Amphotericin B induces apoptosis-like programmed cell death in *Naegleria fowleri* and *Naegleria gruberi*

Roberto Cárdenas-Zúñiga,1 Ángelica Silva-Olivares,1 José D’Artagnan Villalba-Magdaleno,2 Virginia Sánchez-Monroy,3 Jesús Serrano-Luna4 and Mineko Shibayama1,*

**Abstract**

*Naegleria fowleri* and *Naegleria gruberi* belong to the free-living amoebae group. It is widely known that the non-pathogenic species *N. gruberi* is usually employed as a model to describe molecular pathways in this genus, mainly because its genome has been recently described. However, *N. fowleri* is an aetiological agent of primary amoebic meningoencephalitis, an acute and fatal disease. Currently, the most widely used drug for its treatment is amphotericin B (AmB). It was previously reported that AmB has an amoebicidal effect in both *N. fowleri* and *N. gruberi* trophozoites by inducing morphological changes that resemble programmed cell death (PCD). PCD is a mechanism that activates morphological, biochemical and genetic changes. However, PCD has not yet been characterized in the genus *Naegleria*. The aim of the present work was to evaluate the typical markers to describe PCD in both amoebae. These results showed that treated trophozoites displayed several parameters of apoptosis-like PCD in both species. We observed ultrastructural changes, an increase in reactive oxygen species, phosphatidylserine externalization and a decrease in intracellular potassium, while DNA degradation was evaluated using the TUNEL assay and agarose gels, and all of these parameters are related to PCD. Finally, we analysed the expression of apoptosis-related genes, such as *sir2* and *atg8*, in *N. gruberi*. Taken together, our results showed that AmB induces the morphological, biochemical and genetic changes of apoptosis-like PCD in the genus *Naegleria*.

**INTRODUCTION**

The protozoans *Naegleria fowleri* and *Naegleria gruberi* are ubiquitous free-living amoebae, which can be found in soil and freshwater sources; the pathogenic amoeba *N. fowleri* is responsible for the development of primary amoebic meningoencephalitis (PAM), which is a fatal disease that has been reported worldwide, mainly in healthy children and young people with a recent history of swimming activity [1–3]. However, there is currently no specific drug for the treatment of PAM. On the other hand, in some cases of recovery, amphotericin B (AmB) alone or in combination with other drugs has shown clinical efficacy against PAM [4–7]. The mechanism of action of AmB is on the cell membrane, specifically interacting with sterols and more efficiently with ergosterols, forming a hetero-polymeric aggregate that acts as a transmembrane pore that allows cytoplasmic components to leak out, ultimately resulting in cell death [8]. However, the non-pathogenic *N. gruberi* NEG, which was derived from isolated eucalyptus groves at Berkeley, CA, USA in 1959, was the first species of this genus to have its genome described [9]. It is used as a biological model to describe the metabolic versatility of the genus *Naegleria*, and the full genome of *N. gruberi* was recently used to identify novel aspects of *Naegleria* biology. Some authors have shown and identified an extensive repertoire of signalling pathways, and the capacity to alternate between aerobic and anaerobic metabolism, as well as discovering a new set of genes involved in flagellation transformation and cytoskeletal remodelling [10, 11].

Previously, Schuster and Rechthand reported an amoebicidal *in vitro* effect of AmB in *N. fowleri* and *N. gruberi* [12], describing the ultrastructural changes induced by AmB at...
non-lytic doses. The authors observed ultrastructural changes that were suggestive of PCD. However, at that time, this study was not focused on PCD. Apoptosis is the main type of PCD, which is characterized by a series of distinct morphological and biochemical changes [13, 14]. It plays a vital role in normal development in multicellular and unicellular organisms. PCD can be initiated by multiple internal and external stimuli and is coordinated by a complex network of regulator and effector molecules. Onset stimuli include cellular stress, serum or growth factor deprivation, deregulation of cell division, DNA damage and chemotherapeutic agents [15–18]. All of these stimuli are able to induce different pathways to exert a type of PCD, depending on the cellular system. In general, during the PCD of protozoans it has been difficult to identify canonical markers to describe these mechanisms [19–22]. Some differential biochemical or genetic hallmarks have also been used as alternative methods to describe this phenomenon in these microorganisms. In a study of PCD with Entamoeba histolytica, genes such as glutaminyl-tRNA synthase, saposin-like, silent information regulator-2 (sir2) and grainin were overexpressed during apoptosis, mainly due to a lack of caspase homologue genes [23].

