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Cell Death after Photodynamic Therapy Treatment in Unicellular Protozoan Parasite *Tritrichomonas foetus*

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Abstract

Programmed cell death in *T. foetus* does not seem to make sense at first sight; however, different mechanisms of cellular death in this unicellular organism have been observed. This review summarizes the available data related to programmed cell death already published for the cattle parasite *T. foetus* and attempts to clarify some crucial points to understand this mechanism found in non-mitochondriates parasites, as well as assist in future research. Important results with different treatments showed that the *T. foetus* can choose among different pathways how to initiate cell death. Thus, a major challenge for cellular death research remains the identification of the molecular cell death machinery of this protist, such as caspases pathway, nuclear abnormalities, morphology cell changes, cellular death in this parasite and the prospects in the future research. Although, the possibility of the existence of different pathways to cell death in trichomonads is discussed and a model for possible executioners pathways during *T. foetus* cell death is proposed.

Keywords: cell death mechanism, amitochondrial protozoan, photodynamic therapy, apoptosis, necrosis

1. Introduction

The cell death mechanisms used by the parasite *Tritrichomonas foetus* is a matter of an ongoing debate. Many mechanisms have been studied in different treatments, but much remains to be elucidated with respect to the protein machinery developed by these organisms with regard to death pathways. This review summarizes the current knowledge about cell death of *T. foetus* by showing all models. The aim is to show, on the one hand, that there is too much data requiring one or more explanatory model (s), but, the authors proposed specific models, on the other hand that the present data is not sufficient to definitely proof programmed cell death for this organism. Furthermore, we would like to point out perspectives on the proteomic of programmed cell death in this protist.

Besides the significance of the parasite as an etiologic agent, *T. foetus* has been used as a model for the study of drug carriers such as graphene oxide and carbon nanotube oxide (GCN-O) composite. It is still considered fascinating to study the mode of cell death since they do not have mitochondria but possess an unusual anaerobic membrane-bound organelle named the hydrogenosome [1–3].

Cell death has been studied in many organisms: in mitochondriate organisms there are multiple forms of cell death, including the “programmed cellular death” (PCD) types, that will be described below, and depends or not on the presence of family of proteins, which control the mitochondrial membrane permeabilization and the release of some mitochondrial proteins to cytosol, like observed mainly in apoptosis [4]. Besides, other types of programmed death accompanied by changes in morphological and biochemical features like autophagic cell death, for example, have been studied. The amitochondriate organisms, like *T. foetus*, do not have a known usual programmed death machinery [5].

Despite apoptosis has been shown to be the major mechanisms of death observed in *T. foetus* [6], different studies suggest the existence of more than one mechanism of cell death and, it's the type of stimuli and/or the degree of stimuli that determines the mechanism of death chosen by this cell.

Photodynamic Therapy (PDT) and drugs administration used for cancer chemotherapy results in DNA damage in some cells. A variety of injurious stimuli such as heat, radiation, hypoxia and cytotoxic anticancer drugs can induce apoptosis in low doses or result in necrosis at higher doses [7]. It has been assumed that the machinery of PCD is absent in amitochondrial organism, like trichomonads, however, recent studies show that *T. foetus* have the capacity of choose which way will take among many forms of cell death caspases dependent or independent, depending on the stimuli of this parasite, once individuals in the same culture can take different pathways, either inside of the parasite can occur more than one mechanism of cellular death [4]. It has become increasingly apparent that the mechanisms of cell death

