Available online at www.sciencedirect.com
ScienceDirect
Veterinary Parasitology 146 (2007) 175–181
veterinary parasitology
www.elsevier.com/locate/vetpar

Short communication

Ultrastructural changes in *Tritrichomonas foetus* after treatments with $AlPcS_4$ and photodynamic therapy

Newton Soares da Silva *, Claudia de Mello Ribeiro, Aline Helena Araujo Machado, Cristina Pacheco-Soares

Laboratório de Biologia Celular e Tecidual, Instituto de Pesquisa e Desenvolvimento, Universidade do Vale do Paraíba, Av. Shishima Hifumi 2911, 12244-000 São José dos Campos, SP, Brasil

Received 11 September 2006; received in revised form 5 February 2007; accepted 8 February 2007

Abstract

The *Tritrichomonas foetus* is an amitochondrial parasitic protist which causes bovine trichomoniasis, a major sexually transmitted disease in cattle. No effective drugs for this disease have been approved to this date. Photodynamic therapy (PDT) is an experimental treatment that shows great potential for treating bacteria, fungi, yeasts, and viruses. However, the cytotoxic effect of PDT on protozoan has been poorly studied. In this study, PDT with aluminum phthalocyanine tetrasulfonated ($AlPcS_4$) photosensitizer was efficient in killing *T. foetus*. The mode of cell death in *T. foetus* after PDT was investigated by transmission electron microscopy. Morphological changes, such as membrane projections, nucleus fragmentation with peripheral masses of heterochromatin, endoplasmic reticulum proliferation, intense cytoplasmic vacuolization, fragmented axostyle–pelta complex, and internalized flagella could be observed. This is the first report to demonstrate cell death in *T. foetus* after PDT, and thus will open up new lines of investigation to develop new treatments for bovine trichomoniasis.

Keywords: *Tritrichomonas foetus*; Photodynamic therapy; $AlPcS_4$; Cytotoxic effect; Cell death

1. Introduction

The parasitic protozoan *Tritrichomonas foetus* causes bovine trichomoniasis, a major sexually transmitted disease in cattle. Bovine trichomoniasis amounts to considerable economic losses in the United States, Canada, and South America, as it leads to miscarriage and infertility in bovines (Cobo and Campero, 2002). Treatment is difficult because no effective drugs to be used in cattle have been approved to this date (Bondurant, 1997). Thus, it is necessary to develop and test new therapies against this disease.

Photodynamic therapy (PDT) is an experimental treatment, which shows great potential for treating neoplastic and nonneoplastic diseases. In PDT, visible light activates a photosensitizing drug accumulated in cells or tissue. The interaction between the excited photosensitizer and molecular oxygen produces singlet oxygen ($^1O_2$) as well as other reactive oxygen species (ROS) (Dougherty et al., 1998). The generation of singlet oxygen is supposed to play a major role in photodynamic cytotoxicity (Gomer et al., 1989). PDT can induce cell death by necrosis or apoptosis, both *in vitro* and *in vivo* (Fabris et al., 2001). Various cell organelles could be postulated as target for PDT, such as plasma membrane, mithocondria, endoplasmic reticulum, and cytoskeletal structure (Ferreira et al., 2004).

* Corresponding author. Tel.: +55 12 39471143.
E-mail address: nsoares@univap.br (N.S.d. Silva).

0304-4017/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2007.02.006

Oscar Raab (1900) published the first paper about the photodynamic effects of the activities of chemical compounds against microorganisms. He observed that low concentrations of acridines in the presence of light could be lethal to the protozoan *Paramecium*. Antimicrobial PDT research has increased in the last 20 years. Bacteria, as well as fungi, yeasts, and viruses, treated with photosensitizers were shown to be successfully killed by visible light (Wainwright, 1998). However, the cytotoxic effect of PDT on protozoan has been poorly studied.