In the present study, we showed that AmB induces apoptosis-like PCD in N. fowleri and N. gruberi trophozoites, based on several morphological, biochemical and molecular markers. The study of cellular mechanisms, such as apoptosis-like PCD in the genus Naegleria in response to AmB during in vitro treatment, allows us to understand the cell biology of these amoebae in more detail.

METHODS

Naegleria cultures

The pathogenic strain, N. fowleri (ATCC 30808), and non-pathogenic species N. gruberi (donated by Dr Visvesvara, CDC, Atlanta, USA) were cultured axenically at 37 and 25 °C, respectively, in 2% bactocasitone medium (Becton Dickinson) supplemented with 10% foetal bovine serum (FBS). Trophozoites were harvested during the mid-log phase of growth.

Drug preparation

The lyophilized AmB (Amphocil; Lemery) consisted of antibiotic and cholesterol sodium sulfate (1:1 M/M). It was dissolved in sterile water to obtain a concentration of 10 mg ml⁻¹ (main stock). The final concentration of AmB used for all of the in vitro assays was 10 μg ml⁻¹, based on the dose-response of the viability of the amoebae at 1, 10 and 100 μg ml⁻¹, to select a non-lytic concentration.

Viability assay

Untreated amoebae and amoebae that had been treated with AmB (10 μg ml⁻¹) for 12, 24 and 48 h were suspended in 0.5% (final concentration) trypan blue exclusion dye and observed under bright field in an Eclipse 80i microscope (Nikon). The percentage of dead cells was obtained. Samples were processed in triplicate. The statistical significance was calculated using GraphPad Prism statistical software (version 6).

Cell size decrease in trophozoites of N. fowleri and N. gruberi

We analysed the morphological changes in trophozoites at 24 h post-AmB treatment, employing a FACSCalibur (Becton Dickinson). The results were analysed using Summit 5.1 software (Becton Dickinson). The light scattered in the forward direction is roughly proportional to cell size, whereas the light scattered at a 90° angle (side scatter) is proportional to cell density. At least 10,000 events were quantified in each test. Untreated trophozoites were employed as a control.

Intracellular potassium (K⁺) levels

The reduction in cell size during PCD is directly related to the loss of K⁺. For this reason, we evaluated the levels of this ion using potassium-binding benzofuran isophthalate-AM (PBFI-AM; Sigma), which has a high affinity for K⁺. Briefly, after 24 h, untreated or AmB-treated trophozoites were harvested and washed twice with a buffer containing 0.116 M NaCl, 0.0054 M KCl, 0.0008 M MgCl₂, 0.0055 M glucose and 0.05 M MOPS (pH 7.4). Next, the cells were incubated with the same buffer plus 5 μM PBFI-AM for 45 min at 37 °C in the dark. The trophozoites were washed twice and suspended in the same buffer for analysis using a fluorescence spectrometer LS 55 (PerkinElmer) with an excitation wavelength (λ) of 370 nm and an emission λ of 540 nm. The statistical significance was calculated using Sigma Stat statistical software (version 2.0).