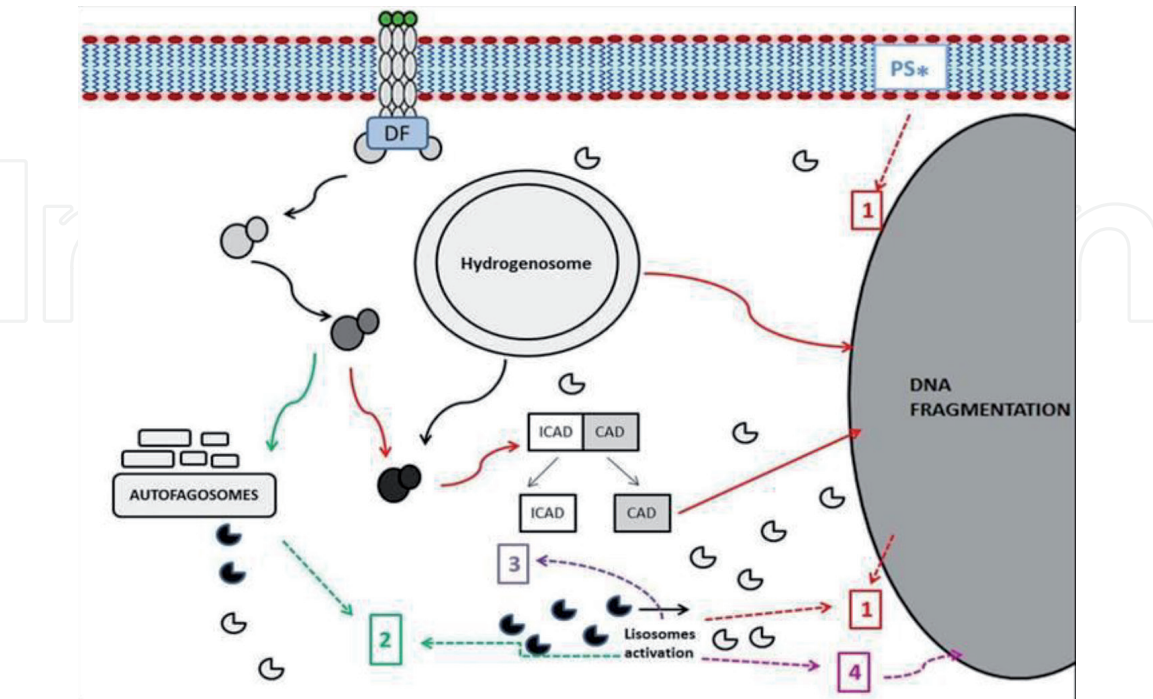


Figure 1. Proposed model for possible executioner pathways during *T. foetus* cell death, which includes the presence of possible apoptosis, autophagy, paraptosis and necrosis. Abbreviations: 1 = apoptosis, 2 = autophagy, 3 = necrosis, 4 = Paraptosis, DF = death receptor, PS* = phosphatidylserine exposure, CAD = caspase-activated DNase, ICAD = inhibitor of CAD.

show a large diversity of phenotypes and cellular mechanisms, and, apparently, a modulation mechanism of cell death may lead to another [8]. Besides, the conservation of the molecular mechanism is relevant to the functional role of PCD process in the biology of protozoa since studies confirm the existence of this process in unicellular eukaryotes of different phylogenetic origins [9]. Hypotheses of alternative pathways of trichomonads cell death are suggested in **Figure 1**.

Other types of cell death may also be considered to be forms of PCD, because they require gene activation and function in an energy dependent manner. PCD is a genetically regulated physiological process, fundamental for multicellular organism development and homeostasis. Studies show that depending on the damage infringed, the cells seem to “choose” how to die [8, 10].

2. Trichomonad hydrogenosomes

According to Müller (1988) [11], *Entamoeba histolytica*, *Giardia lamblia*, *Trichomonas vaginalis*, *Tritrichomonas foetus* and rumen ciliates, they have hydrogenosomes instead of mitochondria. Hydrogenosomes of trichomonads are involved by two membrane layers, as mitochondria, and are organelles related to oxidation of pyruvate and the synthesis of ATP, as well as the storage of Ca^{2+} [12, 13]. As shown previously [14], Succinyl-coenzyme A synthetase (SCS) catalyzes the formation of ATP via substrate-level phosphorylation in hydrogenosome as it does, in mitochondria. Both organelles, mitochondria and hydrogenosome, use pyruvate as a major substrate and form acetyl-CoA (**Figure 2a, b**). Hydrogenosomes convert pyruvate quantitatively to acetate, malate, CO_2 , and H_2 , with acetate as the major product. The electrons from pyruvate:ferredoxin oxidoreductase (PFO) pass through ferredoxin and are transferred to NAD or NADP by ferredoxin:NAD(P) oxidoreductase. This process is accompanied by the phosphorylation of ADP to ATP in presence of

