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APOPTOSIS-LIKE DEATH IN PARASITIC PROTOZOA

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ABSTRACT

Apoptosis is an essential physiological process that plays a critical role in development and tissue homeostasis in multicellular organisms, but which is also observed in several eukaryotic microorganisms such as yeast and protozoa. Here, the authors briefly review the most used techniques to detect apoptosis in mammalian cells, especially those that can be applied to parasitic protozoa after different conditions such as drug-treatment. Apoptosis-like processes have been described in protozoa which present mitochondria, such as members of the Kinetoplastida and Apicomplexa groups as well as in protozoa which do not have a mitochondrion, as *Entamoeba*, *Trichomonas* and *Giardia* do. These observations are of interest from an evolutive point of view, especially due to the fact that the participation of the mitochondria in apoptosis has been extensively analyzed in several biological systems. The authors also reviewed the available data showing that several drugs in use as anti-protozoa agents, as well as others which are in the development phase, kill the protozoa through an apoptotic-like process.

Key words: Apoptosis, chemotherapy, *Leishmania*, phosphatidylserine, protozoa parasites, *Trypanosoma*, trypanosomatids

INTRODUCTION

Studies carried out in multicellular organisms have shown clearly the existence of a suicidal pathway generally known as programmed cell death. This process has clear benefits for multicellular organisms where cells that are damaged, infected or no longer required, are eliminated. Excellent examples of the importance of such mechanism can be found during the evolution of a free living nematode, such as *Caenorhabditis elegans* [19,25,48], or during development of the mammalian nervous system [27]. Considering the importance of programmed cell death in metazoan, it is reasonable to ask if such a predetermined behavior mechanism or operation exists in phylogenetic early ancient single cell organisms such as parasitic protozoa. As early as in 1977, the authors obtained images of epimastigote forms of *Trypanosoma cruzi* incubated in the presence of β-lapachone [18], which belongs to a group of substances that interferes in the biological oxidative process and is used for the treatment of a large number of diseases [6], showing all the morphological changes that are nowadays used as indicative of apoptotic cell death. These alterations included the appearance of plasma membrane blebs, mitochondrial swelling and chromatin condensation, as shown in Figures 1-3. Today, it is known that β-lapachone and its analogues are responsible for topoisomerase inhibition and induction of apoptosis in cancer cells [64].

In general, cell death can be divided into two classes, apoptosis and necrosis [20]. Apoptosis is synonymous to the classic name known as "programmed cell death" which is an essential physiological process that plays a critical role in development and tissue homeostasis. It was first described by Kerr *et al.* in 1972 [36], and is now identified by several morphological and biochemical criteria such as the appearance of the membrane-blebs, chromatin condensation, nuclear fragmentation, loss of adhesion and rounding (in adherent cells), and cell shrinkage [12]. Other characteristics can be observed during apoptosis such as proteolytic cleavage of a number of intracellular substrates [52] and phosphatidylserine (PS) exposure in plasma membranes [53]. Similar alterations are observed in parasitic protozoa submitted to different stress con-

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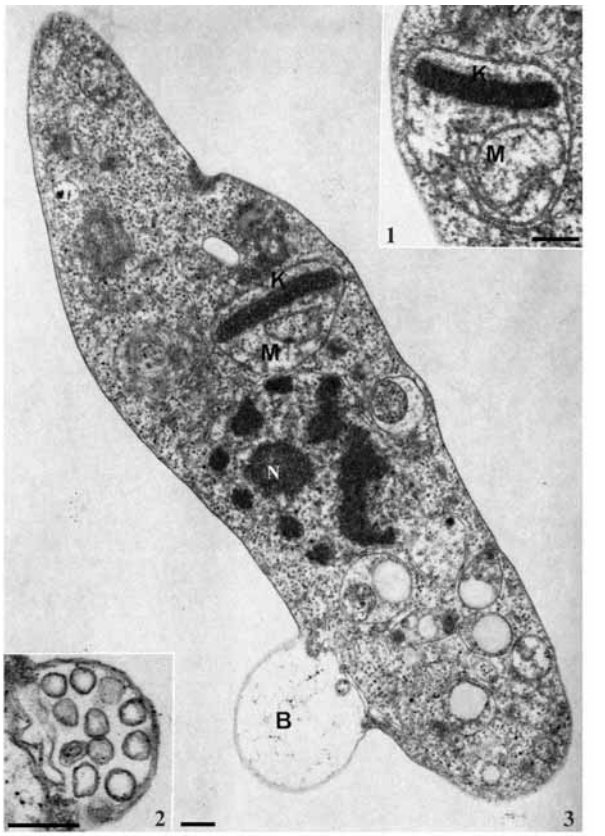
ditions such as contact with chemotherapeutic agents [15,55] and different temperatures [79]. The PS externalization induces the phagocytosis of apoptotic cells by macrophages and this mechanism results in an anti-inflammatory effect and suppression of proinflammatory mediators [35,39]. The PS exposure and clearance of cell corpses are mitochondria-dependent events [81]. There are at least two primary apoptotic mechanisms. The mitochondrion-dependent pathway activated by the release of cytochrome c from the mitochondrial intermembrane space and the activation of surface "death receptors" resulting in a signalling cascade [24]. At present, most of the studies carried out in protozoa point to the first mechanism although the second one can not be eliminated, especially in protozoa which do not have mitochondria.

