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# *Calpain-like*: A $Ca^{2+}$ dependent cystein protease in *Entamoeba histolytica* cell death

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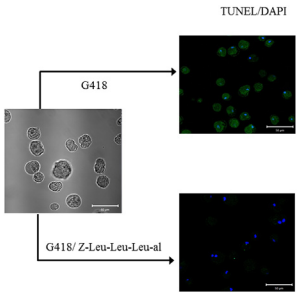
## HIGHLIGHTS

- The *calpain-like* protein activity increase during PCD induced *in vitro* by G418.
- Z-Leu-Leu-Leu-al diminishes the PCD induced *in vitro by G418*.
- The calpain-like protein is involved in the executory phase of PCD.

## GRAPHICAL ABSTRACT

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## ABSTRACT

*Entamoeba histolytica* programmed cell death (PCD) induced by G418 is characterized by the release of important amounts of intracellular calcium from reservoirs. Nevertheless, no typical caspases have been detected in the parasite, the PCD phenotype is inhibited by the cysteine protease inhibitor E−64. These results strongly suggest that $Ca^{2+}$-dependent proteases could be involved in PCD. In this study, we evaluate the expression and activity of a specific dependent $Ca^{2+}$ protease, the calpain-like protease, by real-time quantitative PCR (RTq-PCR), Western blot assays and a enzymatic method during the induction of PCD by G418. Alternatively, using cell viability and TUNEL assays, we also demonstrated that the Z-Leu-Leu-Leu-al calpain inhibitor reduced the rate of cell death. The results demonstrated 4.9-fold overexpression of *calpain-like* gene 1.5 h after G418 PCD induction, while calpain-like protein increased almost two-fold with respect to basal calpain-like expression after 3 h of induction, and calpain activity was found to be approximately three-fold higher 6 h after treatment compared with untreated trophozoites. Taken together, these results suggest that this $Ca^{2+}$-dependent protease could be involved in the executory phase of PCD.



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## 1. Introduction

Programmed cell death (PCD) in protozoan parasites enables the regulation of parasite densities in distinct host compartments and facilitates the avoidance of inflammatory responses, thereby facilitating sustained infection (Reece et al., 2011; Van Zanderbergen et al., 2010).

Several *in vitro* studies have shown that PCD in *Entamoeba histolytica* is induced by nitric oxide, hydrogen peroxide species and by G418 aminoglycoside (Ramos et al., 2007; Nandi et al., 2010; Villalba et al., 2007). The PCD induced by G418 showed morphological and biochemical changes typically described in eukaryotic organisms (Villalba et al., 2007). Interestingly, the parasite displayed a significant increase in cytosolic calcium concentrations, indicating that $Ca^{2+}$ has a pivotal role in this process, as is observed in other systems (Villalba et al., 2007; Sharma and Rohrer, 2004). For example, the overexpression of *grainins*, which are calcium-binding proteins, suggest that its expression could be a compensatory response with the goal of diminishing free intracellular $Ca^{+2}$ and subsequently diminishing the cellular mechanisms activated by this cation (Sánchez et al., 2010, Graidist et al., 2007). Otherwise, caspase-like proteins have not been identified in *E. histolytica*, and the use of E−64, a specific cysteine protease inhibitor, drastically diminished the PCD (Villalba et al., 2007). In amoeba, several types of cysteine proteases have been identified, a portion of which are activated by ligand, metals or ions (Marumo et al., 2014; Tillak et al., 2007; Clark et al., 2007). Considering that after PCD induction, calcium release is concomitant with specific *de novo* protein synthesis (Sánchez et al., 2010), we focused our efforts on investigating the participation of calpain-like protease. Calpains are calcium-dependent cysteine proteinases involved in diverse cellular processes, including apoptosis (Ono et al. 1998). In thymocytes, activated calpain degrades cytoplasmic and nuclear substrates, leading to the breakdown of cellular architecture and finally producing apoptosis (Squier et al., 1994; Momeni, 2011).

