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APOPTOTIC-LIKE ACTIVITY OF STAUROSPORINE IN AXENIC CULTURES OF *Trypanosoma evansi*

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SUMMARY

Trypanosoma evansi is a blood protozoan parasite of the genus Trypanosoma which is responsible for surra (Trypanosomosis) in domestic and wild animals. This study addressed apoptotic-like features in *Trypanosoma evansi in vitro*. The mechanism of parasite death was investigated using staurosporine as an inducing agent. We evaluated its effects through several cytoplasmic features of apoptosis, including cell shrinkage, phosphatidylserine exposure, maintenance of plasma membrane integrity, and mitochondrial trans-membrane potential. For access to these features we have used the flow cytometry and fluorescence microscopy with cultures in the stationary phase and adjusted to a density of 10^6 cells/mL. The apoptotic effect of staurosporine in *T. evansi* was evaluated at 20 nM final concentration. There was an increase of phosphatidylserine exposure, whereas mitochondrial potential was decreased. Moreover, no evidence of cell permeability increasing with staurosporine was observed in this study, suggesting the absence of a necrotic process. Additional studies are needed to elucidate the possible pathways associated with this form of cell death in this hemoparasite.

KEYWORDS: *Trypanosoma evansi;* Axenic culture; Staurosporine; Apoptosis.

INTRODUCTION

Trypanosoma evansi is the most widely distributed among pathogenic animal trypanosomes, affecting domestic and wild animals in Asia, Africa and Central and South America[8,11]. The alterations caused by infection with this parasite are multifactorial, occurring in several clinical signs in hosts, including weakness, weight loss, elevation of body temperature and neurological signs[4,22]. The available drugs against *T. evansi* show significant toxicity in horses. There is an imperative need to develop control measures for trypanosomosis by using the efficacy of the new molecule inhibitors. Very little effort is being made to develop improved methods of prevention, diagnosis and treatment[28]. Therefore, we look for potential targets related to apoptosis in *T. evansi*.

Axenic media has been described as allowing a culture of infective trypanosomes which retain *in vitro* their specific surface antigens and their morphological and biochemical features[2,17]. This kind of culture provides us with information about elucidation of diverse physiological features in the parasite such as growth, control of population density and cell death. *Trypanosoma,* spp. and *Leishmania,* spp. have been shown to exhibit apoptosis-like death as a means of parasite population control or following exposure to drugs[12,13,34,39].

Kinetoplastid parasites share several features with apoptosis of multicellular organisms, including cytoplasmic membrane blebbing and vacuolization, changes in mitochondrial potential ($\Delta\Psi_m$), chromatin condensation and DNA fragmentation. However, trypanosomatids lack of genes, Bcl-2 family genes and the TNF-related family of receptors. The most important advantage of the comprehension of cell death in kinetoplastids would be the design of more-active and less toxic drugs directed towards specific molecular targets of the parasite. In this regard, the compound staurosporine (STS) a protein kinase inhibitor and apoptosis inductor in higher eukaryotes[3] has exhibited significant anti-parasitic activity against *Leishmania major* (IC_{50} 5.30 µM) and *T. brucei* (IC_{50} 0.022 µM)[26]. STS is a prototypical ATP-competitive kinase inhibitor in that it binds to many kinases with high affinity, though with little selectivity[18]. This lack of specificity has precluded its clinical use, but has made it a valuable research tool for investigating cell death processes. Thus, we addressed the apoptotic features in *T. evansi* with the use of STS.

MATERIALS AND METHODS

Parasite culture: Trypanosomes were adapted to axenic culture conditions using a method adapted from ZWEYGARTH *et al*.[41]. TeAp-N/D1 *Trypanosoma evansi* strain[25] were grown *in vitro* in a minimum essential medium (MEM) with Earle's salts supplemented with 1 mg of glucose mL^{-1}, 1% MEM nonessential amino acids (100×), 2.2 mg of $NaHCO_3$ mL^{-1} and 10 mM HEPES. The medium was further supplemented with 2 mM sodium pyruvate, 0.2 mM 2-mercaptoethanol,

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0.1 mM hypoxanthine and 15% heat-inactivated horse serum.