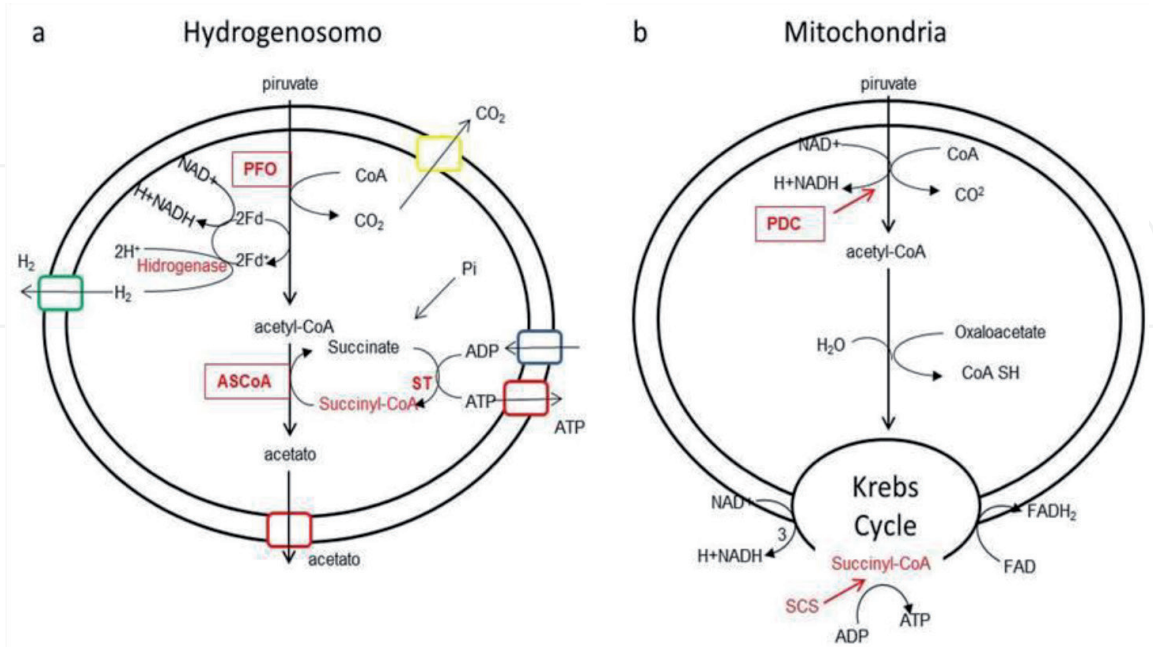


Figure 2.
Comparison of oxidation metabolism of pyruvate between hydrogenosome and mitochondria. (a) In *T. foetus* and probably in all other trichomonad flagellates acetate is formed by the successive action of ASCoA (reaction $\text{acetyl-CoA} + \text{succinate} = \text{acetate} + \text{succinyl-CoA}$) and ST (reaction $\text{succinyl-CoA} + \text{ADP} + \text{Pi} = \text{succinate} + \text{ATP}$). ASCoA = acetate:succinate CoA transferase; PFO = pyruvate:ferredoxin oxidoreductase; ST = succinate thiokinase. (b) Pyruvate oxidation in mitochondria precedes the Krebs cycle. PDC = pyruvate dehydrogenase. SCS = succinyl CoA synthetase.

succinate and acetate:succinate CoA-transferase. The production of H_2 is catalyzed by a hydrogenase which transfers electrons to protons H^+ , a process not available to organisms without hydrogenosomes (**Figure 2a**) [11].

Evidences indicate that hydrogenosomes are anaerobic forms of mitochondria [15] or a specialized form of mitochondria useful in lower O_2 environments [16]. According to Martin (2005) [16] hydrogenosomes and mitochondria are, respectively, anaerobic and aerobic manifestations of the same organelle. Although, unlike mitochondria, the hydrogenosomes lack the DNA [15].

Trichomonad hydrogenosomes possess many proteins in common with mitochondria [15]. Translocation studies using hydrogenosomal ADP/ATP carrier of *T. vaginalis* revealed compatibility in membrane protein import between mitochondria and hydrogenosomes. These hydrogenosomal ADP/ATP carriers utilize the same translocation pathway for translocation into mitochondrial inner membrane [17]. Hydrogenosomes also contain heat-shock proteins which are known to participate in protein translocation and folding in mitochondria [17].