Evidence supports the involvement of mitochondria during cell death of many different cell types, including unicellular organisms, caused by PDT (Wainwright, 1998; Lam et al., 2001). *T. foetus* does not contain mitochondria but has characteristic membrane-bound organelles that are termed hydrogenosomes, which contain enzymes that participate in the metabolism of pyruvate and are the site of formation of ATP and molecular hydrogen (Muller, 1993).

Cell death by PDT in amitochondrial organisms is still to be elucidated. The aim of this study was to evaluate the cytotoxic effect of PDT with aluminum phthalocyanine tetrasulfonated ($AlPcS_4$) photosensitizer, in culture of *T. foetus*.

2. Materials and methods

2.1. T. foetus

T. foetus, K strain, was kindly provided by Dr. Fernando Costa e Silva Filho, from the Institute of Biophysics Carlos Chagas Filho, Brazil (UFRJ-RJ). The parasite was kept in TYM Diamond's medium (Diamond, 1957) supplemented with 10% of fetal calf serum for 48 h at 37 °C in a humidified atmosphere containing 5% of CO_2. The number of parasites was standardized at a number of 1×10^6 cell mL^{-1}.

2.1.1. Photosensitizer

The drug chloroaluminum phthalocyanine tetrasulfonate ($AlPcS_4$) (Porphyrin Products, Inc.), was dissolved in PBS to a stock concentration of 1 mM, and stored in the dark at 4 °C until used.

2.1.2. Treatment of T. foetus with $AlPcS_4$

Parasites were distributed at a number of 1×10^6 cells mL^{-1} as follows: four vials without treatment (control and for light treatment only) and four vials with $AlPcS_4$ (10 μM) were incubated for 60 min in the dark at 37 °C. After this period, they were washed with PBS twice in order to remove the photosensitizer that had not been taken up by cells. Furthermore, 500 μL of fresh TYM Diamond's medium without serum was added for irradiation.

2.1.3. Irradiation

Two vials with parasites that had undergone treatments with $AlPcS_4$ or not were subjected to irradiation in the dark with a semiconductor laser (Thera Lase-DMC), ($\lambda = 685$ nm; $P = 26$ mW; D.E. = 0.5 J cm^{-2}; $t = 35$ s).

The parasite was kept in TYM Diamond's medium (Diamond, 1957) supplemented with 10% of fetal calf serum for 48 h at 37 °C in a humidified atmosphere containing 5% of CO_2. Parasites were counted in Neubauer hemocytometer 24 and 48 h after treatment, using a Leica DMLB photomicroscope.

2.1.4. Transmission electron microscopy

T. foetus was fixed for 1 h at room temperature in 4.0% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. After fixation, parasites were washed in PBS and postfixed in 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.2 with 1% potassium ferricyanide, and 5 mM $CaCl_2$ at room temperature, in the dark. Cells were then washed in PBS, dehydrated in acetone and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and observed in a Jeol 1210 Transmission Electron Microscope or Zeiss EM10 Transmission Electron Microscope.

2.1.5. Statistical analysis

A one-way ANOVA (Microcal™Origin™ 5.0, Microcal Software Inc.) was used. Values are given as mean ± S.E. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Cellular death of T. foetus after incubation with $AlPcS_4$

In this experiment, we investigated the action of $AlPcS_4$ in culture of *T. foetus*. To determine the effect of aluminum phthalocyanine tetrasulfonated photosensitizer alone (regardless of light) on the number of parasites, *T. foetus* was analysed 24 and 48 h after incubation with $AlPcS_4$ (10 μM). The cytotoxic effect of treatment was investigated by counting the parasites in the Neubauer hemocytometer, 24 and 48 h after incubation. The action of $AlPcS_4$ on *T. foetus* resulted in a sharp fall in the number of parasites (~46%), after 24 and 48 h of their incubation with the drug (Fig. 1).