Calpains are heterodimeric proteins, consisting of two subunits of 80 and 28 kDa (Croall and Ersfeld, 2007). The large subunit in classical calpains consists of four conserved domains: an N-terminal anchor helix (Nter), a catalytic protease core domain (CysPc) with the two subdomains PC1 and PC2, a C2-like domain (C2L), and a penta-EF-hand domain (PEF). Non-classical calpains lack both the Nter and the PEF domain and may contain additional domains in combination with CysPc (Strobl et al., 2000; Hosfield et al., 1999, Joyce et al., 2012).

The CysPC catalytic core of the enzyme is highly conserved between different members of the calpain family and is characterized by the presence of the catalytic triad containing the amino acids cysteine, histidine and asparagine. *In vivo*, calpain activity depends on the presence of the three key catalytic amino acid residues; although enzymes with substitutions in these residues do not always display loss of function (Berti and Storer, 1995; Sorimachi and Suzuki, 2001; Goll et al., 2003 Hosfield et al.1999). The CysPc also contains two calcium binding sites that are essential for enzyme activity. The conservation of the calcium binding residues defines an ancestral general mechanism of activation for most calpain isoforms, including a number that lack EF-hand domains. (Moldoveanu et al., 2002).

Atypical or unconventional calpains are described as calpain-like proteins that contain only a CysPc consensus signature with variations in the catalytic triad and no PEF-containing domain is present, and they may also contain additional domains in combination with CysPc (Sorimachi et al., 2010). Calpain-like proteins have mainly been found in invertebrates and lower eukaryotes. In *Trypanosoma* and *Blastocystis hominis*, calpain-like proteins have been involved in the life cycle, the differentiation process and the regulation of PCD (Hertz-Fowler et al., 2001; Yin et al., 2010; Giese et al., 2008).

## 2. Materials and methods

### 2.1. E. histolytica cultures and growth conditions

Trophozoites of clone A (strain HM1: IMSS) were axenically cultured in TYI-S-33 medium (Diamond et al., 1978) at 37 °C. PCD was induced by incubation with10 μg/ml G418 for different periods of time as indicated.

### 2.2. RTq-PCR assays

Three independent biological replicates of trophozoites incubated without treatment or treated with 10 μg/ml G418 for 0.5, 1.5, 3 and 6 h were processed to extract total RNA. Total RNA was isolated using Trizol reagent according to the manufacturer's protocol (Invitrogen). The integrity of RNA samples was checked by electrophoresis in non-denaturing agarose gels. RNA concentrations were spectrophotometrically determined at 260 nm. Isolated RNAs were RQ1RNase-Free DNase treated (Promega) to avoid genomic DNA contamination and were reverse transcribed using the GeneAmp RNA PCR Core Kit (Applied Biosystems) according to the manufacturer's protocol. RT q-PCR was performed in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) by monitoring in real time the increase of fluorescence using the SYBR Green PCR Master Mix (Applied Biosystems). Immediately after amplification, melt curve protocols were performed to ensure that self-complementary primer-dimer formations and other non-specific products were minimized or eliminated. Primer express software for Real-Time PCR ver 3.0 Applied Biosystems was used to design primers for RTq-PCR: calpain S: (5′GTTTCAATATCACAACCT CGTTGTG3′) and calpain AS: (5′AAAGTCTCTCCAGAATCACCTCCA3′) for *calpain-like* and gapdh S: (5′CCGTCCACAGACAATTCGAA3′) and gapdh AS: (5′TTGAGCTGGATCTCTTTCAGCTT3′) for glyceraldehyde-3-phosphate dehydrogenase (*gapdh*). The application of the comparative Cycle Threshold (CT) method using the arithmetic formula $2^{-\Delta C}{}_{T}$ was conducted to validate the effect of treatment on the expression of *gapdh* endogenous control gene (Livak and Schmittgen, 2001). The relative quantification was calculated using the CT method, which uses the arithmetic formula $2^{-\Delta\Delta C}{}_{T}$ (Livak and Schmittgen, 2001). To validate the $2^{-\Delta\Delta C}{}_{T}$ method, we verified that the amplification efficiency of the target gene and the internal control *gapdh* were nearly equal. Statistically significant differences in gene expression between untreated and PCD-induced trophozoites from different periods of time were analyzed by comparisons of the mean using Tukey'stest with Sigma Stat statistical software ver.2.0 ver.2.0 SPSS.Inc.