Transmission electron microscopy

To analyse ultrastructural alterations, AmB-treated and untreated trophozoites were harvested after 24 h, washed twice with PBS (pH 7.2) and fixed with 2.5% glutaraldehyde in cacodylate buffer 0.1 M. Post-fixation was performed with 2% osmium tetroxide. The samples were dehydrated with increasing concentrations of ethanol (70, 80, 90, 100%) and finally with propylene oxide. Subsequently, the amoebae were embedded in epoxy resin. Semi-fine sections (0.5 μm) were stained with toluidine blue for observation using light microscopy in an Eclipse 80i microscope (Nikon) and fine sections (80 nm thick) were contrasted with uranyl acetate and lead nitrate for observation in the transmission electron microscope EM-910 (Zeiss).

DNA fragmentation

Genomic DNA from N. fowleri and N. gruberi, untreated and treated with AmB for 24 h, were obtained using the phenol: chloroform extraction technique. Briefly, cells were lysed in an extraction buffer (0.02 M EDTA, 0.01 M Tris and 0.5% SDS) containing proteinase K (20 μg ml⁻¹) for 20 min at 65 °C, and DNA was obtained with a mixture of phenol: chloroform: isoamyl alcohol (25:24:1) and precipitated with isopropyl alcohol and sodium acetate (3 M) for 3 h at −20 °C. Finally, the DNA was electrophoresed in 3% agarose gel at 75 mA for 1 h and then stained with ethidium bromide for imaging under UV illumination.
TUNEL terminal deoxynucleotidyl transferase dUTP nick end labelling assay

To corroborate the DNA fragmentation, 24 h AmB-treated and untreated trophozoites from both species were fixed with 4 % paraformaldehyde and incubated in 70 % ethanol for 4 h to permeabilize the cells. Next, the cells were labelled with TdT reaction mixture containing FITC-coupled dUTP (ApoAlert DNA fragmentation assay kit; Clontech) for 1 h at 37 °C. Samples were analysed using a FACSCalibur cytometer with an excitation λ of 485 nm and an emission λ of 525 nm.

Phosphatidylserine (PS) externalization

PS externalization to the outer side of the cytoplasmic membrane of the cells was evaluated by FACS (fluorescence-activated cell sorting) using the Annexin V-FITC apoptosis kit (Biovision). Annexin V binds specifically to PS externalized in apoptotic cells, which has been considered to be an important marker during PCD. Briefly, untreated and 24 h AmB-treated trophozoites were incubated in 500 µl of 1× binding buffer containing 5 µl of annexin V-FITC for 5 min in the dark (according to the manufacturer’s protocol). Samples were analysed in a FACSCalibur cytometer (Becton Dickinson) with an excitation λ of 485 nm and an emission λ of 525 nm.

Reactive oxygen species (ROS)

Levels of ROS were determined by employing the dichlorodihydrofluorescein diacetate probe (DCFH-DA; Sigma), a sensitive and rapid quantitation method for ROS. In response to an oxidative agent, DCFH-DA become the highly fluorescent 2,7-dichlorofluorescein (DCF). Untreated and 24 h AmB-treated trophozoites were resuspended in 3 ml of PBS (pH 7.4) containing 25 µM of DCFH-DA, incubated in the dark for 15 min and washed twice with PBS. Finally, the fluorescence signal was detected in a FACSCalibur (Becton Dickinson) with an excitation λ of 485 nm and an emission λ of 525 nm.

Relative gene expression using quantitative real-time PCR (qRT-PCR)