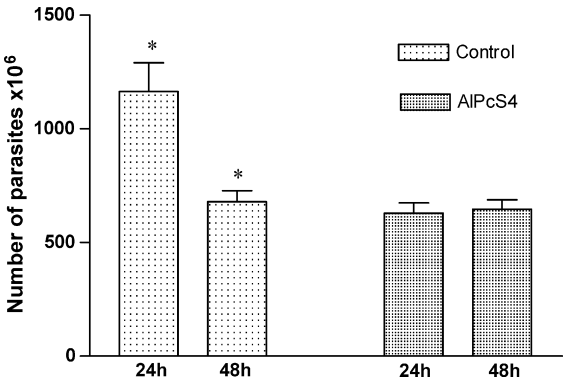

Fig. 1. Number of *T. foetus* 24 and 48 h after incubation with $AlPcS_4$. The means are significantly different: $^{*}P < 0.05$ (mean ± S.E., $n = 12$).

3.2. Cellular death of T. foetus after treatment with $AlPcS_4$ and exposed to laser irradiation (PDT)

We investigated the effect of ROS generated by PDT on *T. foetus*. The parasites were submitted to treatment with $AlPcS_4$ (10 μM) photosensitizer and subjected to irradiation. We evaluated the cytotoxic effect of PDT on *T. foetus* culture by counting the parasites in the Neubauer hemocytometer, 24 and 48 h after treatment. No significant difference was observed between control and parasites treated with light only (Fig. 2A and B). PDT resulted in sharp reduction in the number of these parasites (∼82%) 24 h after treatment (Fig. 2A). Increased number of *T. foetus* was observed 48 h after PDT in relation to what was observed 24 h after treatment (Fig. 2B).

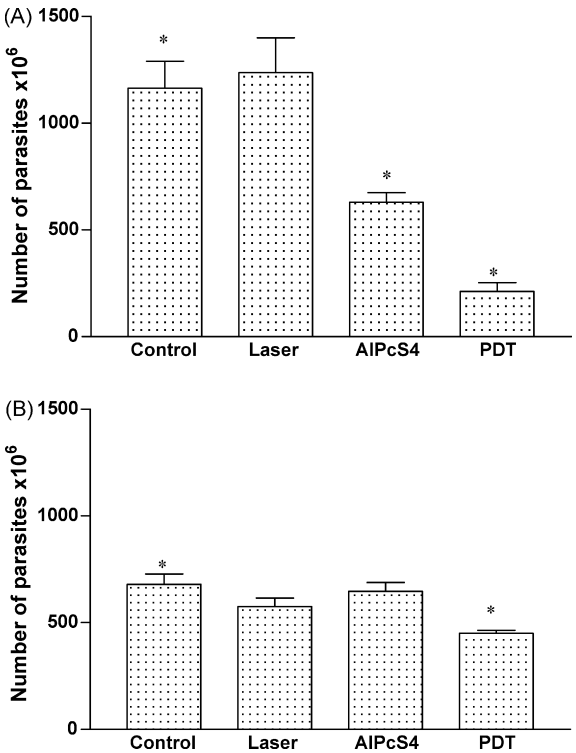

Fig. 2. Number of *T. foetus* 24 h (A) and 48 h (B) after treatment with PDT. The means are significantly different: $^{*}P < 0.05$ (mean ± S.E., $n = 12$).

3.3. $AlPcS_4$ induces morphological changes in T. foetus after incubation in the dark

Parasites were studied by transmission electron microscopy in order to analyze the cytotoxic effect of treatment with $AlPcS_4$ in *T. foetus*. An untreated parasite presents an elongated or pear shape, three anterior flagella and a recurrent flagella, one nucleus, several spherical hydrogenosomes, a prominent Golgi apparatus, and a microtubular axostyle–pelta complex (Fig. 3).

Change in the elongated shape of *T. foetus* was not observed 24 h after treatment with $AlPcS_4$. However, cellular disorder was observed in several cells, such as nucleus fragmentation with peripheral masses of heterochromatin, internalized flagella, fragmented axostyle–pelta complex, and intense vacuoles surrounded by membrane with different contents (Fig. 4).

Analysis 48 h after treatment with $AlPcS_4$ showed that the photosensitizer induced a change in the shape of parasites. *T. foetus* presented rounded shape and some vacuoles with different contents, internalized flagella, and fragmented axostyle–pelta complex were observed (Fig. 5).