### 2.3. Western blot assays

Cell lysates obtained from trophozoites of clone A without treatment or treated with 10 μg/ml G418 for 0.5, 1.5, 3 and 6 h were prepared after three freeze-thaw cycles in 100 mMp-hydroxymercuribenzoic acid (PHMB) (Sigma). Total extracts (30 μg) were analyzed by 10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham, Biosciences). Western blot assays were performed using anti-calpain antibody (H-240, Santa Cruz Biotechnology) (1:200 dilution). Then, nitrocellulose membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG; Zymed; 1: 2000) for 2 h. Antigen-antibody reactions were revealed with a fresh preparation of 5 mg/ml of diaminobenzidine until the color was developed. For internal control, membranes were incubated with polyclonal goat

anti-GAPDH antibody (V-18, Santa Cruz Biotechnology) (1:300 dilution) and (HRP)-conjugated rabbit anti-goat immunoglobulin G (IgG; Zymax; 1: 2000). Western blot assays were performed by triplicate. Densitometry analysis of calpain expression levels, using the gapdh detection for normalization was performed and comparisons expression between untreated and PCD-induced trophozoites from different periods of time were analyzed by comparisons of the mean using Kruskal–Wallis test with Sigma Stat statistical software ver.2.0 SPSS. Inc.

### 2.4. Calpain activity

To determine calpain activity during G418-mediated cell death, *E. histolytica* cells were exposed to 10 μg/ml G418 for 0.5, 1.5, 3 and 6 h and calpain activity was assayed using Suc-Leu-Leu-Val-Tyr-AMC (calpain substrate) as described previously (Kohli et al., 1997). Briefly, cytosolic extracts from untreated and treated cells were prepared and protein concentrations were measured. Forty μl (1 mg protein $ml^{-1}$) of cytosolic protein was added to 160 μl 50 μM Suc-Leu-Leu-Val-Tyr-AMC (30% DMSO in 100 mMTris/HCl, 145 mMNaCl, pH 7.3). Calpain activity was assayed in the presence of 10 mM $Ca^{2+}$. Released AMC (7-amino-4-methylcoumarin) was measured by fluorometry using 360 nm excitation and 430 nm emission filters. Experiments were done by three replicates; data were expressed as the mean ± SD of three experiments. Standard curves were generated for each experiment using AMC of known concentrations. Calcium-dependent calpain activity was expressed as pmol AMC released per mg of cytosolic protein at 10 mM $Ca^{2+}$. Statistically significant differences were analyzed by comparisons of the mean using Tukey's test with Sigma Stat statistical software ver.2.0 ver.2.0 SPSS.Inc.

### 2.5. Z-Leu-Leu-Leu-al inhibition assays

Ten thousand trophozoites of clone A were cultured in TYI-S-33 medium in the presence of 10 μg/ml G418 with or without 50 μM of the calpain inhibitor Z-Leu-Leu-Leu-al (N-benzyloxycarbonyl-leucinyl-leucinyl-leucinal) (Sigma, Aldrich) at 1.5, 3 and 6 h. Finally, viability and TUNEL assays were done as described below.

### 2.6. Viability

The viability of trophozoites was examined by Trypan Blue dye exclusion (0.5 mg $ml^{-1}$) using a hemocytometer under an inverted microscope (Nikon Eclipse TE300). In each test, there were three replicates, and data were expressed as the mean ± SD of the three experiments. Statistically significant differences in viability between trophozoites in presence of G418 incubated with or without calpain inhibitor from different periods of time were analyzed using Student's t-test ($p < 0.05$) with Sigma Stat Statistical software ver.2.0 SPSS.Inc.

### 2.7. Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assays

Trophozoites were fixed in 4% formaldehyde for 45 min at 4 °C. After washing twice with PBS, 50 ml TUNEL reaction mixture (Roche) was added and incubated for 60 min at 37 °C in a humidified atmosphere in the dark. Then, trophozoites were rinsed three times with PBS and nuclei were counterstained with DAPI for 8 min at room temperature. The cells were washed ten times with PBS and were observed using a Zeiss LSM Pascal confocal microscope. As a positive control, trophozoites were treated with 20 μg/μl *DNase I* endonuclease for 10 min, and non-treated trophozoites were used as a negative control.