Apoptosis induction: Apoptosis induction with STS was accomplished in 24-well plates in a total volume of 1 mL with a density of 1 x 10^6 organisms/mL from previously stationary phase cultures (2.3 x 10^6 organisms/mL). Thereafter STS (Sigma) at 20 nM final concentration was added. The concentration of STS that inhibited trypanosome growth by 50% (IC_{50}) was 50 nM as measured by direct counting of survivors using a hemocytometer (data no shown). Previously, it has determined IC_{50} of STS for *T. brucei* (22 nM)[26]. Necrosis control was achieved using 6 µM digitonin in parasite cultures.

Flow cytometric analysis: Phosphatidylserine (PS) exposure on the outer plasma membrane was measured by staining cells with 1 µg/mL of annexin V-Alexa Fluor (Invitrogen). The mitochondrial trans-membrane potential ($\Delta\Psi m$) was measured by incubating cells with 20 nM Mitotracker-Red ® (Invitrogen). Plasma membrane integrity was assessed by using propidium iodide (Sigma) in the absence of cell permeant. Relative DNA content was assessed using propidium iodide after cell permeabilization with 6 µM digitonin. Cells were analyzed using FACScalibur®cell sorter (BD Biosciences)

Fluorescence microscopy: *T. evansi* cells were stained with Annexin V-Alexa Fluor (green fluorescence), thereafter fixed in 3% (w/v) paraformaldehyde in PBS for 10 min. The fixed cells were washed three times in PBS for 10 min each and examined under a fluorescence microscope (magnification 100X, Nikon Eclipse® E-600, NY, USA).

Analysis of results: Flow cytometry data were analyzed with the software Weasel® 3.01(Walter Eliza Hall Institute Medical Research, Victoria, Australia). Statistical analysis of data was analyzed with Graphpad Prism 5.02® (CA, USA). Mann-Whitney test was used to compare statistical differences between histograms with a significance level of 0.05.

RESULTS

Apoptotic features in *T. evansi:* In order to analyze STS-induced cell death in *T. evansi*, a fluorescence-activated cell sorter (FACS) analysis was employed. When treated with STS, the population on the right shifted towards the left, indicating cell shrinkage (Fig. 1a). The decrease in cell volume is an important early event of apoptosis that distinguishes it from necrosis[6].

Though the analysis of light scattering data are limited to suggesting the existence of an apoptotic process, the apparent effect of STS was complemented by checking classical features of apoptotic cell death. Figure 1b shows phosphatidylserine exposure at the outer cell surface with a significant change of histogram median. Additionally *T. evansi* cells in presence of STS disrupted its mitochondrial trans-membrane potential ($\Delta\psi_m$) (Fig. 1c) which was measured through mitotracker red staining.

Cell shrinkage and maintenance of plasma membrane impermeability are two of the major features of mammalian cell apoptosis, which distinguishes it from the passive and chaotic destruction process of necrosis[14]. The propidium iodide (PI), when used in the absence of cell permeant, does not stain apoptotic cells, but it enters and stains necrotic cells, whose plasma membrane is damaged. *T. evansi in vitro* culture for 24 h with STS was not labeled by PI, whereas treatment with digitonin (that induce necrotic death) caused an increase in PI fluorescence (Fig. 2a), moreover DNA degradation was observed in the presence of STS; Fig. 2b shows the fluorescence modification that suggests a nuclear process associated to STS action[1].

Fluorescence microscopy analysis: The fluorescence microscopy (Fig. 3) shows the images of *T. evansi* in the presence of STS 20 nM (b) and digitonin (6 µM). The STS modified the surface, whereas permeant agent (digitonin) completely changed the cells, where annexin V interacted with internal membranes. Additionally the STS changed the morphology of the parasite. This process could be associated with the accumulation of autophagic vacuoles in the cytoplasm[32].