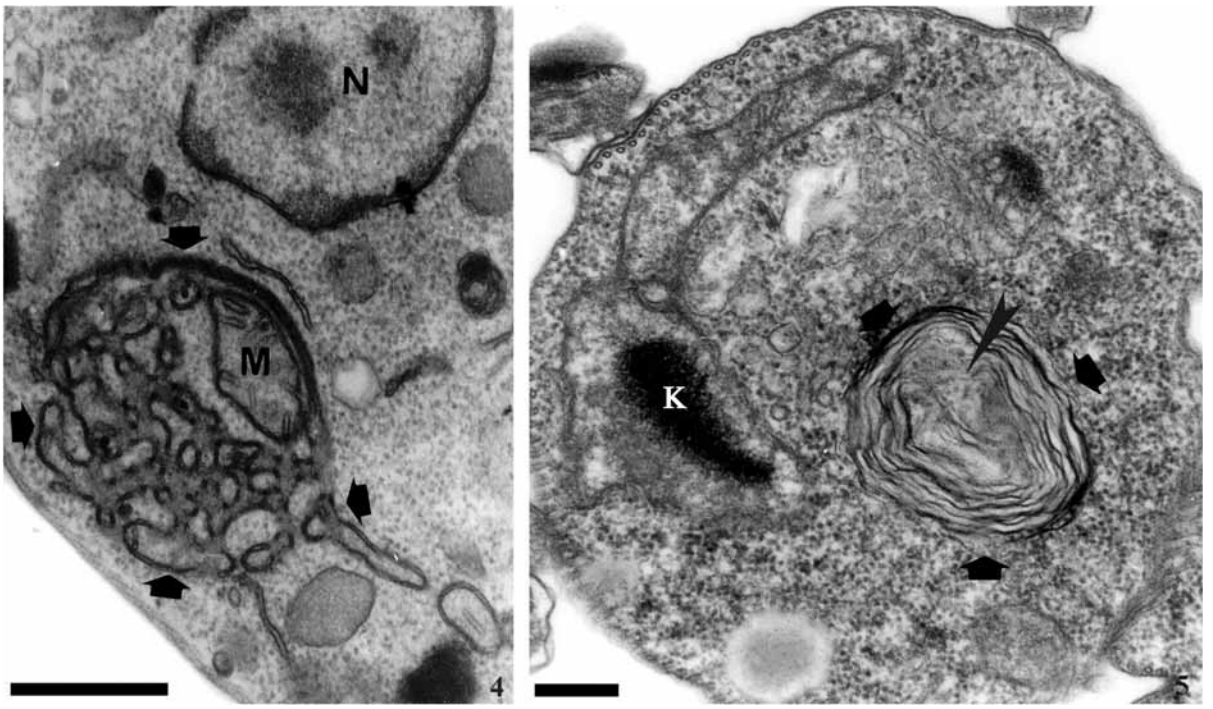
Studies carried out mainly in mammalian cells have shown that the enzymatic machinery of apoptosis begins with the permeabilization of the outer mitochondrial membrane by proapoptotic members of the Bcl-2 family, resulting in a release of proteins from the intermembrane space in the cytosol [13,16]. Among these released proteins is cytochrome c, which interacts with monomeric APAF-1 leading to its oligomerization and recruitment of caspase-9 to form the apoptosome [30]. However, members of the Bcl-2 family have also been localized in the endoplasmic reticulum, indicating a new mitochondrion-independent apoptosis pathway [78]. Caspase are aspartate-specific cysteine proteases that are expressed within proenzymes (zymogens) and activated to fully functional proteases by two cleavage events. The first proteolytic cleavage divides the chain into large and small caspase subunits, and a second cleavage removes the N-terminal prodomain. The active caspase is a tetramer of two large and two small subunits, with two active sites [88]. The most prevalent caspase in the cell is caspase-3, which is the one ultimately responsible for the majority of the apoptotic effects, although it is supported by two others, caspase-6 and -7. Together, these three executioner caspases presumably cause the apoptotic phenotype by cleavage or degradation of several important substrates such as the DNA fragmentation [22,44].