## 3. Results

### 3.1. Calpain-like structure

Homology research within *E. histolytica* genome revealed that calpain-like protein contains a CysPc consensus domain and one additional LIM domain. (Loftus et al., 2005; Zhao et al., 2012). CysPc domain present the catalytic triad containing the amino acids cysteine, histidine and asparagine, which are high conserved. Two non-EF-hand calcium sites are present within the CysPc domain. The conservation of the calcium binding residues defines an ancestral general mechanism of activation for most calpain isoforms, including some that lack EF-hands domains (Moldoveanu et al., 2002). The LIM domain is a zinc binding domain that mediate the protein–protein interaction (Fig. 1).

### 3.2. Development of calpain-like gene expression during PCD induction

Expression levels of *calpain-like* gene were evaluated by RT-qPCR during the development of PCD. PCR efficiencies were nearly equal between the *calpain-like* transcript and the endogenous control *gapdh*. Single peaks were identified in the melt curve for each gene screened, indicating that unique PCR pure products were produced (data no shown). Fig. 2 displays the relative expression levels of transcripts during the course of G418 induction (0, 0.5, 1.5, 3 and 6 h). The results showed that after 0.5 h of PCD induction, *calpain* transcripts decreased, representing only 0.1 of the basal expression from untreated trophozoites. However, after 1.5 h, the relative expression of the *calpain* gene grew up to 4.9-fold. The *calpain* gene expression then decreased slowly, going back to 2.5-fold at 3 h and finally to the basal expression (1.1-fold) at 6 h.

### 3.3. Calpain-like protein expression during PCD induction

Densitometry analysis of Western blot assays (Fig. 3) revealed that trophozoites of clone A without G418 treatment displayed a basal expression of calpain-like protein, while trophozoites treated with G418 showed protein increases of 1.2-, 1.5-, 1.8- and 1.7-fold after 0.5, 1.5, 3 and 6 h, respectively, however not statistically significant differences respect to trophozoites without G418 treatment were detected ($p = 0.118$). GAPDH was used as an internal control for the normalization of results.

### 3.4. Increase of calpain activity during PCD induction

We analyzed the calpain activity during PCD induction and observed that untreated trophozoites displayed calpain activity of 12 pmol AMC released per mg cytosolic protein at 10 mM $Ca^{2+}$, while trophozoites treated with G418 showed calpain activity increases of 15.6, 13.8, 27.6 and 33 pmol AMC released per mg cytosolic protein at 10 mM $Ca^{2+}$ after 0.5, 1.5, 3 and 6 h, respectively (Fig. 4).

### 3.5. Effect of the calpain inhibitor Z-Leu-Leu-Leu-al

#### 3.5.1. Viability

The viability of trophozoites incubated with G418 decreased from 40% after 1.5 h to 10% 6 h later (Fig. 5). However when trophozoites were co-incubated with calpain inhibitor we observed a reduction in the rate of cell death of the least 20% after 3 and 6 h of incubation.