The most accepted hypothesis for the origin of hydrogenosomes and mitochondria is that both organelles share a common ancestral. Phylogenetic studies demonstrated the existence of a typical Hsp 70 gene in the *T. vaginalis* genome DNA that in other eukaryotes codes for a protein located in mitochondria. This suggests that trichomonads have had mitochondria in their early history, and this nuclear sequence could be the result of an ancient gene transfer from mitochondria to nucleus [18]. Many components of classical mitochondria are absent in hydrogenosomes, and they generate molecular hydrogen instead of consume oxygen. It is interesting to study whether hydrogenosomes are involved in the cell death at all [1].

During hydrogenosome formation, they have different forms, and then acquire a spherical structure, which can be changed in stress conditions [19]. Studies proposed that *T. fetus* under treatment with hydroxyurea, zinc or under serum deprivation present endoplasmic reticulum cisternae surrounded by abnormal hydrogenosomes, which have bigger size enlarged peripheral vesicles, and sometimes presenting a degraded aspect [20, 21].

Hydrogenosomes also exhibited altered size and shape and they were randomly distributed within parasites cells after lycorine treatment [2]. The sequence of alterations during the degradation of hydrogenosomes after treatment with lycorine included: matrix swelling, rupture of outer membrane, appearance of flocculent densities, and fragmentation of all membranous structures except the peripheral vesicle [20]. *T. fetus* treated with taxol, nocodazole or colchicine showed modifications in size, shape and distribution of the hydrogenosomes [22]. The presence of hydrogenosomes with altered morphologies was also observed in parasites incubated with different concentrations of thiabendazole [23]. Recent studies showed that alterations in lysosomes and hydrogenosomes were also observed in *T. fetus* after proteasome inhibitors treatment [24]. Other studies suggest that tetracycline disrupts hydrogenosomal function since it reduced the hydrogenosomal energy metabolism efficiency in *T. vaginalis* [25].

3. Morphological features to define programmed cellular death

PCD is not confined to apoptosis but that cells use different pathways for active self-destruction: condensation prominent or apoptosis; autophagy prominent, etc. [26, 27]. Although, there is some resistance to the exclusive use of the term PCD to specifically describe apoptosis [7]. PCD It now generally refers to any cell death that is mediated by the intracellular death program, no matter what triggers it

and whether or not it displays all of the characteristic features of apoptosis. It has become increasingly apparent that cell death mechanisms include a highly diverse array of phenotypes and molecular mechanisms. Because other types of cell death may require gene activation and function in an energy dependent manner, they are also considered to be forms of PCD. There is evidence of other forms of non-apoptotic programmed cell death that should also be considered since they may lead to new insights into cell death programs and reveal their potentially unique roles in development, homeostasis, neoplasia and degeneration. It is probable that all normal cell deaths, as well as many pathological cell deaths, utilize this evolutionarily conserved death program [28].

Apoptosis, autophagy and necrosis was previously named as 'type I, II and III cell death', respectively [29, 30]. Although, several critiques are related to this clear-cut distinction [31, 32]. According to morphological criteria, the cell death modalities during tissue development and homeostasis can be defined with three distinct routes of cellular catabolism.

3.1 Apoptosis

APOPTOSIS is characterized by specific morphological and biochemical changes of dying cells, including cell shrinkage, nuclear condensation and fragmentation, dynamic membrane blebbing and loss of adhesion to neighbors or to extracellular matrix. The biochemical changes include chromosomal DNA cleavage into internucleosomal fragments, phosphatidylserine externalization and a number of intracellular substrate cleavages by specific proteolysis [33].

3.2 Autophagic

AUTOPHAGIC is characterized by the sequestration of cytoplasm and organelles in double or multimembrane vesicles and delivery to the cell's own lysosomes for subsequent degradation, exhibits extensive degradation of Golgi apparatus, polyribosomes and endoplasmic reticulum, which precedes nuclear destruction [27, 34]. Later, swelling of cavities was observed and the dying cells were ultimately fragmented and were phagocytosed by the neighboring cells. The process of autophagy depends on both continuous protein synthesis and the continuous presence of ATP. The molecular mechanisms have been extensively studied in yeast and mammalian orthologues continue to be elucidated [35, 36]. In PDT treatments, a process employing UV-light and photosensitizers to kill cancer cells, as well as accumulation of lysosomotropic agents within the organelle, a process also triggers the lysosomal pathway of cell death [37, 38].