In contrast to apoptosis, necrosis appears to be a passive form of cell death with more similarities to a train crash than suicide [20]. In many cases necrosis is the result of a bioenergetic catastrophe, after a complete ATP depletion, probably initiated



Figures 1-3. *Trypanosoma cruzi* epimastigotes treated with β-lapachone presenting mitochondrion swelling, alterations of the mitochondrial membrane (Figs. 1,3), bleb on the cell membrane with vesicles (Fig. 2), and chromatin condensation (Fig. 1) and all features typical of apoptosis such as mitochondrial, nuclear and cytoplasmic alterations (Fig. 3). **B**: bleb; **K**: kinetoplast; **M**: mitochondrion; **N**: nucleus. Bars = 0.25μm. (After Docampo *et al.*, 1977 [18]).

mainly by cellular "accidents" such as toxic insults or physical damage. In necrosis, different from apoptosis, a vacuolation of the cytoplasm, breakdown of the plasma membrane and an induction of the inflammatory response due to the release of the cellular contents are observed. There is another type of non-apoptotic death that has been classified as a programmed necrosis or autophagic cell death [20,37,45]. This name is coherent, because in programmed necrosis there is a cellular signalling pathway leading to death in response to specific cues, and not due to an "accident". Although there are morphological similarities between necrosis and autophagic cell death, they are two distinct types of death, where in the latter, the name autophagy means, literally, to eat oneself. In this case, transmission



Figures 4-5. *Leishmania amazonensis* promastigotes treated with 22,26-azasterol presenting the mitochondrion (Figure 4, **M**) and a part of the cytoplasm (Figure 5, **arrowhead**) surrounded, by profiles of the endoplasmic reticulum (**arrows**). **K**: kinetoplast; **M**: mitochondrion; **N**: nucleus. Bars = 0.5 μm. (After Rodrigues *et al.*, 2002 [66]).

electron microscopy has shown the presence of a double membrane tubular cisternal engulfing whole organelles, and in many cases, large parts of the same cytoplasm, as can be seen in Figures 4-5, after treatment of the *L. amazonensis* promastigotes with sterol biosynthesis inhibitors [66]. It is important to mention that in autophagic cell death as observed in mammalian cells, there is probably no caspase activation and consequently, no DNA fragmentation [20,37,45]. Figure 6 indicates the connections that exist among the different types of cell deaths with electron micrographs showing the morphological features observed in parasitic protozoa. It should also be mentioned that some authors consider the non-apoptotic programmed cell death as a new process designated as paraptosis [74]. This name was first used in 2000 [74], however few groups have considered this denomination in important revisions concerning cell death [20,37,45].

Apoptosis in unicellular organisms?

In multicellular organisms, programmed cell death (PCD) is important to control cell number for proper development and tissue homeostasis, removal of unwanted cells and functional control of the immune, haemopoietic and nervous systems [86]. The elimination of the apoptotic cells takes place without inflammatory activation in these multicellular organisms [35], thus explaining the "silent death" described in several papers. Recently, apoptosis-like death have been described in several eukaryotic microorganisms such as yeast [28,47], kinetoplastids [2,3,23,43,56,65,80,85-87,89] and apicomplexans [62,63], and even in protozoa which do not present an evident mitochondrial system, as in *Trichomonas* [9,49,50], *Giardia* [9] and *Entamoeba* [34], or where the mitochondrion shows special peculiarities, such as in *Blastocystis hominis* [77]. In most of these organisms the evidence of apoptosis-like death are mitochondrial transmembrane potential disruption (mainly due to mitochondrion permeabilization) and nuclear DNA degradation [3,63]. Two of the major characteristic features of mammalian cell apoptosis, that occur with protozoa and distinguishes it from the passive and chaotic destruction process of necrosis, are cell shrinkage and maintenance of plasma membrane integrity [3]. Studies carried out with *T. brucei* and *T. cruzi* have identified genes coding for metacaspase [38,57,65,76]. In addition, it was shown that *T. brucei*