DISCUSSION

This appears to be the first report showing evidence for the apoptotic-like death of *T. evansi*. The concept of apoptotic death in unicellular organisms is yet to be understood, but it has been thought that several hundreds of parasites could perform as a complex community apparently analogous to the multicellular organisms. Hence contacts among cells could trigger apoptotic signals[13].

Apoptosis in trypanosomatids has been suggested to regulate parasitaemia and differentiation in the insect vector and the coat of procyclin molecules present on the surface of procyclic stages[24,35]. Unlike other trypanosomes species, whose life cycle needs an intermediate host before reaching the adult stage, *T. evansi* does not undergo development in an intermediate host. Most of the studies have reported *T. evansi* as a monomorphic parasite[31,40,41]. However, it has suggested pleomorphism in atypical forms of *T. evansi*[19,31]. This phenomenon depends on the host and environmental conditions of growth of this parasite[11,13,17].

The relevance of apoptosis in this restricted bloodstream parasite remains to be clarified. Nevertheless apoptosis could be a mechanism for regulating cell population which would mitigate the effect of infection in the host[12,13]. In this regard, the host-derived signals could be used to control the parasite density. Different studies have found the presence of *T. evansi* in cerebrospinal fluid and parasite DNA in different areas of the central nervous system from infected animals[4,9,10]. Additionally, PAIM *et al.*[23] have found that infection with *T. evansi* caused changes in the concentrations of 3-nitrotyrosine residues (indirect NO detection) in the CNS of rats infected with *T. evansi.* Oxygen and nitrogen radicals as well as heat shock can induce defined forms of cell death in several parasitic protozoa[7,16,34]. This kind of molecule represents danger signals for the host to regulate parasite population, thereby facilitating the survival of the host but ensuring a sustained infection[13,34],

Our findings indicate that this apoptotic cell death process is triggered in *T. evansi*, as in mammalian cells, by STS. Apoptotic effects of STS in mammalian cells could be produced by different mechanisms: caspase-independent and caspase-dependent. Therefore, this molecule has pleiotropic effects[3]. Nevertheless we can consider the way the STS elicits apoptotic-like death in *T. evansi*. STS is a potent protein kinase C (PKC) inhibitor. WELBURN & MURPHY[36] have reported an upregulation of receptor for activated protein kinase C (RACK) in the bloodstream form of *T. brucei* when cell death is induced with concanavalin A. This suggests the existence of cell death-PKC-related is similar in higher eukaryotes.