To analyse whether the levels of the genes involved in PCD in the genus Naegleria change, as occurs in other protozoans, we searched for and selected some candidates in the genome of N. gruberi, because it is currently the only species in this genus for which the genome has already been described. We employed three sequences from N. gruberi: sir2 NAD-dependent protein deacetylase (ref. XM_002671742.1), autophagy-related protein atg8 (ref. XM_002678882.1) and cytoplasmic cysteine proteinase (cp) (ref. XM_002675417.1) as possible apoptotic markers, and small subunit ribosomal 18S (ref. XM_002682911), which was used as an internal control gene. The primers were designed using Primer Express 2.0 software (Applied Biosystems) and synthesized commercially by custom primers (Invitrogen): ribosomal subunit 18S F: 5’-GTATCTTGCCAAGGATAGCACA-3’ and R: 3’-AGCGTA-GAAAGGACTCTCGGG-5’. Relative gene expression using quantitative real-time PCR (qRT-PCR) was performed in an ABI PRISM 7000 sequence detection system (Applied Biosystems) by monitoring the increase in fluorescence in real time using SYBR Green PCR Master Mix (Applied Biosystems). Melting curve protocols were used to ensure the specificity of the amplification products. The arithmetic formula 2^(-ΔΔC_T) was applied to validate the relative expression of small subunit ribosomal 18S as an endogenous control [24]. The relative quantificatities of sir2, cp and atg8 were calculated using the formula 2^(-ΔΔC_T) [24]. The statistical significance between the untreated and treated trophozoites was calculated using Sigma Stat statistical software (version 2.0).

RESULTS

AmB affects the viability and size of N. fowleri and N. gruberi trophozoites

We analysed the effect of 10 µg AmB ml\(^{-1}\) on the viability of Naegleria species over 12, 24 and 48 h using trypan blue exclusion stain. In N. fowleri, we observed a significant decrease in cell viability in a time-dependent manner, demonstrating a viability of only 55 % at 48 h. However, AmB induced less cell death in the non-pathogenic N. gruberi, with 74 % viability at 48 h after AmB treatment (Fig. 1a). These results suggest that AmB has a weaker amoebicidal effect on the non-pathogenic N. gruberi than it does on the N. fowleri trophozoites. Moreover, shrinkage of AmB-treated amoebae was quantified using FACS. These results showed and confirmed the decrease in cell size after AmB treatment over 24 h in both amoebae (Fig. 1b).

K_\text{r}^+ decrease in AmB-treated trophozoites

Intracellular potassium is involved in PCD, which plays an important role in apoptosis. Reduced levels of this cation result in the loss of cytoplasmic fluids, inducing the shrinkage of apoptotic cells. Thus, we evaluated the changes in the intracellular levels of this ion in both Naegleria species by spectrofluorometric assay using a PBFI-AM probe, which is a potassium-sensitive fluorescent molecule used to measure K_\text{r}^+. These results showed a higher basal level of fluorescence in untreated cells, 645 relative fluorescence units (RFU) in N. fowleri and 582 RFU in N. gruberi (Fig. 2). However, trophozoites treated with AmB for 24 h showed a significant decrease in
fluorescent signal, 453 RFU in \textit{N. fowleri} and 465 RFU in \textit{N. gruberi} (Fig. 2), indicating a decrease in the level of $K^+$ and a reduction in cell size. Negative control samples represent the autofluorescence of the amoebae.

Fig. 1. AmB affects the viability and size of \textit{N. fowleri} and \textit{N. gruberi} cultures. (a) A trypan blue exclusion assay was performed using trophozoites incubated for different times in bactocasitone medium containing 10 $\mu$g ml$^{-1}$ of AmB. The percentage of viability was determined by counting dead cells using light microscopy. An amoebicidal effect was observed in a time-dependent manner ($N=3$). (b) The cell size reductions (shrinkage) of AmB-treated \textit{N. fowleri} and \textit{N. gruberi} trophozoites were analysed by FACS. At least 10000 events were counted. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus untreated samples.

Amoebic ultrastructural changes induced by AmB

To study the ultrastructural changes induced by AmB in \textit{N. fowleri} and \textit{N. gruberi} trophozoites after 24 h, we performed a transmission electron microscopy (TEM) assay. As a control, we used untreated cells, which displayed typical amoebic morphology in both \textit{Naegleria} species; the nuclei exhibited a homogeneous nucleoplasm with a central dense nucleolus, and the formation of pseudopods, evident vacuoles and several mitochondria along the cytoplasm were observed (Fig. 3a, d). However, in trophozoites treated with AmB for 24 h, the most evident changes were the rounded morphology and the reduction in cellular size. The trophozoites presented an irregular nuclear membrane and a decrease in the number of vacuoles. However, the integrity of the cell membrane was apparently intact (Fig. 3b, e). All these changes were observed in both \textit{Naegleria} species. In \textit{N. fowleri}, the presence of blebs (arrowheads) and apoptotic bodies (arrows) was evident (Fig. 3c). Interestingly, in non-pathogenic \textit{N. gruberi} we observed vacuoles that resembled autophagic vacuoles, which presented cytoplasmic content (Fig. 3f).

