. 2005) and anti-helminthic properties (Batatinha et al. 2011). These properties have been attributed to piperidine alkaloids whose structure is compounded by two piperidine rings that are connected with an indolizidine moiety through two aliphatic chains (Ahmad 1986, Ahmad and Mohammad 1979, Ahmad and Qazi 1985, Ahmad et al. 1989c). The main biologically active piperidine alkaloids from *P. juliflora*, 3''''-oxo-juliprosopine, 3-oxo-juliprosine and 3'-oxo-juliprosine, present chemical functional groups (carbonyl radical) at carbon 3'''' of the indolizidine ring or carbon 3 or 3' of the piperidine rings, respectively (Nakano et al. 2004). A synergistic action of piperidine alkaloids has been suggested to be responsible for the neurotoxic damage observed in cattle and goats after intensive ingestion of *Prosopis juliflora* (Câmara et al. 2009, Hughes et al. 2006, Silva et al. 2007, 2013, Tabosa et al. 2000a, 2000b).

Tabosa et al. (2000a) found that the toxic activity observed in mice is chemically related to piperidine alkaloids such as juliprosopine present in the pods of this Leguminosae. We have previously demonstrated that a fraction from leaves composed of the piperidine alkaloid juliprosopine, as the major constituent, amplifies the neurotoxic response induced by a total alkaloid extract (TAE) in neurons and glial cells *in vitro* (Silva et al. 2007, 2013). This response was best characterized in a model of neuron/glial cell co-culture by neuronal death, astrogliosis, microgliosis, cytoskeleton disruption, mitochondrial damage and vacuolation induction (Silva et al. 2013). Maioli et al. (2012) investigated the action of juliprosopine in isolated rat brain mitochondria and determined that it stimulated state-4 respiration, affected the membrane potential and ATP production, and that these changes were mainly attributed to modifications in the inner mitochondrial membrane. However, the involvement of these cellular alterations and the mechanism of death induced by these alkaloids have not yet been clarified. In this context, in this study, we featured the vacuolization induced by alkaloids of *Prosopis juliflora* as vacuolization type autophagy and investigated the involvement of mitochondrial damage and autophagy on the mechanism of cell death induced by these alkaloids using a total alkaloid extract (TAE) and an alkaloid fraction (F32) extracted from leaves of *P. juliflora* in a model of neuron/glial cell primary co-culture.

MATERIALS AND METHODS

OBTAINING THE TOTAL ALKALOID EXTRACT (TAE) AND ALKALOIDAL FRACTION (F32)

Prosopis juliflora leaves were collected at the experimental fields of the Federal University of Bahia (UFBA) in Salvador (BA), Brazil, and dehydrated in a greenhouse at 50°C. The dried leaves (874 g) were extracted three times with hexane (2 L/kg) for 48 h at room temperature with sporadic shaking to remove apolar constituents. The extract was filtered, and the residue was submerged in methanol (1.5 L/kg) using the above

process. An alkaloidal extract was obtained by an acid/base modified extraction as described by Ott-Longoni et al. (1980). The methanol extract was concentrated in a rotary evaporation system at 40°C, and this concentrated residue was agitated with 0.2 N HCl for 16 h, followed by filtration. The solution was agitated with chloroform to eliminate the non-basic material. The aqueous layer was basified with ammonium hydroxide until it reached pH 11 and was then extracted with chloroform. The chloroform phase was evaporated, leading to the production of the TAE. This extract was fractionated by chromatography in a silica gel column using chloroform/methanol (99:1 to 1:1) as a solvent system with a subsequent 100% methanol elution. Thirty-six fractions were obtained from TAE, and after thin layer silica gel chromatography, they were developed with iodine and tested for the presence of alkaloids by the Dragendorf's test (Wagner et al. 1983). Alkaloidal fractions with the same chromatography profile were assembled and designated F21/22/23, F25/26, F27, F29/30, F31/33, F32 and F34/35.

Fraction F32, composed of juliprosopine and juliprosine, was characterized by nuclear magnetic resonance of 1H (500 MHz, CD3OD) and 13C NMR (125 MHz, CD3OD) by Silva et al. (2013). The TAE and F32 were dissolved in dimethylsulfoxide (DMSO, Sigma, St Louis, MO) at a final concentration of 30 mg/mL and stored in the dark at -20°C. In these conditions, the biologically active compounds were stable and there was not significant variation of the results due to TAE or F32 produced at different days.

NEURON/GLIAL CELL PRIMARY CO-CULTURES

Glial cell primary cultures were prepared according to Cookson and Pentreath (1994) and performed according to Brazilian guidelines for production, maintenance and use of animals for teaching activities and scientific research (Brasil 2015) and the local Ethical Committee for Animal

Experimentation, protocol number (0272012, ICS - UFBA). Briefly, cerebral hemispheres from one-day-old postnatal Wistar rat pups were isolated aseptically, and the meninges were removed. The cortex was dissected out and then gently forced through a sterile 75-µm Nitex mesh. Cells were suspended in DMEM HAM's F12 medium (Cultilab), supplemented with 100 IU/mL penicillin G, 100 µg/ml streptomycin, 2 mM L-glutamine, 0.011 g/l pyruvate, 10% FCS, 3.6 g/L HEPES and 33 mM glucose (Cultilab) and cultured in 100-mm Ø plates in a humidified atmosphere with 5% CO_2 at 37°C. Culture medium was changed every two days, and cells were cultured for 15 days. Cells were then trypsinized (Trypsin EDTA) and plated at a density of 670 cells/mm^2 and maintained for 48 h for culture stabilization. At this time, neurons obtained from cerebral hemispheres of 15-18-day-old Wistar rat embryos using the same method described above for glial culture were suspended in supplemented DMEM/HAM's F12 and seeded at half the amount of glial cells (335 cells/mm^2) onto the astroglial monolayer. Cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C for 8 days, when treatments or transfection were performed.