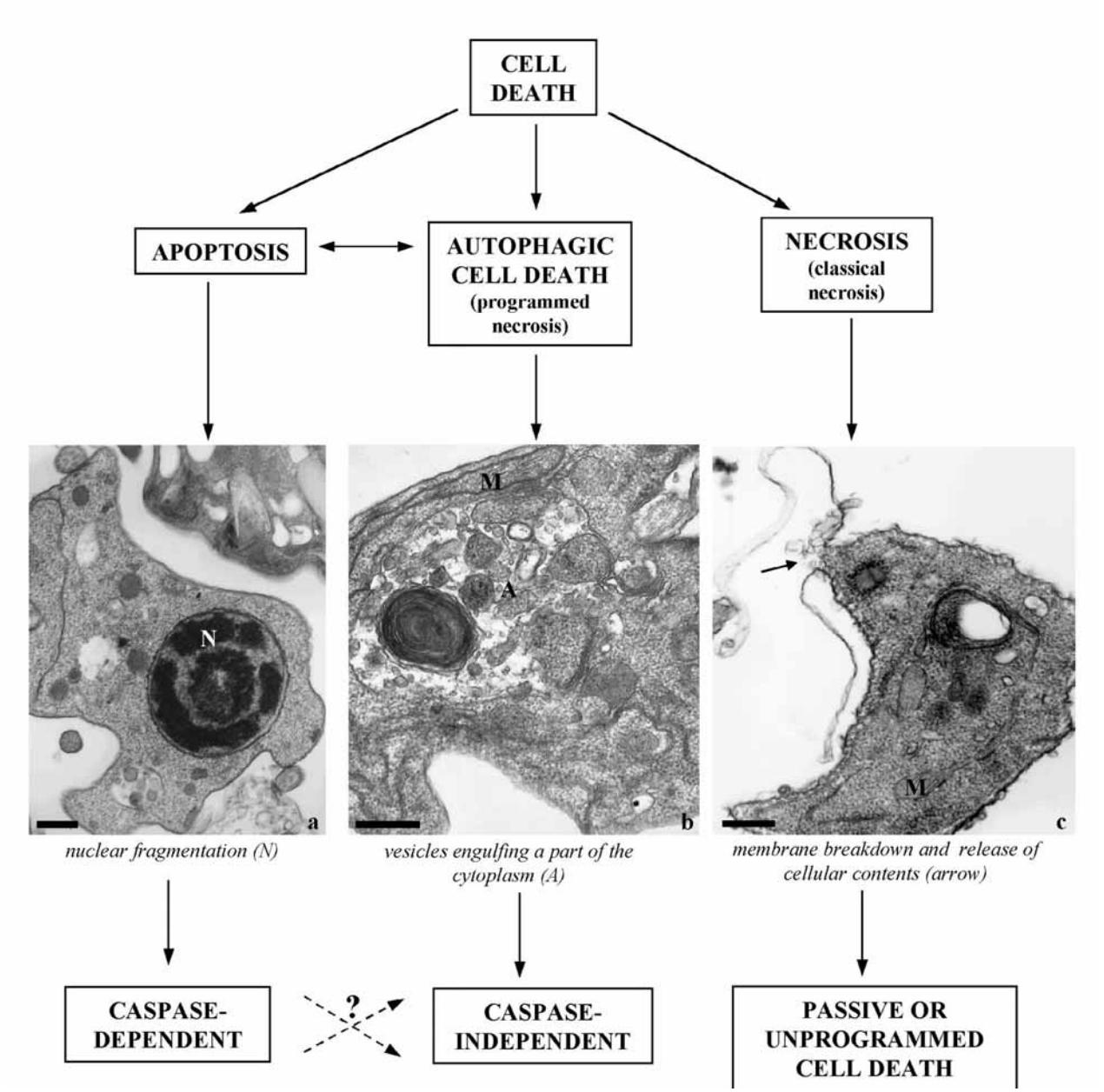


Figure 6. Scheme indicating the connections present in the different types of death, where (**a**) shows an epimastigote of *T. cruzi* presenting an abnormal chromatin condensation, (**b**) a promastigote of *L. amazonensis* with vesicles engulfing a part of the cytoplasm, and (**c**) another promastigote displaying membrane breakdown (**arrow**), and consequently, release of cellular contents. Both protozoan parasites were treated with sterol biosynthesis inhibitors. **A**: autophagic body; **M**: mitochondrion; **N**: nucleus. Bars = 0.5 µm. (After Lazardi *et al.*, 1991 [42], and Rodrigues *et al.*, 2005 [67]).

cultivated in the presence of prostaglandin D_2 displayed features typical of the apoptotic process, where maintenance of plasma membrane integrity, loss of mitochondrial membrane potential, nuclear chromatin condensation and DNA degradation were observed [26]. However, caspase inhibitors did not prevent cell death, indicating a probable caspase-independent process [26]. These observations strongly suggest that a mechanism similar to the programmed cell death can occur during the normal development of protozoa in cultures or when they are submitted to environmental stress conditions. It has been suggested that in order to promote and maintain clonality within the population, protozoa