3.3 Cytoplasmatic death or programmed necrosis

CITOPLASMATIC DEATH or PROGRAMMED NECROSIS is characterized by the swelling of cavities with a membrane border, such as mitochondria, followed by extensive fragmentation of the cells into fragments so small that cell debris can no longer be observed. This type of cell death occurred without the lysosomal system taking part and with recognizable reaction of the neighboring cells and was observed in regions of vacuolated cartilage during mineralization [27].

There is evidence that modulation of one form of cell death may lead to another [7]. Under some circumstances, apoptosis and autophagy can exert synergistic effects, whereas in other situations autophagy can be triggered only when apoptosis is suppressed [33].

4. Biochemical and morphological features to define cell death in parasite trichomonas

4.1 Caspases pathway

Caspases are essential proteins involved in cellular death that exist in cytosol of most cells in its inactive form as a polypeptide. They are, activated by cleavage, and apoptosis is considered a consequence of their activation cascade [39].

In some cases caspases can induce cellular death and in others they seem to be irrelevant in decision between death and life. In both situations, caspases participate in morphology of apoptosis [39]. According to Mariante et al. [4], caspases dependent cell death in trichomonads can occur through different known mechanisms, either by death receptors pathway, or through unknown signaling pathways, like the release of hydrogenosomes molecules, which may have analogous functions of mitochondrial proteins.

Although it is established that the apoptosis regulated by caspases are an important form of PCD, in many instances, PCD is caspase independent and non-apoptotic. Necrosis-like might or might not require caspases to activate cell death, while paraptosis and autophagic/vacuolar cell death traditionally do not call for the participation of caspases [10, 40]. Despite, some studies in mammalian cells indicate that caspases can regulate both apoptotic and nonapoptotic cell death, as autophagy [41].

In eukaryotic organisms, it is known that the caspases possess a fundamental role during the process of cellular death, especially on apoptosis, being the primary site of interaction with Bcl-2 proteins family. However, despite of the lack of mitochondria in trichomonads, Mariante et al. [42] confirm the participation of proteases of the Caspase-3 family in *T. foetus* cellular death, after treatment with H_2O_2 . They showed that hydrogen peroxide may have degenerative effects. Studies suggest that progressive destabilization of the membranes of intracellular organelles, which would be caused for short-term exposure to low concentrations of H_2O_2 may induce lysosomal rupture indirectly by activation of phospholipases [43]. PDT treatment also leads to the release of lysosomes enzymes in *T. foetus* cytoplasm, which by the increase of reactive oxygen species (ROS) activate procaspases that in turn active caspases inducing the occurrence of the cell death process [44].

4.2 Nuclear abnormalities

Trichomonads treated with different concentrations of H_2O_2 showed severe nuclear changes like unusual DNA condensation. Peripheral heterochromatin masses and nuclear DNA fragmentation can be observed in the nucleus of some cells, probably due to the activation of different endonucleases. On the other hand, in mammals the same treatment lead a different nuclear organization [42]. Nuclear changes was observed in *T. foetus* after treatment with H_2O_2 and griseofulvine, which may be related to the presence of activated molecules through caspases pathway, and/or, molecules released from hydrogenosomes [5]. After treatment of *T. foetus* with AlPcS₄, cellular disorder such as nucleus fragmentation was observed in several cells [6]. *T. foetus* PDT treatment associated with the same photosensitizer (AlPcS₄) showed a “ladder pattern” of DNA fragments of *T. foetus* in electrophoresis assay which can be a hallmark of apoptosis. Comet Assay testes confirmed DNA fragmentation in this condition as longer tails was observed (unpublished data). However, taxol and nocodazole induced the formation of multinucleated cells, abnormal distribution of the nuclear contents and, sometimes, nuclear fragmentation [22]. In recent studies utilizing tetracycline, it was observed DNA fragmentation in *T. vaginalis* [25].