TREATMENT

The TAE and F32 stock solutions were diluted in the medium to final concentrations of 30 µg/mL and 7.5 µg/mL, respectively. Cells were treated with TAE or F32 for 4, 12, 16 or 24 h. The negative control group was treated with DMSO diluted in the culture medium at the equivalent volume used in the treated groups (0.1%) and showed no significant effect on the analyzed parameters compared to cells that did not receive diluents.

MEASUREMENT OF ATP

ATP level was measured using a Cell-Titer-Glo® Luminescent Cell Viability Assay (Promega,

Madison, WI, USA). Cells that had been treated with or without TAE or F32 for 12 h were trypsinized and transferred to a 96-well plate in eight replicates. A volume of 100 μL of the reaction reagent was added to each well, and after 10 min, the luminescence signal was detected using an ELISA reader at 562 nm (Varioskan™ Flash Multimode Reader, Thermo Plate). Possible differences between quantities of cells in the different samples were equalized after protein determination by BSA reagents and the subsequent calculation of the ratio between μg of ATP per μg of protein.

DETERMINATION OF MITOCHONDRIAL MEMBRANE POTENTIAL

The cells were incubated as described above for 12 h at 37°C in absence (0.1% DMSO) or in presence of 30 μg/ml TAE or 7.5 μg/ml F32. The cells were incubated in the dark for 40 min at room temperature with freshly prepared 1.5 μg/ml JC-1(5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazo-lylcarbo- cyanine iodide) in PBS and then washed three times with PBS. The cells were observed immediately after labeling using a confocal microscope (Axiovert-100; Zeiss, Göttingen, Germany) to analyze the intensity of fluorescence simultaneously for the green monomeric form at 488 nm (excitation) and 510–525 nm (emission) and for red fluorescent aggregates- JC-1 at 543 nm (excitation) and 570 nm (emission).

NUCLEAR CHROMATIN STAINING

After 16 h of F32 exposure, co-cultures were rinsed three times with PBS and fixed with cold methanol at -20°C for 10 min. Nuclear chromatin was stained with the fluorescent dye DAPI, at a final concentration of 5 μg/mL in PBS, for 10 min at room temperature in a dark chamber. Thereafter, cells were analyzed by fluorescent microscopy (Olympus BX70) and photographed using a digital camera (CE Roper Scientific). The percentage of cells presenting nuclear condensation was

quantified as the ratio of the number of condensed nuclei per total nuclei per photographed field.

FLUORO-JADE B STAINING

Neuronal death was assessed by Fluoro-Jade B staining. After 16 h of TAE and F32 exposure, co-cultures were rinsed three times with PBS and fixed with cold methanol at -20ºC for 10 minutes. After, cells were rehydrated with PBS for 30 min, and permeabilized with Triton X-100 (0.3%) for 10 min. Then, the staining solution (0.001%) was prepared from a 0.01% stock solution of Fluoro-Jade B (Histo-Chem Inc., Jefferson AR) solubilized in distilled water, and the cells were incubated for 30 min at room temperature, that was followed by one rinse with PBS and nuclear chromatin stained with the fluorescent dye DAPI. Thereafter, cells were analyzed by fluorescent microscopy (Olympus BX70) and photographed using a digital camera (CE Roper Scientific). The percentage of non viable neurons was quantified by the ratio between measurements of number of cells stained with Fluoro jade B (green cells) and nuclei stained with DAPI (blue) per photographed field.

GFP-LC3 PLASMID TRANSFECTION

Neuron/glial cell primary co-cultures were performed on glass cover slips in 24-well polystyrene plate (TPP Switzerland) at same density of cells used in other methods (670 cells/mm² glial cells more 335 cells/mm² neuron) were transfected with GFP-LC3 plasmid (gift from Zsolt Tallocky PhD, Columbia University Medical Center). To form the transfection complex, we used 2 μl Fugene HD transfection reagent (Roche Diagnostics) and 0.5 μg GFP-LC3 plasmid DNA in 25 μl total volume of medium for each plate, and it was kept in the dark for 15 min. Thereafter, the transfection complex in 150 μl culture medium was added to each plate and incubated for 24 h. Then, the culture medium was changed and the cells were cultured for more 4 h before treatment with TAE and F32. To control the

success of the transfection process untransfected cells were submitted at same treatment and analysis conditions.

Cover slips were mounted on to slides with fluorescent mounting medium (Dako, Carpinteria, CA) and kept in the dark at 4°C. Confocal microscope model LSM-410 (Axiovert-100; Zeiss, Göttingen, Germany) was used to study the cells. Sample illumination was carried out via a He–Ne laser with 543-nm excitation filter and emission filter over 560 nm.

Nuclear chromatin of fixed cells was stained with the fluorescent dye DAPI as described in section 2.6.

LC-3 II EXPRESSION, CASPASE-9 ACTIVATION AND P62 LEVEL DETERMINATION

LC3-II expression and caspase-9 activation were investigated by western immunoblot. After F32 treatments, cells were rinsed twice with PBS, harvested, and lysed in 2% (w/ v) SDS, 2 mmol/L EGTA, 4 mol/L urea, 0.5% (v/v) Triton X-100, and 62.5 mmol/L Tris-HCl buffer (pH 6.8) supplemented with 0.1% (v/v) of a cocktail of protease inhibitors (Sigma). Protein content was determined by a method adapted from Lowry et al. (1951) with a DC protein assay reagent kit (Bio-Rad, Hercules, CA, USA). For analysis, 15 μg protein, prepared as described above, was loaded onto a discontinuous 4% stacking and 12% running SDS polyacrylamide gel. Electrophoresis was performed at 200 V for 45 min. Proteins were then transferred onto a polyvinylidene fluoride membrane (PVDF, Immobilon-P, Millipore), at 100 V for 1 h. Equal protein loading was confirmed by staining the membranes with Ponceau red (Sigma). Thereafter, membranes were blocked for 1 h at room temperature in 20 mmol/L Tris-buffered saline (pH 7.5) containing 0.05% Tween 20 (TBS-T) and 5% powdered skim milk. Subsequently, membranes were incubated with rabbit anti- LC3B (ab51520) (1:3,000, Abcam) or rabbit anti-caspase-9