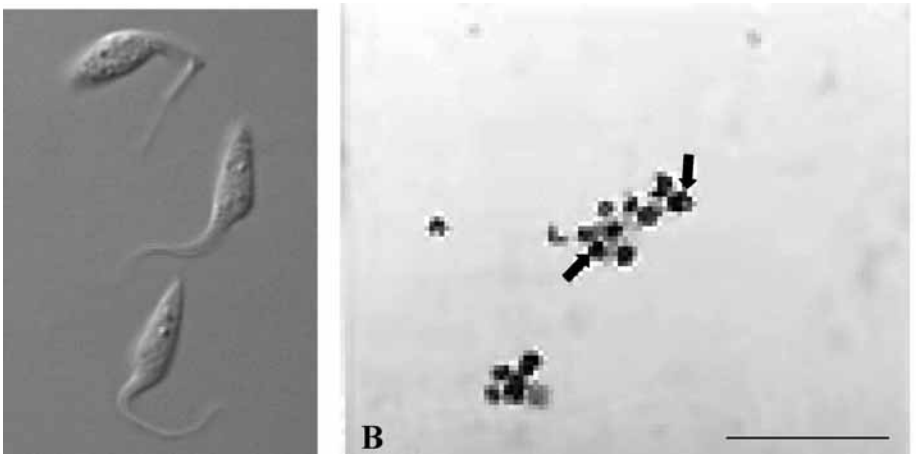


Figure 7. *Trypanosoma cruzi* epimastigote forms treated with *Bothrops jararaca* venom exhibited DNA fragmentation. Parasites were treated for 24 h with 20 µg/ml of venom. The TUNEL technique was used within *in situ* nick-end labeling of cells. (**A**) untreated parasites. Bar =10 µm; (**B**) treated parasites are labeled indicating DNA fragmentation (**arrows**). Note the cytoplasmic condensation of cells. Bar = 17 µm. (After Deolindo *et al.*, 2005 [15]).

develop an altruistic mechanism to control growth via apoptosis-like processes.

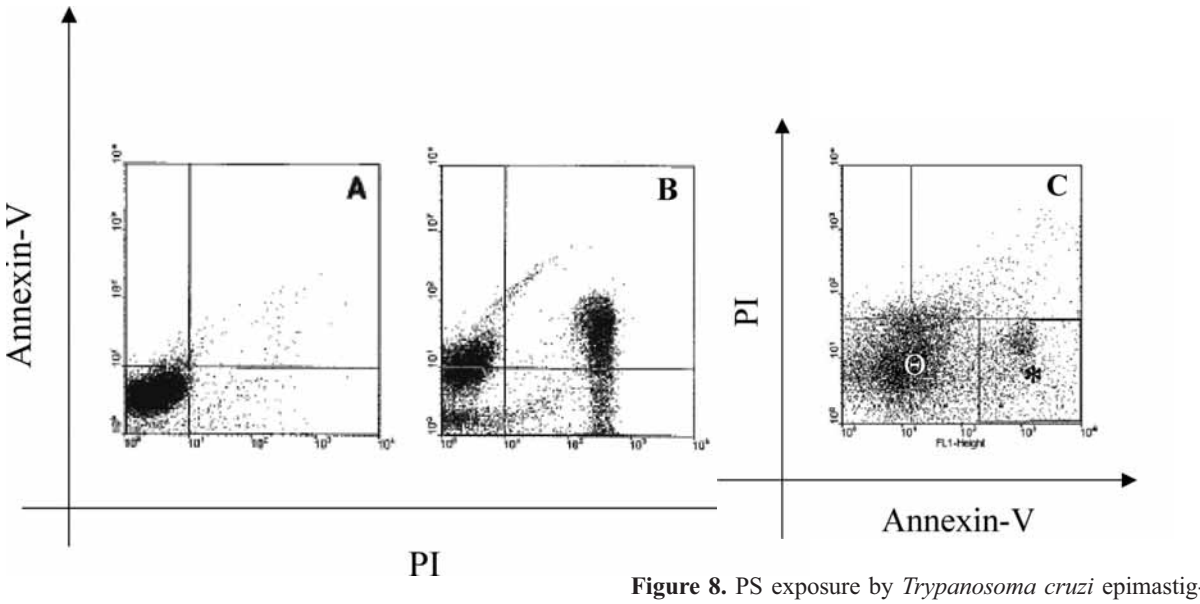
Detection of apoptosis-like death in protozoa