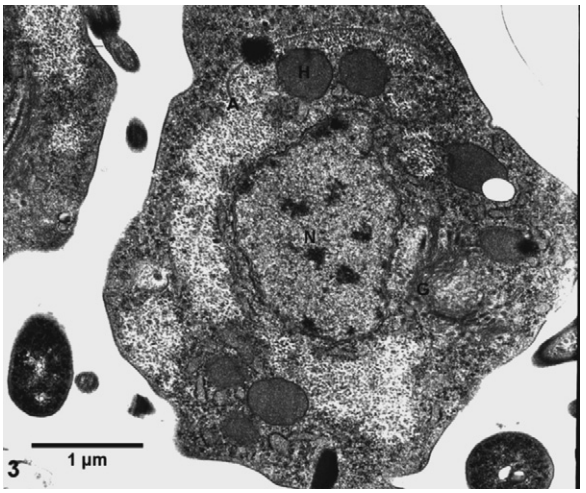

Fig. 3. Transmission electron micrographs of thin sections of untreated *T. foetus* (control): nucleus (N), hydrogenosomes (H), microtubular axostyle–pelta complex (A), and Golgi apparatus (G).

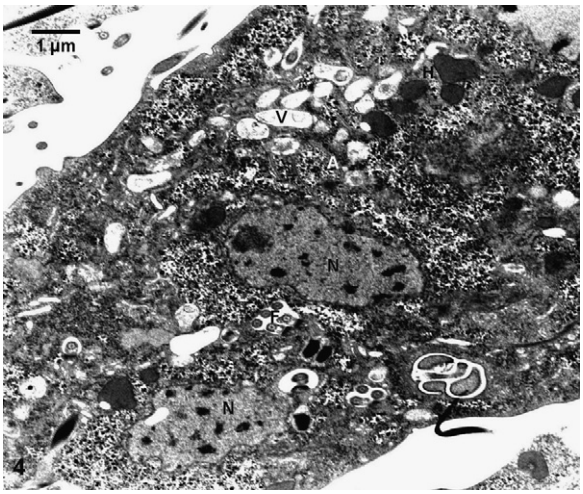

Fig. 4. Section of photosensitizer-treated *T. foetus* 24 h after incubation. Nucleus fragmentation (N), large-sized vacuoles with different contents (V), flagella inside vacuoles (F), fragmented axostyle–pelta complex (A), and hydrogenosomes (H).

3.4. PDT induces morphological changes in *T. foetus*

T. foetus presented morphological changes after treatment with PDT. Some parasites (50–70%) presented rounded shape 24 h after treatment. Cells presented cellular disorder, nucleus fragmentation with peripheral masses of heterocromatin, internalized flagella, intense cytoplasmic vacuolization with different contents, and fragmented axostyle–pelta complex (Fig. 6).

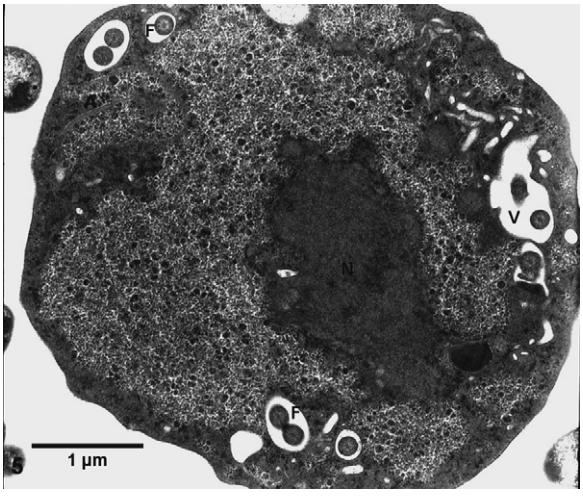

Fig. 5. *T. foetus* 48 h after incubation with photosensitizer. Parasites present rounded shape, nucleus (N), internalized flagella (F), vacuoles with different contents (V), and fragmented axostyle–pelta complex (A).