(ab25758) (1:200, Abcam), diluted in TBS-T containing 1% powdered skim milk, overnight. Mouse anti-Alpha tubulin antibody (1:1,000, Santa Cruz Biotechnology) was used as an internal standard band. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG (1:10,000 in TBS-T, Bio-Rad) was used as a secondary antibody. Immunoreactive bands were visualized with Rx exposition film by chemiluminescence immunoassay using the Immune Start HRP substrate kit (Bio-Rad). Quantification was obtained by scanning densitometry (ScanJet 4C, Hewlett Packard) of three independent experiments, and analyzed with ImageJ 1.33u software (Wayne Rasband, National Institutes of Health, USA).

Ubiquitin-binding protein p62 level was determined by Dot-Blot. A PVDF membrane was placed on *Minifold I 96-Well System* (GE) and 100 μL 20 mmol/L Tris-buffered saline (pH 7.5) was added to each well prior addition of 25 μg protein determined by a method adapted from Lowry et al. (1951) with a DC protein assay reagent kit (Bio-Rad, Hercules, CA, USA). Vacuum was applied and when the PVDF membrane was dried, the membrane was incubated in 20 mmol/L Tris-buffered saline (pH 7.5) containing 0.05% Tween 20 (TBS-T) and 5% powdered skim milk. Subsequently, membrane was incubated with rabbit anti-SQSTM1 antibody (sc-25575) (1:500, Santa Cruz Biotechnology), followed by washes whit TBS-T and incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10,000 in TBS-T, Bio-Rad). Mouse anti-Alpha tubulin antibody (1:1,000, Santa Cruz Biotechnology) was used as an internal standard spot. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG (1:10,000 in TBS-T, Bio-Rad) were used as a secondary antibodies. Immunoreactive spots were visualized with Rx exposition film by chemiluminescence immunoassay using the Immune Start HRP

substrate kit (Bio-Rad). Quantification was obtained by scanning densitometry (ScanJet 4C, Hewlett Packard) of three independent experiments and analyzed with ImageJ 1.33u software (Wayne Rasband, National Institutes of Health, USA).

CELL VIABILITY ASSAY

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) test and membrane integrity analysis using Trypan Blue staining method. The MTT test was performed on 96-well plates (TPP Switzerland) with neuron/glial cell co-culture. The cells were incubated with 7.5 μg/mL F32 or 0.025% DMSO (control) for 4, 12, 16 or 24 h. The cell viability was measured using the conversion of yellow MTT by mitochondrial dehydrogenases of viable cells to purple MTT formazan as an index. Treated and control cell cultures were incubated with MTT at a final concentration of 1 mg/mL for 2 h. After that, cells were lysed with 20% (w/v) sodium dodecyl sulfate (SDS) and 50% (v/v) dimethylformamide (DMF) solution (pH 4.7). Plates were incubated overnight at room temperature to dissolve formazan crystals. The optical density of each sample was measured at 540 nm using a spectrophotometer (Varioskan™ Flash Multimode Reader, Thermo Plate). The results from the MTT test were expressed as percentages of the viability of the treated groups compared to the control groups.

Moreover, floating and adherent cells, cultured in 35-mm Ø plates (TPP Switzerland), were harvested after trypsinization (trypsin 0.025%, EDTA 0.50%) and centrifuged for 5 minutes at 1,300 g. The cells were suspended in 200 μL PBS and stained with Trypan blue at a final concentration of 0.1% (v/v). The viable and non viable (blue) cells/μL were determined after 12 or 24 h exposure to 7.5 μg/mL F32 by counting the number of cells in 10 μL of cell suspension for each experiment.

The effects of autophagy inhibition on cell viability of neuron/glial cell co-cultures exposed to TAE or F32 were analyzed using preincubation with 1 μM bafilomycin A1 (a V-ATPase inhibitor) for 2 h, or preincubation with 10 μM vinblastine (a microtubule depolymerizing agent) for 24 h. The effects of autophagy induction on cell viability of neuron/glial cell co-cultures exposed to TAE or F32 were analyzed using preincubation with 10 μM ramapycin (a m-TOR inhibitor) for 24 h, or preincubation in serum deprivation conditions for 1 h.

STATISTICAL ANALYSIS

The results of DAPI staining and Fluoro-Jade B staining were expressed as the median. Kruskal-Wallis test followed Dunns Test was used to determine the significant differences among F32 and DMSO groups. The results of other assays were expressed as the mean ± standard deviation. Student's t-Test, for independent samples, was used to compare two small sets of quantitative data. One-way ANOVA followed by the Student-Newmann-Keuls test was used to determine the significant differences among groups differing in only one parameter. Values of $p < 0.05$ were considered to be significant. All analyses were performed with three independent experiments carried out with eight replicate wells.

RESULTS

MITOCHONDRIAL DYSFUNCTION

Measurement of mitochondrial membrane potential, by JC-1 staining demonstrated that neurons/glial cell primary co-cultures in control conditions (DMSO 0.1%) presented the JC-1 as a monomer in the cytosol, which stains green, and was also accumulated in the mitochondria, which stains red, as aggregates, indicating that cells had a normal mitochondrial membrane potential. Moreover, cells exposed to 30 μg/mL TAE or 7.5 μg/mL F32 for 12 h did not shown JC-1 accumulated as aggregates

in the mitochondria which indicates loss of mitochondrial membrane potential. Densitometric analysis by ImageJ 1.33u software indicated a reduction of 52.1% of arbitrary units of red/green ratio by TAE treatment and reduction of 61.2% of arbitrary units of red/green ratio by F32 treatment (Figure 1a).