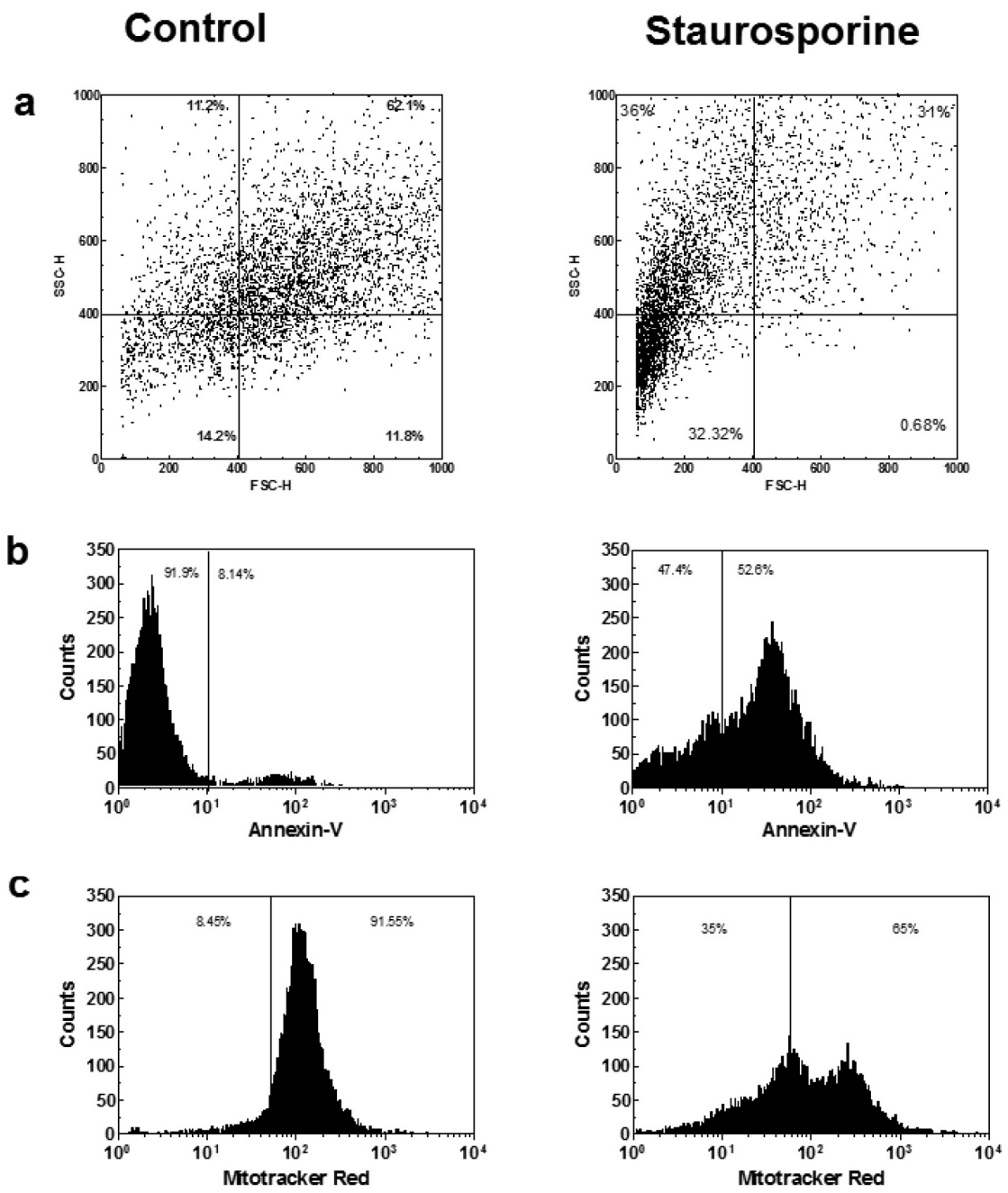

Fig. 1 - Flow cytometry analysis of the apoptotic features of *T. evansi* death. *T. evansi* were incubated in the absence (Control) or presence of staurosporine (20 nM) for 24 h. (a) light scattering properties, (b) phosphatidylserine exposures (PS) and (c) mitochondrial membrane potential (ΔΨm).

The presence of cysteine proteinases and metacaspases has been reported in *T. evansi*[28,37], but there is no consistent data in trypanosomatids regarding the caspase implication in apoptotic-like death[13,34]. However, apoptosis mediated by proteases dissimilar to classical caspases have been reported by numerous authors[5,30]. Cysteine proteinases of *T. evansi*, resemble the mammalian lysosomal cathepsin B and L, which are implicated in apoptotic processes[20,37]. Future studies to elucidate the role of *T. evansi's* proteases in apoptosis will be made.

Microscopy analysis data suggest the vacuolation process. It has been reported that apoptosis induces vacuolation. We find morphological modification in the presence of staurosporine. Previously YIN *et al.*[38] have shown the vacuolation process to be induced by STS in *Blastocystis,* spp. On the other hand the feasibility of the other death process (autophagy) could be settling during STS induction[33].