The principal method utilized for the detection of apoptosis-like death in protozoa is the nuclear DNA fragmentation, as revealed using two different approaches: one is agarose gel electrophoresis where leader formation of bands are observed [3,43,63] and the other is the TUNEL label technique where DNA fragmentation can be revealed by fluorescence microscopy (Fig. 7) [15] or flow cytometry, in this case more usual to quantify the fragmentation. Transmission electron microscopy of thin section has also been used to show condensation of the nuclear chromatin, mitochondrial swelling and blebs of the plasma membrane as shown in Figures 1-3 [18]. This technique also permits visualization of cytoplasmic vacuolation, as well membrane breakdown, both features observed in autophagic cell death and necrosis (Fig. 6). The exposition of phosphatidylserine on the cell surface, evaluated by flow cytometry or fluorescence microscopy using labeled annexin-V, has also been used as a criterion to identify apoptotic mammalian cells [82]. However, studies carried out with parasitic protozoa which are able to interact with host cells, especially with phagocytic cells show that exposure of PS on their surface can be used to avoid host cell inflammatory responses (Fig. 8) [15,68]. In these organisms PS exposure mimics apoptosis and regulates the microbicide action of macrophages via TGF-β production, regulating nitric oxide production. This mechanism has been described for *Leishmania amazonensis* [5], *Toxoplasma gondii* [68] and *Trypanosoma cruzi* (unpublished data), and configures a common mechanism for survival in mammalian hosts. It is important to point out that there are other examples in mammalian cells of phosphatidylserine exposure without any relationship to cell death. Examples include T lymphocytes that express low levels of the transmembrane tyrosine phosphatase [21], sperm cells in the process of capacitation [29], and myotube formation [83].

Apoptosis-like death in protozoa induced by chemotherapeutic drugs

One of the main topics of research in protozoology is the development of new drugs which are able to kill the parasitic protozoa without interference in host cells. The data obtained with these drugs show that they kill the parasites by three distinct mechanisms: (a) changes in structures such as the nucleus and the mitochondria similar to those described in apoptotic cells (Fig. 1-3; Fig. 6a); (b) induction of autophagy, where profiles of the endoplasmic reticulum surround damaged organelles such as mitochondria as well as the cytoplasm (Fig. 4-5; Fig. 6b), and (c) induction of necrosis of the protozoa (Fig. 6c).

Programmed cell death (PCD) in the unicellular organisms, mainly analysed in *Leishmania* spp., has attracted considerable attention, because several



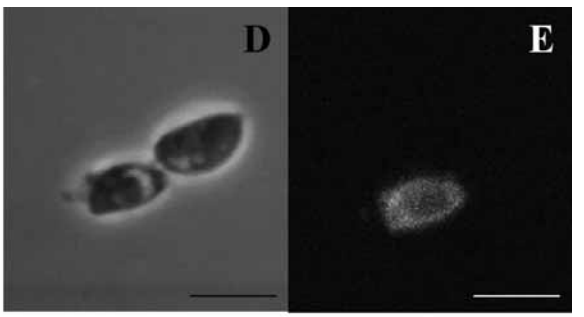


Figure 8. PS exposure by *Trypanosoma cruzi* epimastigote forms control (**A**), treated with *Bothrops jararaca* venom for 8 h (**B**) or *Toxoplasma gondii* control (**C**) analyzed by flow cytometry and light microscopy (**D**, **E**). Note that epimastigote forms do not expose PS in control cells (**A**). However, a PS positive population is seen after venom treatment. PS exposure is also detected on the surface of *Toxoplasma gondii*. A population of this protozoan contains both PS positive (Θ) and PS negative (*), an observation also confirmed by microscopy visualization (**D**, **E**). **D** – Phase contrast microscopy. Bar = 10 μm, E – Fluorescency microscopy. Bar = 10 μm. (After Deolindo *et al.*, 2005 [15], and Seabra *et al.*, 2004 [68]).

studies have shown that different drugs, some of them in the development phase as future chemotherapeutic agents in the treatment of leishmaniasis, induce the parasite to die an apoptosis-like death. At present, the first option for treatment of leishmaniasis consists in the administration of pentavalent antimonials. Although the mechanism of action of these drugs is not yet well established, recent studies showed that at low concentrations, potassium antimonyl tartrate, a Sb(III)-containing drug, induces DNA fragmentation in axenic amastigotes of *Leishmania infantum*, as visualized both by in situ TUNEL assay and agarose gel electrophoresis [71]. Experiments carried out on *L. donovani*, showed that this antimonial induced an increase in the generation of ROS in the macrophage, a process dependent on drug concentration and the time of incubation. However, this increase in the ROS-concentration was also observed inside the parasitophorous vacuole, indicating that the amastigotes are able to produce ROS after exposure to antimonial. The production of ROS in parasites treated with antimonial [75], or in presence of H_2O_2 [58] is accompanied by the loss of the mitochondrial membrane potential ($\Delta\Psi$), and an increase in the cytosolic Ca^{2+} pool. All these changes are usually associated with cell death by apoptosis.