The measurement of ATP revealed that after 12 h exposure ATP levels were 10.4 ± 1.2 and 9.3 ± 0.8 µg ATP per µg of protein in control cultures with the medium alone or exposed to 0.1% DMSO (vehicle control), respectively, showing that DMSO did not interfere in this parameter. However, after 12 h exposure to TAE (30 µg/mL) and F32 (7.5 µg/mL) it was detected a reduction in ATP levels to 6.6 ± 0.6 µg and 1 ± 0.5 µg, respectively (Figure 1b).

INDUCTION OF PROGRAMMED CELL DEATH (PCD)

It was found that 30 µg/mL TAE or 7.5 µg/mL F32, for 16 h, induced caspase-9 activation, as determined by an increase in the arbitrary densitometric units of fully cleaved caspase-9 (25 kDa) and procaspase-9 (45 kDa) bands and complete disappearance of the cleaved large fragment (37 kDa bands) in neuron/glial cell primary co-cultures (Figures 2a and 2b). Moreover, an increase in the number of condensed chromatin visualized by DAPI fluorescence stain in cells exposed to 30 µg/mL TAE or 7.5 µg/mL F32 for 16 h was found (Figures 2c and 2d), that was accompanied by an increase in the number of neuronal cell death visualized by Fluoro-Jade B fluorescence stain (Figures 2e and 2f).

MTT test, which measures the reduction of the tetrazolium salt (MTT) to the purple formazan by cellular dehydrogenase enzymes in living cells, demonstrated that 7.5 µg/mL F32, for a 4 h exposure, did not reduce dehydrogenase activity in neuron/glial cell co-culture. However, exposure for 12, 16 and 24 h reduced dehydrogenase activity to $52.2 \pm 4\%$; $33.8 \pm 1\%$ and $25.8 \pm 3\%$, respectively (Figure 2g). On the other hand, trypan blue staining, which assess membrane integrity, demonstrated that 7.5 µg/mL AF, for a 4 h exposure, did not reduce the number of viable cells; however, exposure for 24 h reduced the number of viable cells to 28% (Figure 2h).
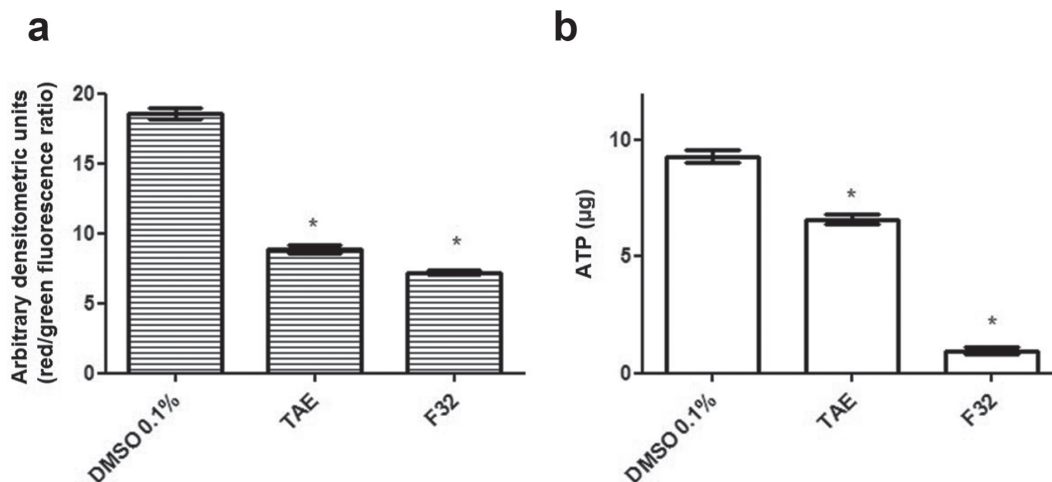