Independent of the molecular nature of the effectors involved, our

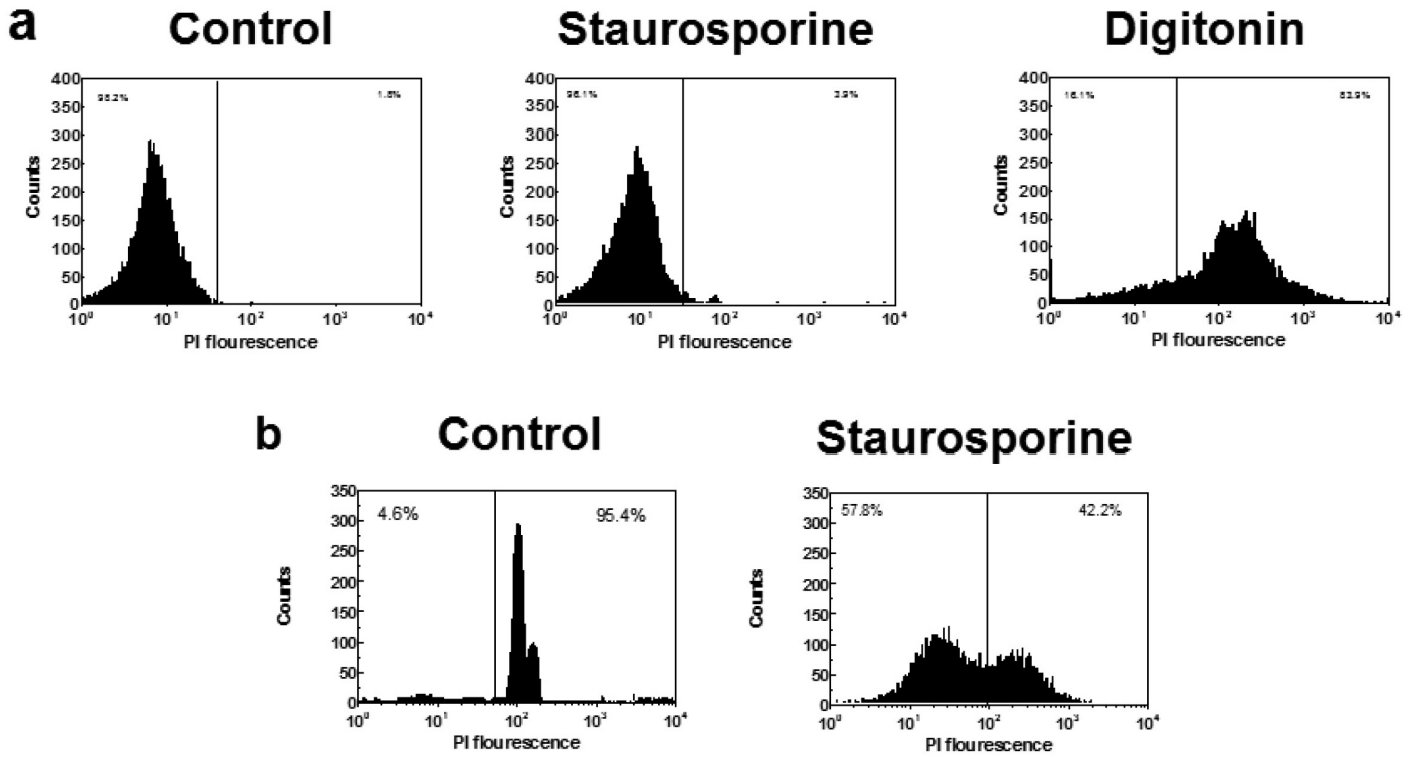

Fig. 2 - Cell permeability and DNA content in *T. evansi* in absence and presence of staurosporine. a) Fluorescence of PI and b) DNA content in absence and presence of STS in *T. evansi* cells previously permeabilized with digitonin 6 µM at 30 min.