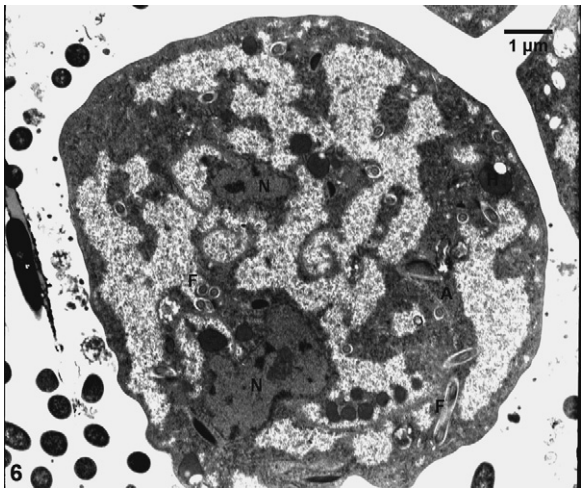

Fig. 6. *T. foetus* 24 h after PDT. The parasites display rounded shape, nucleus fragmentation (N), flagella inside vacuoles (F), and fragmented axostyle–pelta complex (A).

Forty-eight hours after PDT, morphological changes in *T. foetus* were similar to the ones in other cells studied in this experiment, which included nucleus with peripheral masses of heterocromatin, internalized flagella, membrane projections, cytoplasmic vacuoles with different contents, and hydrogenosomes with highly electron dense matrix (Fig. 7).

4. Discussion

Several drugs have been used to investigate the cytotoxic effect on *T. foetus*, such as colchicine,

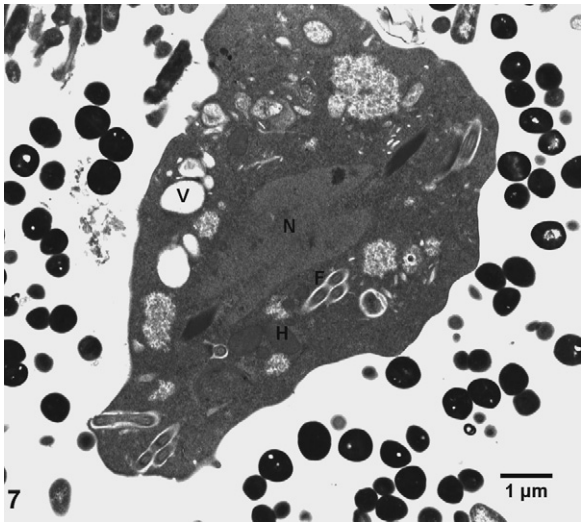

Fig. 7. *T. foetus* 48 h after PDT. Observe nucleus (N), vacuoles (V), internalized flagella (F), and hydrogenosomes (H).

vinblastine, cytochalasin B (Silva-Filho and De Souza, 1986), taxol, nocodazole, griseofulvin, lactacyst, and hydrogen peroxide (Vancini and Benchimol, 2005). In this experiment, we analyzed the cytotoxic effect produced by PDT with aluminum phthalocyanine tetrasulfonated ($AlPcS_4$) photosensitizer on *T. foetus* culture.

At first, we investigated the effects of the photosensitizer without exposing this protist to light. A decrease in the number of *T. foetus* was observed when counting the parasites in the Neubauer hemocytometer 24 h after incubation. This data suggests that $AlPcS_4$ in the dark is toxic to parasites. Similar results have been observed in protozoa *Trypanosoma cruzi* and *Plasmodium falciparum*. The silicon phthalocyanine (Pc4) (2 μM) photosensitizer, without illumination, inactivates *T. cruzi* trypomastigote in fresh frozen plasma after 30 min of incubation (Gottlieb et al., 1995). The parasitemia in culture of *P. falciparum* incubated with *d*-aminolevulinic acid (ALA), decreased after 3 days (Smith and Kain, 2004). Hence, current evidence suggests that $AlPcS_4$ without light exposure might be useful against bovine trichomoniasis for chemotherapeutic purposes.