Figure 1 - TAE (30 µg/mL) and F32 (7.5 µg/mL) induce mitochondrial dysfunction after 16 h exposure in neuron/glial cell primary co-cultures. **(a)** Changes in mitochondrial membrane potential determined by JC-1 staining, confocal microscopy and quantify red/green ratio fluorescence by densitometric analysis. The values represent the means ± SD (n = 3) of the ratio between red/green fluorescence arbitrary densitometric units. **(b)** Decrease ATP production by luminescence. The values represent the means ± SD (n = 3) of the ratio between µg of ATP per µg of protein. In both, **(a)** and **(b)**, the statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Newman-Keuls (*p < 0.05).
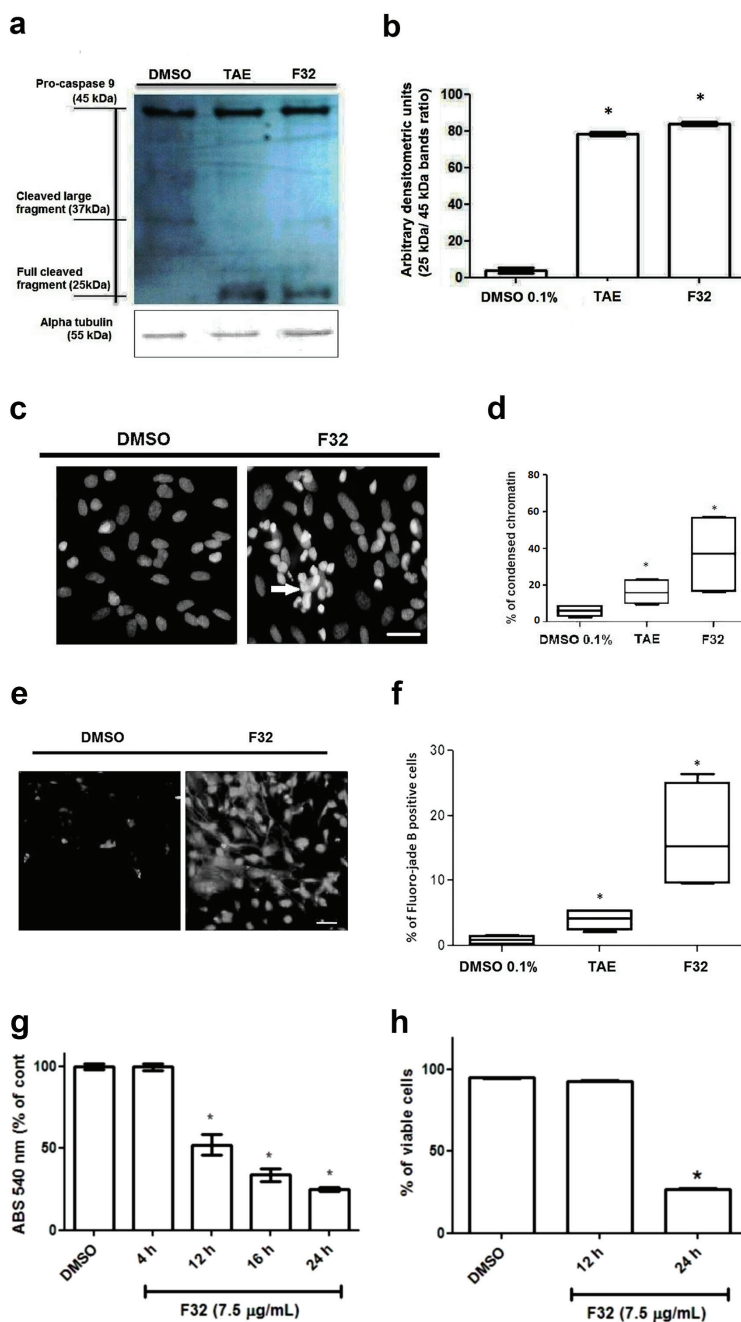
Figure 2 - TAE (30 μg/mL) and F32 (7.5 μg/mL) induce programmed cell death via caspase-9 activation after 16 h exposure in neuron/glial cell primary co-cultures. **(a)** Caspase-9 activation determined by western blotting analysis. **(b)** Densitometry analysis of immunoreactive bands using ImageJ 1.33u. The graphic represents the ratio of relative expression of fully cleaved caspase-9 (25 kDa) to pro-caspase-9 (45 kDa). **(c)** Photomicroscopy of nuclei stained with DAPI. The arrow indicates condensed nuclear chromatin. **(d)** The ratio of condensed nuclear chromatin to chromatin total number in neuron/glial cell co-cultures. **(e)** Photomicroscopy of neurons stained with Fluoro-Jade B. **(f)** The percentage of Fluoro-Jade B-positive cells. **(g)** Time-dependent effect of F32 on succinate dehydrogenase activity in neuron/glial cell co-cultures analyzed by MTT test. **(h)** Time-dependent effect of F32 on cell viability in neuron/glial cell co-cultures analyzed by trypan blue exclusion test. In **(b, h, g)**, the values represent the means ± SD (n = 3). One-way ANOVA followed by the Student-Newmann-Keuls test was used to determine the statistical significance (*p < 0.001). In **(d, f)**, the values represent the median (n = 3). Kruskal-Wallis test followed Dunns was used to determine the statistical significance (*p < 0.05). In **(c, e)**, Obj. 20x0.70, scale bars = 50 μm.

DETERMINATION OF AUTOPHAGY

We analyzed the formation autophagosome by transfecting neurons/glial cells primary co-cultures with a plasmid coding for LC3-GFP that produce green fluorescence. Incubation of transfected cells with 30 µg/mL TAE or 7.5 µg/mL F32 for 16 h induced an increase in GFP-LC3 positive fluorescence of $8 \pm 4\%$ and $30 \pm 11\%$, respectively (Figure 3a) compared to cells under control conditions (0.1% DMSO). Untransfected cells presented no GFP fluorescence.

MAP LC3β levels in the control were determined by high levels of an 18-kDa immunoreactive band (LC3-I) and low levels of a 16-kDa immunoreactive band (LC3-II) by western blot. However, in cultures exposed to 7.5 µg/mL F32 for 4 h, a change in the pattern of MAP LC3β migration was observed. There was a reduction in the levels of LC3-I and an increase in the levels of LC3-II expression (Figures 3b and 3c).

Neuron/glial cells primary co-cultures exposed to 30 µg/mL TAE or 7.5 µg/mL F32 for 4 h presented a decrease of 0.15 ± 0.06 and 0.52 ± 0.08 pixels on densitometric analysis of p62/Alpha tubulin protein level compared to cells under control conditions (0.1% DMSO) (Figures 3d and 3e).

ROLE OF AUTOPHAGY IN PIPERIDINE ALKALOIDS INDUCED CELL DEATH

To study the role of autophagy and piperidine alkaloids-induced cell death, we treated cells with TAE or F32 after autophagy inhibition or after autophagy induction. Alive and dead cells were counted with a phase contrast microscope after staining with trypan blue. A significant decrease in cell viability was observed in neurons/glial cell primary co-cultures incubated for 24 h with 30 µg/mL TAE and 7.5 µg/mL F32 (Figures 4 a-d). Interestingly, an increase of cell death was observed in neuron/glial cell primary co-cultures exposed for 24 h to 30 µg/mL TAE when they were pre-incubated with vimblastin or bafilomycin (Figure 4a). Moreover, inhibition of cell death was observed in neuron/glial cell primary co-cultures exposed for 24 h to 7.5 µg/mL F32 when they were pre-incubated with the autophagy inductor rapamycin or serum deprivation conditions (Figure 4d).