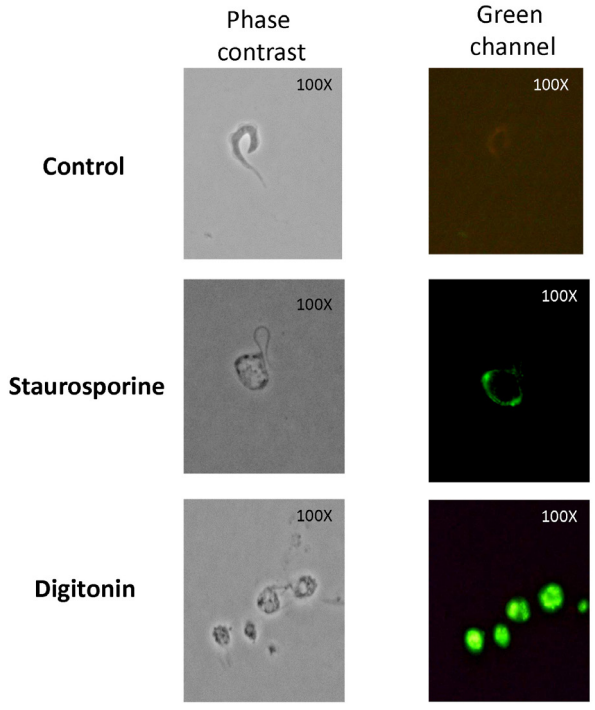

Fig. 3 - Fluorescence microscopy in *T evansi* cells. Apoptosis-inducing activity was evaluated by morphological observation. Annexin-V green fluorescence signal in control (a), staurosporine (20 nM, at 24h) and digitonin 6 µM.

finding suggests that *T. evansi* has the capacity to achieve cytoplasmic features of apoptosis, which could have implications for the pathogenesis of this parasite. Numerous findings suggest that the ingestion of apoptotic cells by macrophages or dendritic cells induces a downregulation of the macrophage capacity to secrete pro-inflammatory cytokines and the dendritic cell capacity to activate an immune response to foreign antigens. Consequently it limits the potential for neutrophil-mediated tissue damage during progression of inflammatory responses[15,27]. Drugs elicit apoptosis in parasites that could be favorable for the host. Future findings in this area could help to elucidate this feature.

The continuous emergence of drug resistance is a pressing need for new chemotherapeutic agents. MUREGI *et al.*[21] found that apoptosis can be modulated in resistant *Plasmodium berghei* since the cost of fitness resulted in a greater degree of internucleosomal DNA fragmentation. Thereby the apoptosis-like cell-death mechanism in *T. evansi* can be beneficial to respond to the increasing problem of drug resistance in this trypanosomosis.

RESUMEN

Actividad proapoptótica de la estaurosporina en cultivos axénicos de *Trypanosoma evansi*

Trypanosoma evansi es un hemoparásito, el cual es el agente causal de la surra (tripanosomiasis) en mamíferos, perteneciente al orden Kinetoplastidae. Este estudio se oriento a caracterizar la muerte celular similar a apoptosis en cultivos *in vitro* de *Trypanosoma evansi* a través del uso del inductor esturosporina. Este efecto se evaluó a través de

diversos aspectos fenotípicos de la apoptosis: el encogimiento celular, la exposición de fosfatidilserina, el mantenimiento de la integridad de la membrana plasmática y el potencial de membrana mitocondrial. Para evaluar estas características se utilizaron técnicas de citometría de flujo y microscopía de fluorescencia con cultivos en fase estacionaria ajustados a una densidad de 10^6 células/mL. El efecto apoptótico de la estaurosporina en *Trypanosoma evansi* fue evaluado a una concentración de 20 nM. Se evidenció un aumento de la exposición a fosfatidilserina, mientras que el potencial mitocondrial disminuyó. Por otra parte, no hay evidencias de aumento de la permeabilidad celular con estaurosporina, sugiriendo la ausencia de un proceso necrótico. Estudios adicionales son necesarios para dilucidar las posibles vías asociadas con esta forma de muerte celular en este hemoparásito.

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AUTHOR CONTRIBUTIONS

The work presented here was carried out in collaboration with all the authors. G. Bruges and A. Mijares defined the research theme and interpreted the results and wrote the paper. G. Bruges and M. Betancourt designed methods and experiments and analyzed the data. M. Betancourt, M. March and E. Sánchez carried out the laboratory experiments and strain *T. evansi* maintenance. All the authors have contributed in viewing and approving the manuscript.

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