4.3 Morphology cell changes

T. foetus has a simple life cycle that consists of only a trophozoitic form, which is characterized by a pear-shaped (PS) body, three anterior flagella and one recurrent flagellum [24, 45]. The trophozoite undergoes profound morphological alterations and takes on an endoflagellar form (EFF), also known as pseudocyst, under unfavorable environmental conditions, such as abrupt changes in temperature or in the presence of drugs, e.g. colchicine [22]. In *T. foetus* treated with AlPcS₄, the internalized flagella and fragmented axostyle-pelta complex was seen, whereas changes in the elongated shape were not observed after 24 h. Although, after 48 h of treatment was observed changes in the shape of parasites. The same changes were observed in *T. foetus* after treatment with PDT [6]. However, after treatment with H₂O₂, the parasites exhibit a change in their morphology, changing from elongated shape to spherical one [42]. After treatment with taxol, nocodazole or colchicine, a large number of cellular changes and drastic effects were observed such as the loss of the cells original shape, the flagella were gradually internalized and the cells assumed the pseudocyst form. During treatment with taxol cell size was increased, and giant, multinucleate and abnormal cells were observed, as well as the presence of zooids and membrane blebbing formation [22]. In *T. vaginalis* tetracycline-treatment the cell exhibited a round shape, a not disrupted plasma membrane, and a rough cell surface [25]. However, the parasites treated with mepoxomicin and bortezomib showed the appearance of wrinkled or rounded cells with externalized flagella, membrane blebbing, cell lysis, intense cytosolic and nuclear vacuolization, cytoplasmic disintegration and abnormal Golgi reduction [24]. The flagella were internalized after treatment of *T. foetus* with thiabendazole, and about 70% of the parasites were observed as giant form. Furthermore, after treatment with mebendazole, more than 90% of these parasites presented drastically altered morphologies [23]. The EFF occurs in living cells able to undertake nuclear division to form multinucleated cells and able to provoke damage to host cell. It is a reversible form of parasite and can have the ecto-phosphatase activities responsible to signaling this process [45, 46].

5. Possible cell death modalities in trichomonas

The biochemical basis for alternative forms of cell death morphologically distinct from PCD continues unknown. Understanding the mechanisms for these forms has implications for the understanding of evolutionary aspects of cell death programs, developmental cell death, neurodegeneration, and cancer therapeutics and for the design of novel therapeutic agents for diseases featuring these alternative forms of cell death [30].

Death of *T. foetus* was observed after higher concentrations of hydroxyurea or zinc, or longer times of serum deprivation [12, 21]. Recent studies show that the molecular events governing tetracyclines induced cell death in trichomonads provided via activating of several specific pathways and genes families [25] which leads to a programmed type of death.

The parasite *T. foetus* submitted to treatment with H₂O₂ and griseofulvine, demonstrated the induction of mechanisms of a second pathway of cellular death related to autophagy machinery with formation of autophagosomes [4]. When subjected to different treatments such as griseofulvine and PDT, the parasite cytoplasm either shows the formation of autophagic vacuoles, which can be related to the mechanism of autophagic cell death [4, 6]. The process of autophagy is suggested also after treatment of *T. vaginalis* with lycorine, owing to fragments of

	Apoptosis	Paraptosis	Autophagy	Treatment	Presence	References
Nuclear Fragmentation	YES	YES	YES (lack of chromatin condensation)	H ₂ O ₂ PDT Griseofulvine AlPcS ₄ Taxol Nocodazole Tetracycline (<i>T. vaginalis</i>)	YES	[42] [6] [4] [6] [22] [22] [25]
Caspases Activation	YES	*	YES	H ₂ O ₂ Griseofulvine	YES NO	[42] [4]
PS Externalization	YES	NO		Licorine (<i>T. vaginalis</i>)	NO	[2]
Citoplasmic vacuolization	NO	YES	YES	H ₂ O ₂ PDT Licorine (<i>T. vaginalis</i>) Proteasome inhibitors Mepoxomicin and Bortezomib	YES	[42] [6] [2] [24] [24] [23]
Cellular shaped changes	YES			H ₂ O ₂ PDT Licorine (<i>T. vaginalis</i>) Mepoxomicin and Bortezomib Mebendazole	YES	[42] [6] [2] [24] [23]
Lysosomal enzyme release	YES		YES	PDT	YES	[44]
Flagellar internalization	*	*	*	AlPcS ₄ Taxol, Nocodazole and Colchicine Thiabendazole	YES	[6] [20] [23]
Plasmatic Membrane Projections (Blebs)	YES	NO	NO	H ₂ O ₂ Licorine (<i>T. vaginalis</i>) Taxol, Nocodazole and Colchicine	YES NO YES	[42] [2] [20]