#### 3.5.2. DNA fragmentation

Analysis of PCD by TUNEL assays showed that trophozoites

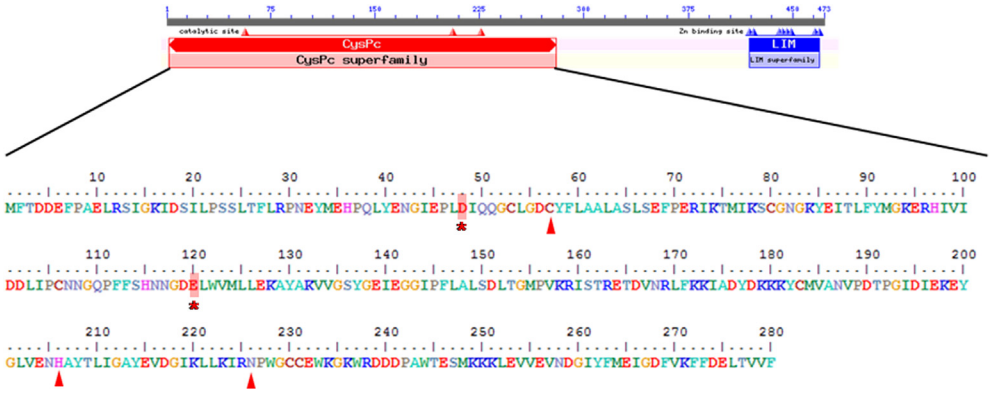


**Fig. 1.** Calpain-like protein in *E. histolytica*. (blast http://blast.ncbi.nlm.nih.gov/Blast.cgi). The arrows point to C, H, and N residues within the catalytic site, and residues that coordinate $Ca^{+2}$ through their side chain are highlighted with * (D, E).

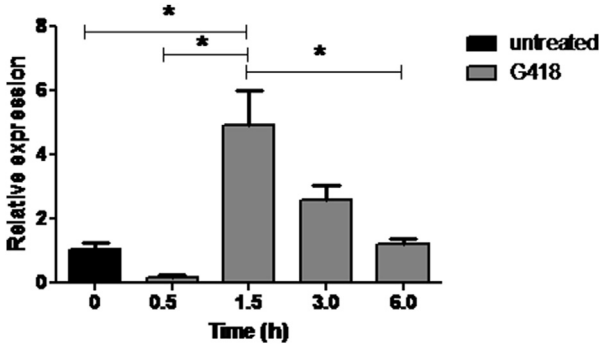


**Fig. 2. Relative expression of *calpain-like* in trophozoites incubated with and without G418**. The graph shows the relative expression of *calpain-like* in trophozoites incubated without G418 (untreated) and with 10 ug/ml of G418 during different periods of time (0.5, 1.5, 3.0 and 6.0 h). * indicate trophozoites that showed statistically significant ($p < 0.05$) in relative expression.

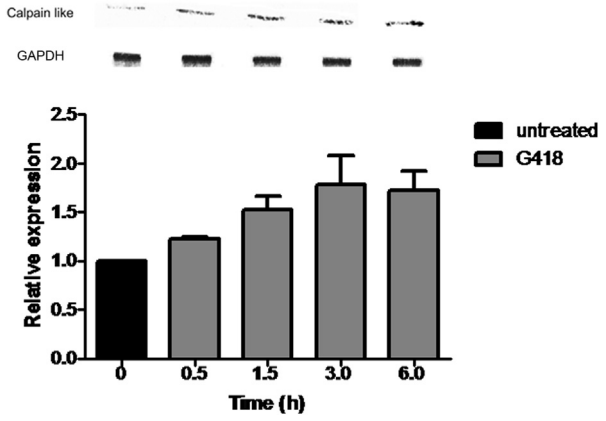


**Fig. 3. Western-blot analysis**. Calpain like protein expression (anti-calpain antibody) in trophozoites incubated without G418 (untreated) and with 10 ug/ml of G418 during 0.5, 1.5, 3.0 and 6.0 h. Anti-GAPDH antibody was used as an internal control. The graph shows the densitometry analysis of calpain expression levels, using the GAPDH detection for normalization.

induced to death displayed nuclear TUNEL staining when trophozoites were incubated with G418. At 1.5 and 3 h, most of nuclei were also counterstained with DAPI, however in some nuclei the double staining were less evident or did not overlap. Sixth hours after PCD induction the fluorescence was less evident and the double stained

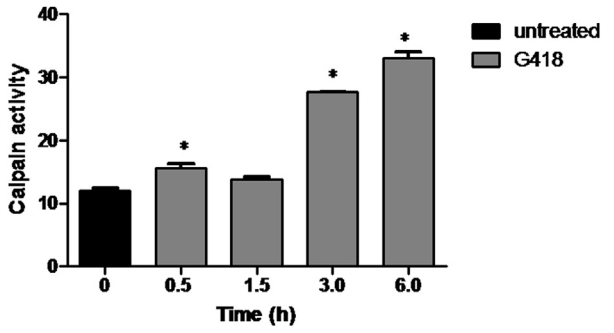


**Fig. 4. Calpain activity introphozoites incubated with and without G418**. The graph shows calpain-like activity express as pmol AMC released per mg cytosolic protein at 10 mM $Ca^{2+}$in trophozoites incubated without G418 (untreated) or with 10 ug/ml of G418 during different periods of time (0.5, 1.5, 3.0 and 6.0 h). * indicate treated trophozoites that showed statistically significant respect to untreated trophozoites ($p < 0.001$).