Pentamidine is a second class of drugs used in Leishmaniasis treatment, mainly in those cases where treatment with pentavalent antimonials failed. Vercesi and Docampo showed that pentamidine induces a rapid collapse of the mitochondrial inner membrane potential of *L. donovani* promastigote [84]. Previous morphological studies using a different approach have already showed that pentamidine induced extensive mitochondrial disruption, with membrane and cristae fragmentation [11]. These studies are consistent with a recent work that demonstrated the effect of pentamidine inhibiting the respiratory chain in the complex II and, consequently, inducing the apoptotic death of the

promastigote forms of *L. donovani* [54]. In addition, the use of antioxidants and Ca^{2+} channel blockers revealed the role of the Ca^{2+} intracellular pool in the apoptotic death in this parasitic protozoan.

Miltefosine, an alkylphosphocholine originally developed as an anticancer drug, has been recently introduced with success for the oral treatment of visceral Leishmaniasis caused by *L. donovani* in Indian. This drug shows a low toxicity. Although the mechanism of action of miltefosine is not well defined, different works have showed that it is able to induce apoptosis-like cell death [60,85]. It was also shown that caspase inhibitors interfered with the effect of miltefosine, thus supporting the notion that apoptosis-like death is in some way involved in the protozoan killing. These data also suggest that at least part of the apoptotic machinery operating in the parasites involves proteases [60,85].

Camptothecin (CPT), an inhibitor of DNA topoisomerase I, which is in phase III clinical trials for colon cancer, has been used to induce apoptosis under experimental conditions [4,59]. It has been previously shown that DNA topoisomerases play an important role in the viability of *Leishmania* spp. indicating their potential as targets for therapeutic agents [7,55]. CPT has been shown to inhibit type I DNA topoisomerase of *Leishmania donovani* [7]. Several studies in the last three years have showed the ability of CPT, and other Topo-I and Topo-II inhibitors, to induce apoptotic cell death in *Leishmania* spp. and *Trypanosoma brucei* [10,51,69,70,73]. All drugs induced changes characteristics of an apoptosis process, including the inhibition of oxygen consumption and the release of cytochrome c, both as a consequence of the collapse of the inner mitochondrial membrane potential, and disruption of the outer mitochondrial membrane, respectively [70]. In addition, these drugs induced dyskinetoplastidy, as previously shown for the other topoisomerase inhibitors [72], and many cells displayed chromatin condensation, as visualized by transmission electron microscopy.

In contrast with the significant amount of information on *Leishmania* spp. there are few studies dealing with apoptosis-like death in *Trypanosoma* spp. In the case of *Trypanosoma cruzi* it was shown that *Bothrops jararaca* venom is able to induce PCD in epimastigote forms, leading to mitochondrion swelling and kinetoplast disorganization. Furthermore, the venom activated the caspase-like protein and induced DNA fragmentation after 24 h of treatment [15].

Apoptosis-like death in parasitic protozoa by other factors

In the absence of chemotherapeutic agents inducing apoptosis-like processes in different parasitic protozoa, some characteristics of the programmed cell death can be observed in normal conditions, following stress conditions such as low and high temperatures and nutrient depletion or in some cases during interaction of the parasites with host cells.

Studies carried out on *Trypanosoma brucei* showed the participation of a proapoptotic protein Bax, already characterized in mammalian cells, causing the release of cytochrome c, depolarization of the mitochondrial membrane potential, as well mitochondrial fission. However, in contrast to mammalian cells, the three events are well separated indicating, in principle, that it is reversible [23]. Another study with *T. brucei* showed the effect of different cell culture conditions and low-temperature stress in the induction of PCD after inhibition of the Trypanosome Alternative Oxidase (TAO). This is an alternative catalytic activity that occurs in the cyanide insensitive electron transport system in the inner mitochondrial membrane and that is necessary for the reoxidization of NADH generated during glycolysis. It is well known that in the bloodstream form mitochondrion activity is almost quiescent and glucose is the major source of energy [79].

Although much less studied, apoptosis-like processes have been described in several parasitic protozoa species. It is important to point out that apoptosis-like death has been described in organisms which present a modified mitochondrion, as is the case of *Blastocystis hominis* [77], and in anaerobic protozoa which do not have the mitochondrion, an organelle which plays a fundamental role in the induction of apoptosis, as in *Trichomonas* [9,49,50] and *Giardia* [9]. This feature is of high interest from the evolutionary point of view, since hydrogenosomes present some characteristics of mitochondria, such as the presence of two membranes and the involvement in Ca^{2+} control [Review in 9].

One situation which deserves a special comment is the ability of some parasitic protozoa to induce the apoptosis of cells from the host or even host cells while others avoid the apoptosis of the host cell. Indeed, increasing viral, bacterial and protozoan

pathogens have been shown to modulate the host apoptotic response. For instance, *T. gondii*-infected cells are resistant to apoptosis. The protozoan inhibits the apoptotic cascade by manipulating the activity of the caspase cascade [61]. NFkβ also plays a role in this process [61]. Recent work showed that *T. gondii* is also able to manipulate the central role of mitochondrion in the apoptosis process at multiple levels, an important feature that could explain the resistance of host-cells to apoptosis after *T. gondii*-infection [8].