ROS generated by antiparasitic agents or macrophages can kill intracellular parasites and are therefore important regulators of protozoal infection (Das et al., 2001). *In vitro* studies revealed decreased viability *of Trichomonas vaginalis* exposed to various concentrations of hydrogen peroxide (H_2O_2) (Davis and Lushbaugh, 1993). Inhibited motility and increased cellular death incidence were observed after treating cultures of *T. foetus* with H_2O_2, both of which depended on the H_2O_2 concentration (Mariante et al., 2003). Treatment of *Leishmania donovani* promastigotes with H_2O_2 resulted in a dose-dependent inhibition of cell motility and cell death. Nevertheless, loss of motility did not coincide with cell death, as membranes were intact even in immotile cells, suggesting that H_2O_2 was the source of stress that caused metabolic changes within the cells (Das et al., 2001).

In PDT, interaction between the excited photosensitizer and molecular oxygen caused ROS to induce cell death (Dougherty et al., 1998). Nonetheless, information concerning the effect of ROS generated by PDT in parasites protozoan is scant.

Photodynamic activation derivatives of ALA are able to inactivate *P. falciparum in vitro* (Smith and Kain, 2004). PDT with silicon phthalocyanine (Pc4) photosensitizer inactivates *T. cruzi* trypomastigote in fresh frozen plasma and red blood cell concentrates (Gottlieb et al., 1995).

In order to better analyze the cytotoxic effect of ROS generated by PDT on *T. foetus*, parasites were treated with $AlPcS_4$ and exposed to laser irradiation. When counting *T. foetus* in the Neubauer hemocytometer 24 h after treatment, a decrease in the number the parasites was observed, demonstrating that this type of therapy is efficient against *T. foetus in vitro*. *T. foetus* count 48 h after PDT demonstrated that there was an increase in the number of parasites. This fact can be explained by the life cycle of these protozoan, which lasts around 4–6 h. Although the number of parasites decreased, some protozoan demonstrated not to be affected 24 h after PDT, and thus had food and space available in the culture medium. Due to this fact, it is thought that these parasites divided themselves, increasing their number.

The mode of *T. foetus* cell death after treatments with photosensitizer and PDT was also investigated by transmission electron microscopy. Similar morphological changes in parasites after these treatments, such as cellular disorder, membrane projections, nucleus fragmentation with peripheral masses of heterochromatin, intense vacuoles with different contents, fragmented axostyle–pelta complex, and internalized flagella were observed through ultrastructural analyses.

T. foetus submitted to stressful conditions internalized their flagella to form pseudocysts which could be a defensive strategy against environmental changes (Mariante et al., 2006). The induction of pseudocysts by chemicals can lead to an irreversible process that ends up with the death of cells, depending on the exposure time and/or the concentration of the drug employed (Mariante et al., 2003).

The mode of cell death by PDT may be either apoptosis or necrosis, depending on the nature and concentration of the photosensitizer and the amount of irradiation (Moor, 2000). However, cell death after PDT is often by apoptosis, and substantial evidence supports the involvement of mitochondria in this process. It has been proposed that ROS induce the opening of the mitochondrial membrane permeability transition pores, which results in the dissipation of the mitochondrial membrane potential ($\Delta\psi_m$) and mitochondrial swelling, essential conditions for the escape of cytochrome *c* from mitochondria into the cytosol (Chiu and Oleinick, 2001; Zeiss, 2003).

Several trichomonad species, including *T. foetus*, contain no mitochondria but have another type of membrane-bound organelle, which was termed hydrogenosomes. The hydrogenosomes are bound by a double membrane, generating ATP and molecular hydrogen. However, this organelle does not contain genome, oxidative phosphorylation, tricarboxylic acid

cycle, cytochromes, cardiolipin, or F0F1 ATPase activity (Muller, 1993; Bui et al., 1996).

