

Review Article

Indian J Med Res 123, March 2006, pp 233-244

Apoptosis in *Leishmania* species & its relevance to disease pathogenesis

Chandrima Shaha

National Institute of Immunology, New Delhi, India

Received February 28, 2005

Apoptosis is a morphologically distinct form of cell death necessary for embryogenesis, tissue homeostasis and disease control in metazoans. Earlier, it was thought that apoptosis is the prerogative of multicellular organisms. However, it is now evident that unicellular organisms are also capable of undergoing apoptosis. In the context of *Leishmania* spp., a unicellular eukaryote responsible for causing leishmaniasis, the process of apoptosis is important for successful survival. The flagellated promastigote form of the parasite resides in the midgut of the insect vector, the female sandfly and at this niche; the cell fittest to survive to pass onto the pharynx of the fly is selected by eliminating unfit cells through apoptosis. Within the mammalian host, inside the macrophage, apoptosis is necessary to regulate cell numbers and to minimize immune reactions. With most apoptosis inducing stimuli, *L. donovani* shows typical features of apoptotic death like cell shrinkage, nuclear condensation and DNA fragmentation. Agents capable of precipitating apoptosis in this parasite include anti-leishmanial drugs like antimony, amphotericin B, pentamidine and miltefosine. Other agents like heat shock, treatment with staurosporine, knocking out centrin gene also precipitate apoptosis of the parasites. A pivotal role in cellular apoptosis is played by the single mitochondrion of *Leishmania* spp., where a fall or increase in mitochondrial potential leads to cell death by apoptosis. Ca^{2+} appears to be a vital ion involved in *Leishmania* apoptosis and partial inhibition of cytosolic Ca^{2+} increase achieved by chelating extracellular or intracellular Ca^{2+} during oxidative stress results in significant rescue of the fall of the mitochondrial membrane potential and consequently apoptosis. Elucidation of the molecular events linked to apoptotic death of *Leishmania* spp. might help define a more comprehensive view of the cell death machinery in terms of evolutionary origin and identify new target molecules for chemotherapeutic drug development and therapeutic intervention.

Key words Apoptosis - calcium - *Leishmania* - mitochondria - *Trypanosoma*

Apoptosis, a morphologically distinct form of cell death is crucial for embryogenesis, tissue homeostasis and disease control in metazoans¹. Although it was initially assumed that apoptosis arose with multicellularity and would have been counterselected in unicellular organisms², several

findings have indicated that a similar process of apoptosis operates in single-celled eukaryotes^{3,4}. Till date, various forms of apoptotic death have been described in at least nine species of unicellular eukaryotes that belong to four different branches of the phylogenetic tree. The phylogenetic divergence of

these species is believed to range from around 2 billion years ago to 1 billion years⁴. Among the unicellular eukaryotes where apoptosis has been observed, are the kinetoplastid parasites of the genera *Trypanosoma* and *Leishmania*, which are believed to be among the earliest diverging eukaryotes. These kinetoplastid parasites are the causative agents of trypanosomiasis and leishmaniasis, respectively, and these tropical diseases affect around 30 million people worldwide. Leishmaniasis can manifest itself as self-healing cutaneous lesions, mucocutaneous lesions or fatal, generalised visceral infection. *Trypanosoma* spp. and *Leishmania* spp. are the most well studied parasites in terms of apoptotic features and conditions that precipitate apoptosis. Unicellular organisms in which apoptosis has been described include slime mold *Dictyostelium discoideum*⁵, the kinetoplastid protozoans *Trypanosoma brucei*⁶ and *Leishmania amazonensis*⁷, the ciliate *Tetrahymena thermophila*⁸ and the dinoflagellate *Peridinium gatunense*⁹. This review will be confined to a detailed analysis of data available in *Leishmania* spp. and mention *Trypanosoma* spp. apoptosis for comparison purposes as it is a related parasite.

The process of apoptosis

Apoptosis was first described by Kerr, Wyllie and Currie in the early 1970s¹⁰ and is defined by the morphologic appearance of the dying cell, which includes blebbing, chromatin condensation, nuclear fragmentation, rounding and cell shrinkage. Biochemical features associated with apoptosis include high molecular weight DNA fragmentation into an oligonucleosomal ladder, phosphatidyl serine externalization, and proteolytic cleavage of a number of intracellular substrates. These biochemical features are the result of the operation of complex pathways involving different kinds of proteins. Among many other components, the aspartate-specific cysteine protease (caspase) cascade is now believed to be the main pathway by which cellular death is orchestrated. Several different caspases are constitutively expressed in cells and reside in the cytosol in an inactive form. The most prevalent caspase in the cell is caspase-3. This caspase is ultimately responsible for the majority of the effects

finally leading to dismantling of the cells. Other than caspase 3, there are several other caspases taking part in different parts of the pathway, caspase 3 being the terminal caspase. In general, caspase activation requires a cleavage, preferentially after an aspartate residue, leading to the formation of the active enzyme from the inactive proform¹¹.