DISCUSSION

Neurohistologic and ultrastructural lesions in cattle experimentally intoxicated with *Prosopis juliflora* were characterized by vacuolation and swollen mitochondria, with the mitochondrial cristae displaced peripherally, disoriented and disintegrating in neurons of the motor cranial nerve nucleus (Tabosa et al. 2006). Studies in isolated mitochondria of rat brains showed that juliprosopine stimulates stage-4 cellular respiration and alters the mitochondrial membrane potential and ATP production by inhibiting complex I of the respiratory chain (Maioli et al. 2012). In our previous studies, we demonstrated that subtoxic doses of alkaloidal extract (TAE) induce alteration of mitochondrial shape and size and that the alkaloidal fraction (F32) induces shortened and disintegrating mitochondria cristae in neuron/glial cell co-cultures (Silva et al. 2013). Mitochondria play a fundamental role in triggering programed cell death (PDC) because mitochondrial calcium (Ca^{2+}) overload is one of the pro-apoptotic signals to induce the swelling of mitochondria, with perturbation or rupture of the outer membrane, and these responses in turn stimulate the release of mitochondrial apoptotic factors into the cytosol (Giorgi et al. 2012). In the present study, we show that TAE and F32 induce neural cell death with caspase-9 activation and nuclear condensation at 16 h, suggesting that alkaloids from *P. juliflora* induce a cell death with initial features of PCD in response to mitochondrial dysfunction.
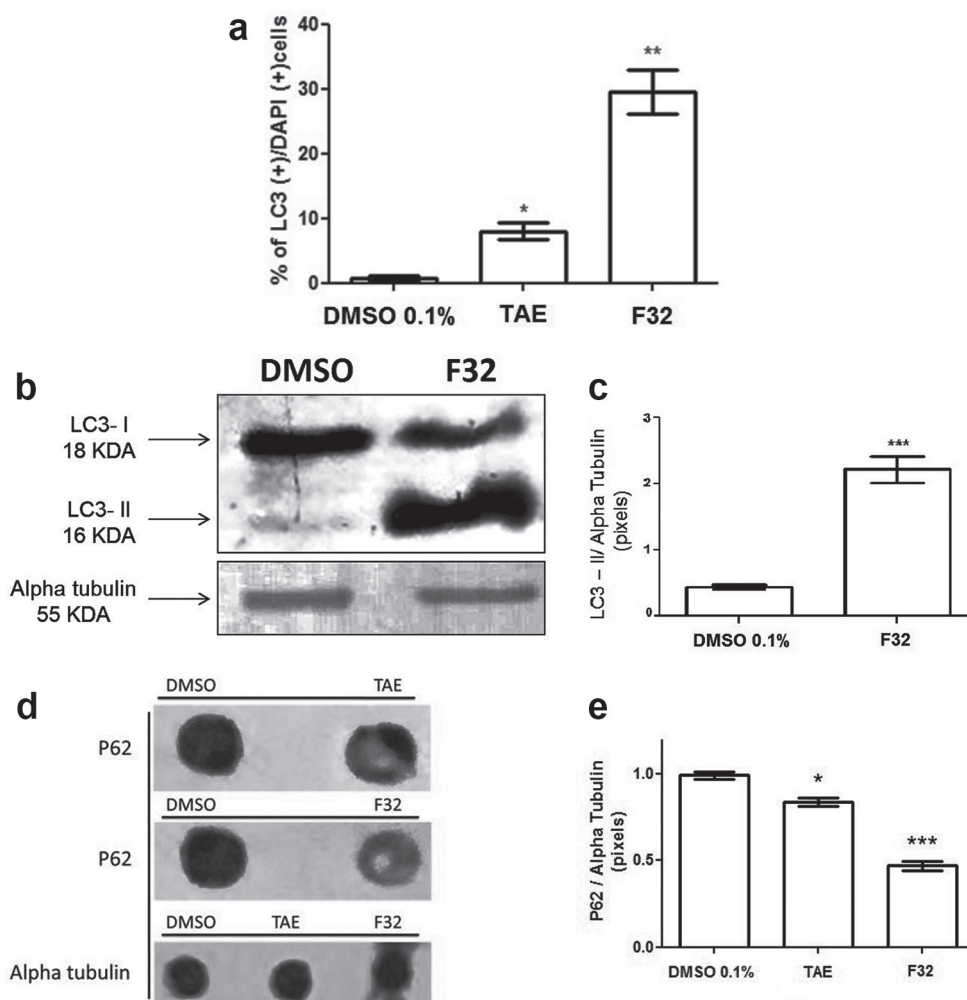
Figure 3 - TAE and F32 induce autophagy in neuron/glial cell primary co-cultures. **(a)** Determination of autophagosome vesicles in neurons/glial cell primary co-cultures transfected with GFP-LC3. Transfected cells were treated with 0.1% DMSO, 30 μg/mL TAE, 7.5μg/mL F32 for 16 h, after sample were illuminated with 543-nm excitation filter and emission filter over 560 nm to see green fluorescence. The quantification of number of cells DAPI (+) with autophagic vacuoles (LC3+) was plotted. The values represent the means ± SD (n = 3). One-way ANOVA followed by the Student-Newmann-Keuls test was used to determine the statistical significance (*p < 0.05; *p < 0.001). **(b)** Determination of LC3-II expression by western blotting analysis. Non-transfected cells were treated with 0.1% DMSO or 7.5μg/mL F32 for 4 h, after protein sample were obtained to western blotting analysis. **(c)** Densitometry analysis of immunoreactive bands using ImageJ 1.33u. The graphic represents the ratio of relative expression of LC3-II (16 kDa) to alpha tubulin (55 kDa). Student's *t*-test was used to determine the statistical significance (***p < 0.0001). **(d)** Determination of p62 expression by dot-blot analysis. Non-transfected cells were treated with 0.1% DMSO or 7.5μg/mL F32 for 4 h, after protein sample were obtained to dot-blot analysis. **(e)** Densitometry analysis of immunoreactive spots using ImageJ 1.33u. The graphic represents the ratio of relative expression of p62 to alpha tubulin. ANOVA for multiple comparisons and the Newman-Keuls test were used to determine the statistical significance. * (p < 0.05; ***p < 0.0001). The values represent the means ± SD (n = 3).