AmB induces DNA fragmentation

DNA fragmentation is a typical hallmark of PCD. To confirm whether AmB is able to induce this event in both \textit{N. fowleri} and \textit{N. gruberi}, we performed agarose gel
electrophoresis with DNA obtained from both *Naegleria* species. We observed that untreated control samples showed undegarded genomic DNA, whereas that of trophozoites treated with AmB for 24 h demonstrated a degraded pattern of DNA, appearing as a smear in both species (Fig. 4a).

**Detection of positive TUNEL signalling in AmB-treated trophozoites**

TUNEL is usually performed to analyse DNA degradation in apoptotic cells. We performed the TUNEL assay using FACS in AmB-treated and untreated trophozoites. These results showed a 35 % increase in positive signal for *N. fowleri* at 24 h after AmB treatment compared with untreated amoebae (Fig. 4b). In the non-pathogenic *N. gruberi*, the results showed that this amoeba had a lower positive signal of 22 % compared with the untreated amoebae (Fig. 4c).

**PS externalization in trophozoites treated with AmB**

PS is a membrane phospholipid contained in the inner side of the lipid cytoplasmic bilayer and its externalization to the outer side of the cell membrane is also used as a marker for PCD. To evaluate this characteristic, we used annexin V-FITC in non-permeabilized trophozoites. In the control cells, a basal signal (2 %) positive for FITC was detected in both species (Fig. 5a, b). However, we observed an increased fluorescence signal in trophozoites treated with AmB. The percentage of positive cells was 58 % in *N. fowleri* (Fig. 5a) and 35 % in *N. gruberi* (Fig. 5b) 24 h post-treatment.

**Increment of ROS induced by AmB**

Overproduction of ROS in apoptotic cells has been widely studied, and thus an increase in the intracellular levels of ROS was used as an additional marker for PCD in this study. These results showed that *N. fowleri* treated with AmB for 24 h exhibited an increase in the positive signal for ROS of approximately 77 % compared with the control (Fig. 6a). In contrast, *N. gruberi* treated with AmB presented a 40 % increased fluorescence signal compared with their respective untreated control (Fig. 6b). Taken together, these results showed that AmB induces higher production of ROS in *N. fowleri* than in *N. gruberi*.

**Relative expression of apoptotic-related genes induced by AmB**

Analysis of the expression of genetic markers in apoptosis has been employed in many protozoans. In this study, we analysed the *cp*, *sir2* and *atg8* genes of *N. gruberi* as...
alternative indicators of PCD. These results showed that the expression of \textit{sir2} in AmB-treated \textit{N. gruberi} trophozoites was non-significant at 1.5 and 3 h. However, at 6 h post-AmB treatment, a fivefold higher expression compared to the control was observed (Fig. 7). On the other hand, \textit{atg8} showed a significantly high expression at 3 and 6 h, with three- and sevenfold higher expression, respectively, compared with the control at 0 h (Fig. 7). In contrast, the expression of \textit{cp} was not significant at any evaluated time point (Fig. 7).