There are two pathways through which death process can be initiated. The first involves death receptors such as the tumour necrosis factor (TNF) receptor and the Fas receptor, and the second one depends on the participation of the mitochondria. The death receptors of the tumour necrosis factor receptor (TNFR) family include TNFR1, Fas receptor, DR3/WSL, and the TRAIL/Apo-2L receptors (TRAIL-R1/DR4, TRAIL-R2/DR5). When these death receptors are bound by their ligands [TNF or lymphotoxin, Fas-ligand (FasL), the ligand for DR3, or TRAIL/Apo-2L, respectively] apoptosis can occur. Ligation of death receptors results in the rapid formation of an intracellular death-inducing signaling complex through an amino acid stretch within the carboxy terminus of the receptor called the "death domain". This domain is responsible for coupling the death receptor to either a cascade of caspases, leading to induction of apoptosis, or to the activation of kinase-signaling pathways, resulting in gene expression through NF- κ B or activator protein 1¹².

Other than the death receptor pathway which can work independent or dependent of the mitochondria, a mitochondrial pathway capable of responding to various stimuli independent of death receptors exist. Mitochondrion acts by releasing apoptogenic factors, such as cytochrome c, from the intermembrane space into the cytoplasm, which activates the downstream execution phase of apoptosis. In living cells, mitochondrial changes are predominantly prevented by anti-apoptotic members of the Bcl-2 family of proteins. Bcl-2 was first discovered as a proto-oncogene in follicular B-cell lymphoma¹³. Subsequently, it was identified as a mammalian homologue to the apoptosis repressor ced-9 in *Caenorhabditis elegans*¹⁴. Since then, at least 19 Bcl-2 family members have been identified in mammalian cells. Apaf-1 is a protein contained in the cytosol, and cytochrome C binds and induces it to

oligomerize. This then recruits an initiator caspase, procaspase-9. The apoptosome now recruits procaspase-3, which is cleaved and activated by the active caspase-9 and released to mediate apoptosis. Recently, a new protein with the dual name Smac/DIABLO has been discovered, which is also released from the mitochondria along with cytochrome c¹⁵. The mitochondrial pathway involving Bcl-2 family members, mitochondria, cytochrome c, Apaf-1, and caspase-9 is fundamentally distinct from that of the death receptors. Cells lacking caspase-8 or FADD do not respond to death ligands but are capable of undergoing apoptosis that is induced by other agents, such as cellular stress. Cells lacking caspase-9 or Apaf-1 are incapable of undergoing apoptosis that is induced by such stressors, but they readily die in response to death ligands¹⁶.

The above is an overview of how metazoan apoptosis works, however, pathways operative in unicellular organisms are not available in such details and are slowly coming to light through the study of various models using different apoptogenic agents.

The relevance of study of apoptosis in Kinetoplastid parasites

Apoptosis, being a process that can clear cells without causing an immune response is very important in the context of pathogenesis of a disease. Also, this process might contribute to successful survival of the fittest cell in a given colony. The promastigote form of the parasite, flagellated and extracellular, resides in the midgut of the insect vector, the female sandfly (*Phlebotomus*) and at this niche, the cells fittest to survive and pass onto the pharynx of the fly is probably selected by eliminating unfit cells through apoptosis¹⁷. The survival within the macrophage is also an important issue in disease pathogenesis in the mammalian host. An understanding of cell death processes in the macrophages, might offer new possibilities for controlling the parasitic diseases. In addition to this, the extent of overlap between effectors and regulators of apoptosis in these parasites and mammalian hosts might also help to identify specific pathways leading to cell suicide that could be exploited for parasite control. Apart from the understanding of the

apoptotic process in the *Leishmania* spp. in the context of a disease, these cells serve as interesting models to dissect apoptotic pathways that can be used for analysis of apoptosis in higher eukaryotes.

Life cycle of *Leishmania* spp.