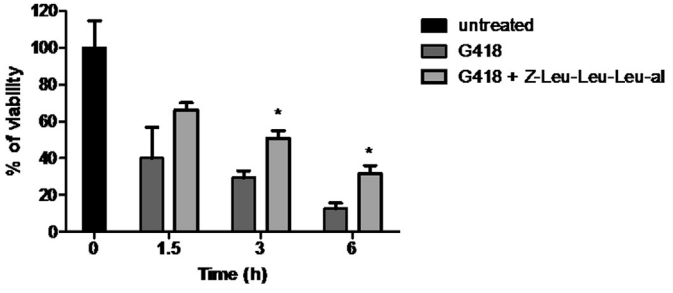


**Fig. 5. Viability percentages by effect of calpain inhibitor Z-Leu-Leu-Leu-al**. Viability percentages were evaluated in trophozoites incubated with 10 ug/ml of G418 during different periods of time (1.5, 3.0 and 6.0 h) co-incubated with and without Z-Leu-Leu-Leu-al. * indicate statistically significant differences in viability between trophozoites growth in presence of G418 incubated with or without calpain inhibitor from different periods of time ($p < 0.05$).

nuclei diminished. It is important to note that TUNEL staining also marked in cytoplasm, specially, after 3 h of PCD induction. When trophozoites were co-cultured with calpain inhibitor an important TUNEL staining reduction was found in all times assayed, in both, nuclei and cytoplasm (Fig. 6).