It was already known that intoxication of animals by alkaloids from *P. juliflora* induces cytoplasmic vacuolation in neurons (Tabosa et al. 2000b, 2006, Câmara et al. 2009). In a previous *in vitro* study, we characterized autophagy in astrocytes and neurons after exposure to TAE and fraction F32. We observed, after Rosenfeld staining, that exposure of neuron/glial cell cultures to TAE and F32 induced structural changes in astrocytes and an important dose-dependent
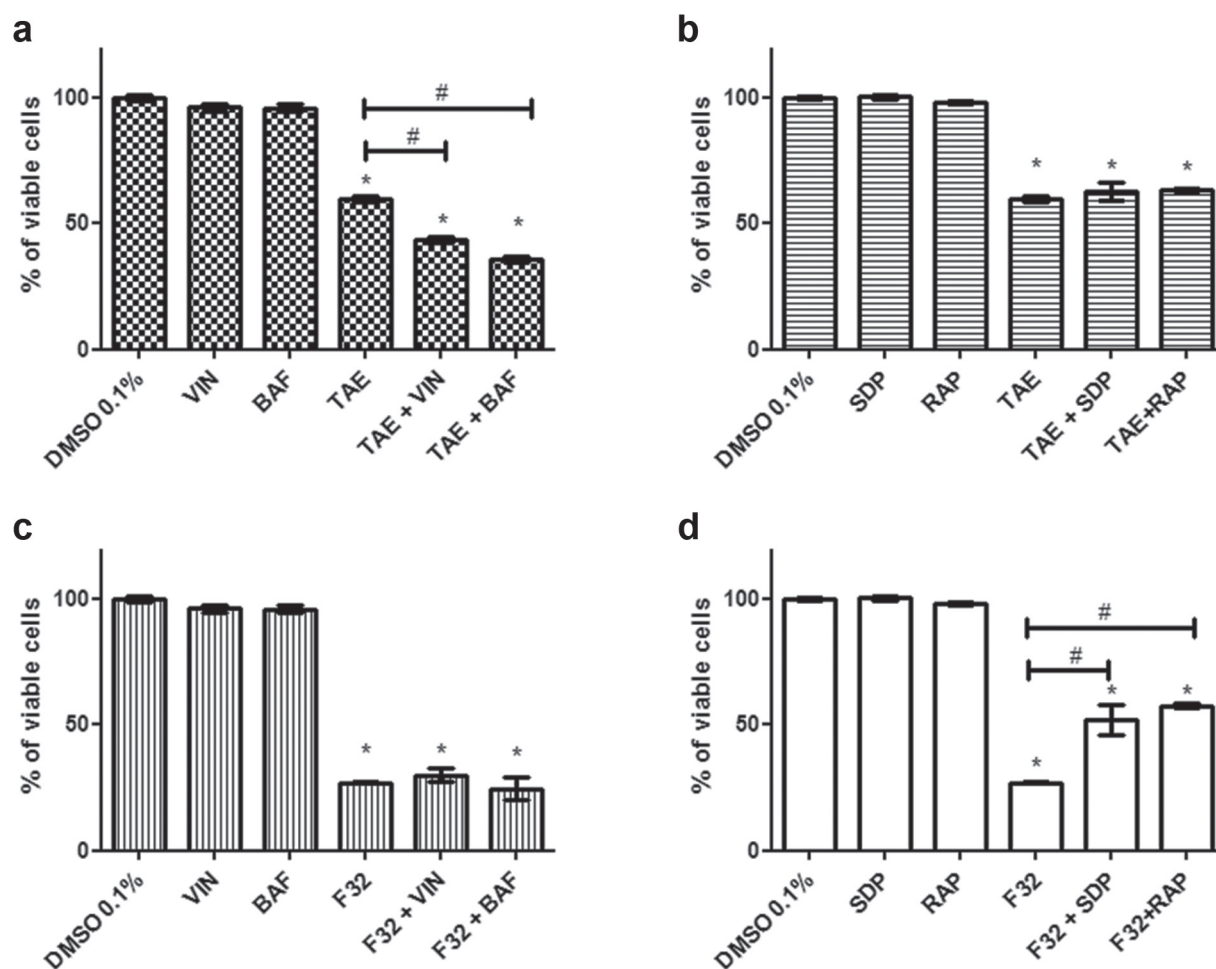
Figure 4 - Role of autophagy on TAE (30µg/mL) and F32 (7.5 µg/mL) induced cell death after 24 h exposure measured by trypan blue assay. **(a)** The effect of autophagy flux inhibited using pretreatment with 10 µM vinblastine (VIN), a microtubule depolymerizing agent, for 24 h or 1 µM bafilomycin A1 (BAF), a V-ATPase inhibitor, for 2 h, on TAE-induced cell death. **(b)** The effect of induced autophagy using pretreatment with serum deprivation conditions (SDP) for 1 h or 10 µM ramapycin (RAP), a m-TOR inhibitor, for 24 h on TAE-induced cell death. **(c)** The effect of autophagy flux inhibited by the pretreatment with VIN or BAF on F32-induced cell death. **(d)** The effect of autophagy induced by pretreatment with SDP or RAP on F32-induced cell death. ANOVA for multiple comparisons and the Newman-Keuls test were used to determine the statistical significance. * ($p < 0.05$) represents statistical significance compared to the DMSO group, and # ($p < 0.05$) represents statistical significance compared to the TAE or F32 group. The values represent the means ± SD (n = 3) of ratio of viable to nonviable cells.

cytoplasmic vacuolation, as well as a disruption in the neurite network and intense vacuolation in neurites. Immunocytochemistry for β-tubulin III, the main component of the neuronal cytoskeleton in primary cultures, confirmed the presence of neuronal vacuolation with interruptions in β-III-tubulin staining in cultures exposed F32 (Silva et al. 2013).