**DISCUSSION**

Programmed cell death (PCD) is an important phenomenon that was discovered in multicellular organisms [16],
although it has also been described in some protozoans, such as *Dictyostelium discoideum*, *Plasmodium berghei*, *Blastocystis hominis*, *Entamoeba histolytica* and trypanosomatids [17, 19, 21, 25–29]. The physiological role of PCD in unicellular parasites has been discussed for many years, but some studies have demonstrated that this process is relevant in the control of parasitic populations, which is important during the infection process of *Leishmania major* and *Trypanosoma brucei* [15, 25, 27, 30, 31]. Studies by Kim and colleagues showed that *N. fowleri* can induce cell death in human microglial cells in a non-contact system formed by a tissue-culture insert with a membrane pore size of 0.2 µm [32]. However, the PCD mechanism in the genus *Naegleria* has not yet been completely described. In this study, we analysed the effect of AmB at a non-lytic dose (10 µg ml\(^{-1}\)) in cultures of *N. fowleri* and *N. gruberi*. This drug was able to induce cell death in a time-dependent manner in both *Naegleria* species; the AmB-treated cultures showed that 55% of *N. fowleri* and 74% of *N. gruberi* maintained their membrane integrity at 48 h. Importantly, cell membrane impermeability is the main difference between apoptosis and necrosis, and it can be observed using light microscopy and TEM [14, 16]. Light microscopy analysis showed that AmB-treated trophozoites presented shrinkage, which was corroborated by FACS studies. The shrinkage was directly related to the loss of cytoplasmic fluid, which was due to an imbalance in the homeostasis of K\(^+\). Moreover, the levels of K\(^+\) decreased significantly in AmB-treated trophozoites compared with untreated amoebae. This phenomenon has been shown to be important during PCD in other microorganisms [17, 19, 33–35].

Ultrastructural studies showed the presence of a safe mechanism, such as autophagy, which could be activated by pharmacological stress. This phenomenon was more evident in *N. gruberi*. Importantly, all of the *N. fowleri* results showed greater susceptibility to the AmB effects than those for *N. gruberi*. The higher susceptibility of *N. fowleri* to AmB compared with *N. gruberi*, was reported previously by Schuster and Rechthand; these authors observed ultrastructural changes, such as an alteration in nuclear shape, an increase in rough and smooth endoplasmic reticulum, the swelling and degeneration of mitochondria, a decrease in the number of vacuoles and, importantly, the presence of blebs. They also described the presence of autophagic vacuoles in *N. fowleri* when treated with AmB [12, 36]. However, they did not describe PCD. Interestingly, our ultrastructural results revealed important changes in the morphology of trophozoites, such as shrinkage, alterations in the nuclear membrane and mitochondrial structure, and loss of typical vacuoles, in both species. In addition, in *N. fowleri* the formation of blebs and apoptotic bodies was observed; in contrast, autophagic vacuoles containing

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**Fig. 5.** PS externalization was induced by AmB. PS externalization was evaluated by FACS using annexin V-FITC. (a) AmB-treated *N. fowleri* showed an increase of 58% in positive labelling. (b) *N. gruberi* presented a 35% increase in positive labelling compared with the untreated amoebae (2%).
electron-dense material and cytoplasmic residues were observed in \textit{N. gruberi}. Some studies have reported the activation of an autophagy mechanism during PCD. It has also been proposed that autophagy can act as an anti-apoptotic mechanism [14, 37–39]. These observations strongly suggest that \textit{N. gruberi} can display the autophagy pathway [40, 41].

DNA fragmentation has also been considered to be a hallmark of apoptosis [42, 43]. Thus, DNA extracts from control and AmB-treated trophozoites were analysed using agarose gel electrophoresis. DNA degradation induced by AmB did not result in evident total ladder DNA fragmentation in either set of the amoebae. Furthermore, some reports have suggested that DNA degradation can occur in non-nucleosomal fragments, which generate a non-evident ladder pattern during agarose gel electrophoresis, which is correlated with findings obtained in other micro-organisms, such as \textit{B. hominis} and \textit{D. discoideum} [19, 20, 29]. In addition, we observed a positive signal for DNA fragmentation using a TUNEL assay, which was higher in the pathogenic strain \textit{N. fowleri} than in the non-pathogenic \textit{N. gruberi}. However, DNA degradation during PCD is not considered to be the only criterion to evaluate PCD [13]. For this reason, we evaluated some biochemical parameters related to PCD [22, 44]. Our results showed an increase in the externalization of PS phospholipid in non-permeabilized AmB-treated trophozoites, indicating that AmB induces an apoptotic mechanism in trophozoites of both species. As previously described, the involvement of K$^+$ in PCD is considered to be an important hallmark. Deregulation of this cation is related to damage to the Na$^+$-K$^+$ ATPase.