Apoptosis-like processes have also been described in *Plasmodium* spp. An important study in *P. berghei* demonstrated the ability of this parasite to regulate its numbers using the programmed cell death. This work showed all features present in metazoan apoptotic cells including condensation of chromatin, fragmentation of the nuclear DNA and movement of phosphatidylserine from the inner to the outer lamellae of the cell membrane [1]. In addition, the presence of caspase-like activity in the cytoplasm of the ookinete was observed suggesting once again the presence of a cellular mechanism, similar to those found in mammalian cells [1]. On the other hand, the ability to induce apoptosis during the epithelial invasion of the vector midgut in *Plasmodium* spp. was also observed. Studies of the interaction between malaria and mosquitoes allowed an improved understanding of the mechanisms involved in establishment of the parasite and the vector response to infection [14,33]. In a different study, the authors showed that *P. chabaudi* is able to decrease the expression in the spleen cells of important molecules involved in apoptosis, consequently inhibiting this process in the cells during the infection, in some cases affecting the immune system modulation [40].

Theileria induces T cells to proliferate through interference with NFkβ that is constitutively activated in the infected cells [17] and protects parasite-infected T cells against apoptosis [32]. In addition, an important study showed that upon elimination of the *Theileria parva* from the host cell by treatment with theilericidal drug, cells become increasingly sensitive to Fas/FasL-induced apoptosis [41]. In the case of *Entamoeba histolytica*, apoptosis of host cells requires contact with the parasites with the involvement of the Gal/GalNAc lectin exposed on the protozoan surface. Caspase 3-like activity was observed within minutes of amoeba contact [34].

It has also been shown that activation-induced

Table 1. Comparative analysis of programmed cell death (PCD) features can be observed by different morphological approaches in many organisms.

Type of death	Multicellular organisms	Trypanosomatids	*Giardia Entamoeba*	*Trichomonas*
Apoptosis	- phosphatidylserine exposure	- not depend of apoptosis activation*	- not reported	- phosphatidylserine exposure
	- mitochondrial alterations	- mitochondrial alterations	- absence of mitochondria**	- absence of mitochondria
	- membrane blebs	- membrane blebs	- membrane blebs	- membrane blebs
	- caspase activation	- caspase activation	- no reported	- caspase activation
	- DNA fragmentation	- DNA fragmentation	- DNA fragmentation	- DNA fragmentation
Paraptosis or non-apoptotic programmed cell death	- cytoplasmic vacuolation	- cytoplasmic vacuolation	- not reported	- cytoplasm vacuolation
	- mitochondrial swelling	- mitochondrial swelling	- absence of mitochondria	- absence of mitochondria
	- no caspase activation	- no caspase activation	- not reported	- not reported
	- no DNA fragmentation	- no DNA fragmentation	- not reported	- not reported
Necrosis	- breakdown of the plasma membrane	- breakdown of the plasma membrane	- not reported	- not reported

* The PS-exposure is controversy in case of trypanosomatids, because there is important works describing the exposure in physiological conditions. About this, see the text.

** In *Giardia* was described the mitosome also considered as a mitochondria-like/organelle.

CD4+T cell death by apoptosis is a prominent feature of experimental infection with *T. cruzi* and may play a role in immunosuppression and parasites persistence in infected hosts [46]. Another important study about apoptosis in *T. cruzi* showed that it is able to use a specific inhibitor (FLICE inhibitory protein), a known inhibitor for death receptor-mediated apoptosis in mammalian, for the inhibition of Fas-mediation apoptosis by posttranscriptional up-regulation [31]. This data suggests a possible mechanism that could allow the parasites to persist in the host cell.

CONCLUSION

After a detailed description about the physiology of the apoptosis and its occurrence, it is possible to conclude that the programmed cell death in protozoan parasites such as *Leishmania* spp. and *Trypanosoma* spp. is the most common mode of death for the parasites after treatment with different drugs, some of them used as current treatment, mainly in infections with *Leishmania.* On the other hand, we can affirm that different parasitic protozoan were able to use the cell machinery and physiology to control the host cell to its advantage. To finalize our considerations about PCD, Table 1 shows a summary of the features that have been analyzed in some parasitic protozoa. However, for a better understanding of the PCD it is necessary to study the biochemical and molecular mechanisms involved in this process further as an approach to develop new therapeutic agents for the treatment of diseases caused by protozoan parasites.

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