In the present study, we show that TAE and F32 induce a significant increase in LC3-II expression. The protein LC3 is initially synthesized in an unprocessed form, proLC3, which is converted into a proteolytically processed form lacking amino acids from the C terminus, LC3-I, and is finally modified into the phosphatidylethanolamine-conjugated form, LC3-II, which is the only

protein marker that is reliably associated with completed autophagosomes and is also localized to phagophores (Klionsky et al. 2012). Similar, the p62 protein serves as a bond between LC3 and ubiquitinated substrates and befall integrated into the completed autophagosome. In this sense, p62 is degraded in autolysosomes, thus serves as marker of autophagic degradation and autophagic flow (Larsen et al. 2010). It is known that autophagy plays an important role in the elimination of damaged organelles such as mitochondria and constitutes protection against programmed cell death induced by mitochondrial dysfunction in neuronal cells (Paris et al. 2011). The presence of LC3-II expression, decrease of p62 levels and absence of cell death in cultures exposed for 4 h to F32 and mitochondrial dysfunction, followed by caspase-9 activation, suggest an effective protective mechanism by autophagy against the cytotoxicity induced by piperidine alkaloids from *P. juliflora* (Figure 5).

Autophagy has been shown to be activated in neuronal cells in response to injury and is suggested to have a cell-protective role in neurodegenerative diseases (Paris et al. 2011, Sarkar et al. 2007). We demonstrated that cytotoxicity induced by F32 was inhibited by induced autophagy when the cells were pre-treated with rapamycin or serum deprivation conditions. Both, rapamycin and serum deprivation lead to induction of autophagy via mTORC1, which is an autophagy-suppressive regulator that integrates growth factors, nutrients and energy signals (Feldman et al. 2009, Klionsky et al. 2012). Besides, cytotoxicity induced by TAE
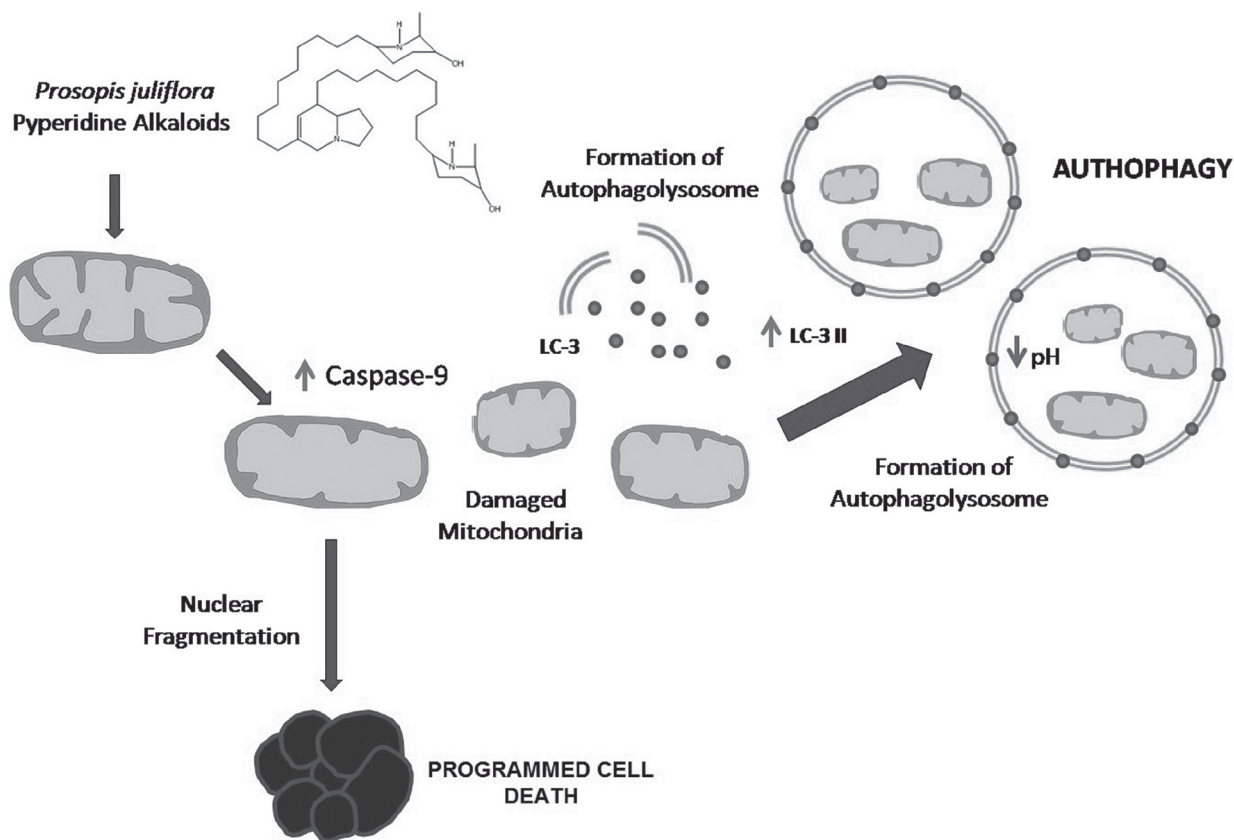

Figure 5 - Mechanisms of cell death induced by piperidine alkaloids from *P. juliflora*. We suggest that piperidine alkaloids from *P. juliflora* induce programmed cell death via the caspase-9 pathway in response to mitochondrial dysfunction. Moreover, autophagy is activated as a main mechanism to promote neural cell survival.

was amplified by inhibition of autophagy flux when the cells were pre-treated with vinblastine or bafilomicyn. These results support our hypothesis that autophagy plays a protective role against the deleterious effects of piperidine alkaloids from *P. juliflora*. We suggest that the differences in our data found in treatments with TAE and F32 was caused by a major concentration of juliprosopine and juliprosine in F32.

CONCLUSIONS

In conclusion, our results identify autophagy as the main mechanism of protection against neural cell death induced by piperidine alkaloids juliprosopine and juliprosine from *P. juliflora* at concentration adopted, associated with programmed cell death in response to mitochondrial damage.

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