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**Fig. 6.** AmB increased the levels of ROS in \textit{N. fowleri} and \textit{N. gruberi}. Intracellular levels of ROS were determined by FACS using a DCFH-DA probe. (a) \textit{N. fowleri} treated with AmB for 24 h showed an increase of 77 % in positive labelling for ROS, (b) AmB-treated \textit{N. gruberi} showed an increase of 40 % in positive labelling for ROS. Both \textit{Naegleria} were compared with control trophozoites.

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**Fig. 7.** Relative expression of apoptosis-related genes in \textit{N. gruberi}. Analysis of the relative expression of genetic markers in \textit{N. gruberi} was evaluated using qRT-PCR. In AmB-treated \textit{N. gruberi} trophozoites, \textit{sir2} showed overexpression at 6 h. The \textit{atg8} expression increased significantly at 3 h and 6 h compared with the control (time 0 h). Randomly selected cytoplasmic cysteine proteinase (\textit{cp}) did not display differences during AmB treatment. Bars display the standard deviation of three independent assays. *$P \leq 0.05$ versus control amoebae.
pump, which is caused by an increase in ROS levels during apoptosis [21, 34, 45, 46]. Intracellular levels of ROS were evaluated in both Naegleria species, demonstrating that AmB can increase oxidative stress in AmB-treated trophozoites, as previously reported during PCD in yeast and protozoans [21, 22, 47].

Genetic regulation of PCD is an important characteristic of this pathway, and the destruction of apoptotic cells is controlled at the level of genes. However, the typical genes employed as genetic markers have not been identified in the N. gruberi genome, because it is the only species of this genus for which the genome has been sequenced [9, 10]. Nevertheless, in the parasitic protozoan E. histolytica, the overexpression of sir2 has been described to have an anti-apoptotic role during PCD induced by aminoglycoside G-418 [23]. Although the precise function of this enzyme during PCD is unknown, its disruption by deletion of the gene induces PCD in L. major [48]. Our results showed a significant increase in sir2 in AmB-treated N. gruberi. Moreover, based on our ultrastructural observations of autophagic vacuoles, we examined the expression of Atg8, which is the most representative molecule involved in the labelling of autophagy vacuoles [49, 50]. Using qRT-PCR, a significant increase in atg8 transcription was observed in AmB-treated N. gruberi trophozoites. These results confirmed that some anti-apoptotic mechanisms are activated that try to avoid the PCD process induced by AmB at non-lytic doses. In a similar vein, the degradation of all cellular components is the most representative biochemical function in PCD, and it is performed in a controlled manner by caspases, a specific type of cysteine proteinase which are activated during PCD [18, 34, 51]. In protozoa, a specific caspase has not been described [20, 22, 52]. However, some cysteine proteinases have been described as caspase-like molecules [20, 22, 52]. In this study, we assayed a randomly selected cp of N. gruberi. However, the expression of this cp was not significant during AmB treatment, suggesting that this randomly selected cp does not participate during PCD in N. gruberi. Thus, it is necessary to perform further studies to obtain a wider screening of the cps described for N. gruberi to evaluate their contribution during PCD. This is the first study to describe PCD in detail in these free-living amoebae, and it presents new mechanisms that further our understanding of the biology of these protozoa. Taken together, these results showed that AmB induces morphological, biochemical and genetic changes that are typical of apoptosis-like PCD in the genus Naegleria.

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Conflicts of interest
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