In this study, after treatment with photosensitizer or PDT, *T. foetus* presented some morphological aspects similar to apoptosis such as nuclear fragmentation and chromatin condensation; aspects of paraptosis were also present, a form of programmed cell death that is distinct from apoptosis by the criteria of morphology, biochemistry, and response to apoptosis inhibitors (Sperandio et al., 2000) such as cytoplasmic vacuolization and chromatin condensation.

Nevertheless, another form of cell death, autophagy, presents features of cellular components degradation that start in the autophagic vacuoles of the dying cell. The morphological characteristics of autophagy include vacuolization, degradation of cytoplasmic contents, and slight chromatin condensation. The autophagic pathway begins with the sequestration of cytoplasmic material in double-membrane vesicles known as autophagosomes. Autophagosomes then fuse with lysosomes in a process that depends on the microtubules, and the contents are then degraded (Fink and Cookson, 2005).

T. foetus presented intense cytoplasmic vacuolization after treatment with photosensitizer and PDT. The vacuoles are surrounded by membranes with different contents including membrane profiles, and disorganized flagellar-axonemes. The same results were observed by Mariante et al. (2006) in *T. foetus* treatment with griseofulvin. These authors also found positive reaction for tubulin in these vacuoles, possibly corresponding to internalized flagella and/or the axostyle–pelta complex and detected autophagic vacuoles with acridineorange, suggesting the presence of an autophagic process.

Interestingly, similar morphological aspects of cell death in trichomonads have been described after treatments with drugs. Chose et al. (2002) observed morphological aspects of *T. vaginalis* treated with pro-apoptic drugs such as rounded shape, nuclear fragmentation, apoptotic-like bodies, and cytoplasmic vacuolation. After treatment with different H_2O_2 concentrations, Mariante et al. (2003) observed spherical shape *T. foetus*, nucleus with peripheral masses of heterocromatin, hydrogenosomes elongated with an electron dense matrix, myelin-like figure, large-sized vacuoles with different contents, endoplasmic reticulum proliferation, internalized flagella, and disorganized axostyle.

The data obtained in this study is not sufficient to understand cell death in *T. foetus*. Additional molecular studies are necessary to determine the overall mode of cell death in *T. foetus* after PDT.

In conclusion, we demonstrated that PDT killed an amitochondrial organism such as *T. foetus*. In this parasite, PDT induces a type of cell death other than necrosis. Apoptosis or autophagic cell death after PDT in *T. foetus* may benefit bovines by limiting the inflammatory response, which is detrimental and could even be lethal to these animals.

Acknowledgements

The authors thank Pedro Duarte Novaes, PhD, FOP-Unicamp, for letting us use the Zeiss EM10 Electron Microscope. We also thank Elisa Aparecida Gregório, PhD, CME-UNESP for letting us use the Jeol 1210 Electron Microscope.

References

Bondurant, R.H., 1997. Pathogenesis, diagnosis, and management of Trichomoniasis in cattle. Vet. Clin. N. Am. Food Anim. Pract. 13, 345–361.

Bui, E.T.N., Bradley, P.J., Johnson, P.J., 1996. A common evolutionary origin for mitochondria and hydrogenosomes. Proc. Natl. Acad. Sci. U.S.A. 93, 9651–9656.

Chiu, S.M., Oleinick, N.L., 2001. Dissociation of mitochondrial depolarization from cytochrome *c* release during apoptosis induced by photodynamic therapy. Br. J. Cancer 84, 1099–1106.

Chose, O., Noel, C., Gerbod, D., Brenner, C., Viscogliosi, E., Roseto, A., 2002. A form of cell death with some features resembling apoptosis in the amitochondrial unicellular organism *Trichomonas vaginalis*. Exp. Cell. Res. 276, 32–39.

Cobo, E.R., Campero, C.M., 2002. Nuevos aspectos inmunologicos y vacunales de la tricomoniasis Bovina. Rev. Med. Vet. 83, 203–208.

Das, M., Mukherjee, S.B., Shaha, C., 2001. Hydrogen peroxide induces apoptosis-like death in *Leishmania donovani* promastigotes. J. Cell. Sci. 114, 2461–2469.