## 4. Discussion

Calcium signaling has long been known to be critically involved

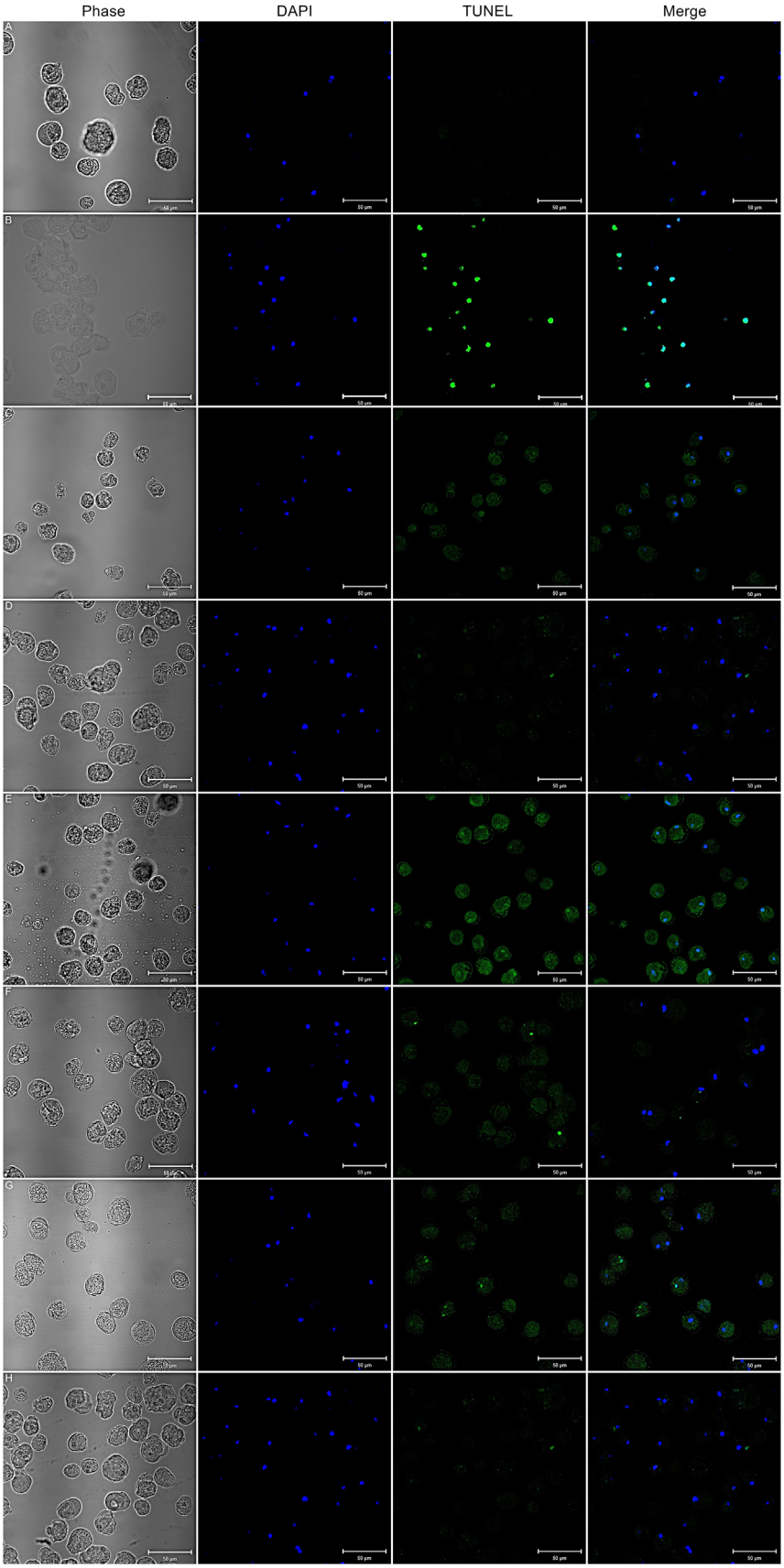


**Fig. 6. DNA fragmentation in trophozoites after G418 and G418/Z-Leu-Leu-Leu-al treatments**. Confocal microscopy analysis of trophozoites showing TUNEL staining counterstained with DAPI. A) negative control, untreated trophozoites; B) positive control, trophozoites were treated with 20 ug $ul^{-1}$ *DNase I* endonuclease for 10 min; C) and D), trophozoites after 1.5 h of incubation with G418 or with G418/Z-Leu-Leu-Leu-al, respectively; E) and F), trophozoites after 3.0 h of incubation with G418 or with G418/Z-Leu-Leu-Leu-al, respectively; G) and H), trophozoites after 6.0 h of incubation with G418 or with G418/Z-Leu-Leu-Leu-al, respectively. Bars, 50 μm.

in both the initiation and execution phases of cell death (Nicotera, 2003). Thus, necrosis was found to be associated with a perturbation of intracellular $Ca^{2+}$ homeostasis, and key events in the apoptotic process are known to be triggered by $Ca^{2+}$ signals (Orrenius et al., 2003). Recent studies in *Leishmania* and *Toxoplasma* parasites have also demonstrated the crucial role of the $Ca^{2+}$ ion in the initiation and regulation of cell death (Dolai et al., 2011; Peng et al., 2003). Our previous reports of PCD in *E. histolytica* showed that intracellular $Ca^{2+}$concentrations increased in trophozoites treated with G418, from 20 nM at the beginning of incubation to 48 nM after 120 min, suggesting that an important pro- and anti-apoptotic genetic program is triggered by free cytosolic calcium. For example, the overexpression of *grainins* 1 and 2, thirty minutes after G418 PCD induction strongly indicate that cellular mechanisms are turned on trying to re-capture free cytosolic calcium (Sánchez et al., 2010). On the other hand, the lost of $K^{+}$ ion by the inactivation of Na/K ATPase and the progressive TUNEL staining of nuclei (Villalba et al., 2007) also suggest the activation of apoptotic mechanisms and the participation of putative $Ca^{2+}$-dependent proteases in this process. In fact, our previous results showed that a general cysteine protease inhibitor blocked DNA fragmentation (Villalba et al., 2007). Taking into consideration all these results, in this work, we explored the relevance of calpain-like cysteine protease in *in vitro* PCD.