	Apoptosis	Paraptosis	Autophagy	Treatment	Presence	References
Hydrogenosomes shaped changes	*	*	*	H ₂ O ₂ PDT Hidroxiuréia Zinc Serum deprivation Taxol, Nacodazole and Colchicine Licorine (<i>T. vaginalis</i>) Lactacystin Mebendazole	YES	[42] [6] [21] [20] [20] [20] [2] [24] [23]
Citoplasmatic components degradetion	YES (Fragmention of citoplasm)	YES	YES	Licorine (<i>T. vaginalis</i>) Mepoxomicin and Bertezomid	YES	[2] [24]
Major structural changes and pathways in cell death of the parasite T. foetus. *Unreferenced data.						

Table 1.
Ultrastructural changes leading to cell death pathways on T. foetus.

endoplasmic reticulum seen in close contact with abnormal hydrogenosomes [2]. Furthermore, parasites treated with proteasome inhibitors exhibit the appearance of an uncommon enlarged endoplasmic reticulum and concentric membrane whorls, which resembled autophagic vacuoles [24]. The round-shaped mebendazole-treated parasites presented an intense cytoplasmic vacuolization, and profiles of endoplasmic reticulum were frequently seen in association with abnormal hydrogenosomes and vacuoles [23]. Another autophagy way can be necessary for caspases activation and induction of apoptosis in trichomonads. However, cellular death induced by griseofulvine in trichomonad seems to not involve the path of caspases autophagy way. Since both the mechanisms, apoptosis and autophagy, can lead to release of lysosomal components after treatment of *T. foetus* with PDT [44], it is not known which of these mechanisms the parasite is using in different treatments.

The absence of apoptotic bodies and the characterization of a non-apoptotic-like cell death which fails to fulfill the criteria for apoptosis suggest paraptosis mechanism, once the cell death features shown by lycorine treatment in *T. vaginalis* differ from the apoptosis-like characteristics reported for this protist [2]. Although *T. foetus* presents some morphological aspects similar to apoptosis such as nuclear fragmentation and chromatin condensation, it also features aspects of paraptosis such as cytoplasmic vacuolization and chromatin condensation [6]. The main feature of paraptosis consists of extensive cytoplasmic vacuolation without significant cell membrane blebbing, nuclear shrinkage, or pyknosis. To date the most defining feature that differentiates it from autophagic cell death is the absence of autophagic vacuoles in paraptosis (**Table 1**) [29, 30, 42, 47].

Although, recent studies showed the “ladder pattern” compatible internucleosomal genomic DNA fragmentation characteristic of apoptosis, in *T. foetus* submitted to PDT treatment (unpublished data).

The different treatments and the different results obtained with *T. foetus* suggest the existence of more than one mechanism of cell demise in these parasites (**Figure 1**).

6. Implications and future directions

The evidence of that alternative, non-apoptotic, PCD in unicellular organisms has important implications for understanding cell dynamics. The environmental stimuli can produce different types of cell death depending on the intensity of stimulus, and that classic apoptosis and necrosis may represent only two extremes of a continuum intermediate form of cell death, applicable to also unicellular organisms [10]. Comparatives analyses of proteome maps from parasites exhibiting such pathogenic characteristics may provide valuable data to understand the pathogenic mechanisms involved in urogenital trichomoniasis. Besides, the metabolic pathways that are different from those of their mammal hosts, given that Trichomonads possesses a hydrogenosomal/cytosolic compartmentalization of metabolism and metabolic pathway, the identification of proteins involved in such metabolic pathways could reveal good targets for drugs development [1, 48].

Several laboratories have contributed to understand the protein expression of Trichomonads, but despite numerous research and efforts to unravel the mechanisms of cell death, detailed description of the molecular mechanisms is still unknown. Identification of proteins related to the machinery of death of these cells should be the main focus of studies in the coming years. Studies related to molecular biology and biochemistry are still needed because little is known about the overall proteomic expression profiling of this parasite.

Acknowledgements

We gratefully acknowledge the Brazilian agency FAPESP (São Paulo Research Foundation) (2013/20054-8) for financial support.

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