Davis, S.R., Lushbaugh, W.B., 1993. Oxidative stress and *Trichomonas vaginalis* effect of hydrogen peroxide in vitro. Am. J. Trop. Med. Hyg. 48, 480–487.

Diamond, L.S., 1957. The establishment of various trichomonads of animals and man in axenic cultures. J. Parasitol. 43, 488–490.

Dougherty, T.J., Gomer, C.J., Henderson, B.W., Jori, G., Kessel, D., Korbelik, M., Moan, J., Peng, Q., 1998. Photodynamic therapy. J. Nat. Cancer Inst. 90, 889–905.

Fabris, C., Valduga, G., Miotto, G., Borsetto, L., Jori, G., Garbisa, S., Reddi, E., 2001. Photosensitization with zinc(II) phthalocyanine as a switch in the decision between apoptosis and necrosis. Cancer Res. 61, 7495–7500.

Ferreira, S.D.R.M., Tedesco, A.C., Sousa, G., Zângaro, R.A., Silva, N.S., Pacheco, M.T.T., Pacheco-Soares, C., 2004. Analysis of mitochondria, endoplasmic reticulum and actin filaments after PDT with $AlPcS_4$. Lasers Med. Sci. 18, 207–212.

Fink, S.L., Cookson, B.T., 2005. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infect. Immun. 73, 1907–1916.

Gomer, C.J., Rucker, N., Ferrario, A., Wong, S., 1989. Properties and application of photodynamic therapy. Radiat. Res. 120, 1–18.

Gottlieb, P., Shen, L., Chimezie, E., Bahng, S., Kenney, M.E., Horowitz, B., Ben-hur, E., 1995. Inactivation of *Trypanosoma cruzi* trypomastigote forms in blood components by photodynamic treatment with phthalocyanines. J. Photochem. Photobiol. 62, 869–874.

Lam, M., Oleinick, N.L., Nieminen, A.L., 2001. Photodynamic therapy-induced apoptosis in epidermoid carcinoma cells. J. Biol. Chem. 276, 47379–47386.

Mariante, R.M., Guimarães, C.A., Linden, R., Benchimol, M., 2003. Hydrogen peroxide induces caspase activation and programmed cell death in the amitochondrial *Tritrichomonas foetus*. Histochem. Cell. Biol. 120, 129–141.

Mariante, R.M., Vancini, R.G., Benchimol, M., 2006. Cell death in trichomonads: new insights. Histochem. Cell. Biol. 125, 545–556.

Moor, A.C.E., 2000. Signalling pathways in cell death and survival after photodynamic therapy. J. Photochem. Photobiol. 57, 1–13.

Muller, M., 1993. The hydrogenosome. J. Gen. Microbiol. 193, 2879–2889.

Raab, O., 1900. Ueber die Wirkung fluorizierender Stoffe auf Infusorien. Z. Biol. 39, 524–546.

Silva-Filho, F.C., De Souza, W., 1986. Effect of colchicine, vinblastine, and cytochalasin b on cell surface anionic sites of *Tritrichomonas foetus*. J. Protozool. 33, 6–11.

Smith, T.G., Kain, K.C., 2004. Inactivation of *Plasmodium falciparum* by photodynamic excitation of Heme-cycle intermediates derived from d-aminolevulinic acid. J. Infect. Dis. 190, 184–191.

Sperandio, S., Belle, I., Bredesen, D.E., 2000. An alternative, non-apoptotic form of programmed cell death. Proc. Natl. Acad. Sci. U.S.A. 97, 14376–14381.

Vancini, R.G., Benchimol, M., 2005. Appearance of virus-like particles in *Tritrichomonas foetus* after drug treatment. Tissue Cell 37, 317–323.

Wainwright, M., 1998. Photodynamic antimicrobial chemotherapy (PACT). J. Antimicrob. Chemother. 42, 13–28.

Zeiss, C.J., 2003. The apoptosis–necrosis continuum: insights from genetically altered mice. Vet. Pathol. 40, 481–495.