Expression levels of *calpain-like* gene evaluated by RT-qPCR during the development of PCD suggest the upregulation of the *calpain* gene from 1.5 to 3 h, after an initial decrease of transcripts, very early during PCD induction. The overexpression of the calpain gene peak correlates in time with the time release of cytosolic calcium after PCD induction (Villalba et al., 2007). To correlate the increase of transcript with the increase of protein synthesis and/or its accumulation, we searched for calpain protein expression in the same PCD periods of induction. The results showed that the calpain protein is expressed basally and it increased after 0.5 h of induction, accumulating very slowly to the end of the evaluated time. On the other hand, calpain activity was also assayed in untreated and treated trophozoites, and it was found that activity was approximately three-fold higher 6 h after treatment compared with untreated trophozoites**.** Protein and activity results together also suggest that besides a gene regulation mechanism, enzyme activity could be regulated by calcium and low protein level found could be probably due to the autolysis of the enzyme, which has been well documented to have an important role in its function (Cong et al., 1993). The concept that the calpains were pro-enzymes was widely accepted in the 1990s, and some authors have described the autocatalytic calpain activity as synonymous of "activation" (Suzuki et al., 1981).

Nandi et al. (2010) found in *E. histolytica* increased calpain activity during hydrogen peroxide PCD induction. However, no further studies were performed to associate the protein activity with the induction of gene expression. In other parasites, such as *Blastocystis* and *Leishmani*a, PCD is augmented when calpain is inhibited (Marinho FA et al. 2014; Yin J et al. 2010). In mammals, the participation of calpain in the promotion and/or the suppression of apoptosis has been contradictory (Carragher, 2006) because calpains have a large influence over many apoptotic processes. Thus, its specific role during apoptosis may differ depending on the cell type and the nature of the apoptotic stimulus (Carragher, 2006). Considering that the activities of the calpain gene and protein are induced during PCD induction, we explored the biological effect of the specific calpain inhibitor Z-Leu-Leu-Leu-al (Tsubuki et al., 1996) and determined the viability percentages of cells following G418 treatment, with or without Z-Leu-Leu-Leu-al. We observed higher viability percentages in trophozoites co-incubated with calpain inhibitor in all times analyzed, suggesting that the inhibitor reduces cell death. To support evidence that calpain could participate in the executory process of PCD, we assessed changes in DNA fragmentation of nuclei for the biological effect of Z-Leu-Leu-Leu-al. Results showed nuclear TUNEL staining in the times assayed. In contrast, the nuclei staining from trophozoites co-incubated with the inhibitor diminished importantly, correlating with the increased in cell viability. Trophozoites induced to PCD also displayed cytoplasmic TUNEL staining specially in longer incubation times. It is possible that due cultures were not synchronized, trophozoites were in different phases of cell death process, some of them in early PCD phases, others in late phases and even though others in a necrotic process, delivering DNA molecules to cytoplasm. On the other hand, it is also possible that the presence of cytoplasmic DNA reported in *E. histolytica* (Orozco et al., 1997; Ghosh et al., 2000) would be giving the positive cytoplasmic TUNEL staining. In this parasite, genome-wide homology searches have identified more than 50 *E.* histolytica genes encoding for cysteine proteases (Clark et al., 2007); therefore, it is possible that other cysteine-proteases could participate in this process. Our results are in concordance with evidence reported by Nandy et al. (2010) using hydrogen peroxide as an inductor. However, both results contrast with the findings of Ramos et al. (2007), who suggested that *Entamoeba* PCD induced by nitric oxide species occurs without the participation of cysteine proteases. Therefore, evidence supports the idea of both dependent and independent cysteine protease pathways in this parasite, depending on the stimuli.

Taken together, the results presented here suggest that this $Ca^{2+}$-dependent protease could be involved in the executory phase of PCD, assays currently under progress using other calpain inhibitors and also using antisense strategy, will allow us to determine the specific role of calpain-like gene and other genes that we have found to be important as putative pro- or anti-apoptotic signals in *E. histolytica*.

Knowledge of the molecules involved in cell suicide may offer new possibilities for controlling parasitic diseases because several calpain inhibitors are under development and a portion of them could be useful agents against important human pathogens, such as amoeba (d'Avila-Levy et al., 2006; Sangenito et al., 2009).

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