The *Leishmania* has two morphological forms in its life cycle, the immotile amastigotes and the motile promastigotes. The amastigotes live inside phagolysosomes present in phagocytic cells of the vertebrate host, namely monocytes and macrophages, which are ingested by the female phlebotomine sandfly when it takes an infecting bloodmeal^{18,19}. Diptera of the subfamily Phlebotominae are the only vectors of the various *Leishmania* species, with the genus *Phlebotomus* hosting the Old World species and *Lutzomyia* the species of the New World²⁰. In these blood-sucking insects, the parasite develops a complex series of morphological modifications^{21,22}. Here, the parasite differentiates from a dividing procyclic promastigote stage that is attached to the gut wall, to a non-dividing metacyclic promastigote stage that is unable to attach to the midgut and migrates to the mouth parts²¹⁻²³. Coincidentally, the maximum infective capacity occurs at the time of a new bloodmeal. When the infected sandfly probes a new host, it regurgitates about 1 to 1000 metacyclics, closing the life cycle¹⁸. It seems clear that this coordination of events takes the parasite to an optimum opportunity to infect the vertebrate host and perpetuate its cycle.

Possible life-cycle phases where apoptosis is relevant

The life cycle pattern shows that the guts of the sandfly harbouring the promastigotes and the macrophages where the parasites exist in very close contact with each other are possible points at which apoptosis might occur. When the procyclic promastigotes divide in the sandfly gut, a control mechanism for regulation of absolute number of cell is required so that excessive division does not compromise the survival of the sandfly. Similarly, within the macrophages, the amastigotes divide and might generate superfluous cell numbers. In addition to controls in cell division, induction of apoptotic

death would be relevant in these stages of life cycle to eliminate unfit cells and control cell numbers which is vital for the survival of the promastigotes within the insect gut and amastigote survival within the macrophages. In the bloodstream or at the site of inoculation, apoptosis is not logically relevant and no data exist on the issue.

The apoptotic process in *L. donovani*

Various models of apoptosis induction have been tested in *L. donovani* which were developed using a variety of apoptosis inducing agents. In this review, the analysis of data available is divided into apoptosis caused by antileishmanial drugs and other agents. A section is dedicated to mitochondrial changes because kinetoplastids are single-celled eukaryotes that belong to one of the most ancient diverging branches of the eukaryote phylogenetic tree²⁴ and are amongst the first mitochondrial eukaryotes containing only one giant mitochondrion. By virtue of having a single giant mitochondrion kinetoplastids provide an interesting model to investigate primarily the mitochondrial changes that occur during apoptosis.

Induction of apoptosis by antileishmanial drugs in *L. donovani*

Treatment available for visceral leishmaniasis is far from ideal and the classic first line of treatment is pentavalent antimony Sb(V), traditional second line drugs being pentamidine and amphotericin B whose use are limited by toxicity and availability²⁵. Pentavalent antimony is generally regarded as a pro-drug requiring conversion to a trivalent form in order to be cytotoxic and this reduction takes place in the parasite or the host cells or both^{26,27}. Antimonials are thought to act by inhibiting metabolic pathways although the mode of action is poorly understood²⁸. The clinical value of antimony therapy is now threatened because of emergence of drug resistance²⁹ and co-infection with human immunodeficiency virus³⁰, in addition to side effects of high doses of antimony. There are several reports showing that *Leishmania* apoptosis occur in response to antileishmanial drugs. Most of these reported studies were carried out *in vitro*. One of the systems that are commonly used are the axenic amastigotes generated

in the laboratory by subjecting the promastigotes to changes in pH and temperature. Although not totally resembling intracellular amastigotes, these organisms are frequently used as drug testing models²⁶.

During the late log growth phase of *L. donovani* axenic amastigotes, treatment with increasing concentrations of Sb(V) shows a significant induction of caspase-like activity³¹. On the contrary, no significant caspase-like activity is observed in promastigotes upon treatment with Sb(V) even at a higher concentration or when treated for a longer period of time³¹, showing that there is a distinct difference into how the different forms of the parasite respond to Sb (V). In contrast to the above observations reported by Lee *et al*³¹, no caspase-like activity upon treatment of axenic amastigotes of *L. infantum* with trivalent antimonial compound Sb(III) was detected although presence of apoptotic features such as DNA fragmentation was seen³². However, in studies of Lee and co-workers, promastigotes were found to show DNA laddering after antimony exposure without any increase in caspase activity³¹. Therefore, these observations provide possibilities of existence of a caspase independent apoptotic pathway in the *Leishmania* spp. Caspase-independent cell death in multicellular organisms is known^{33,34}, where release of death factors from mitochondria has been shown to cause apoptosis without involving caspases. While all the above data are with free-swimming forms, some reports are available on the immotile intracellular amastigotes. The capability of the *L. donovani* to survive within the host cell parasitophorous vacuoles as non-motile amastigotes determine disease pathogenesis, therefore, apoptosis in the intracellular forms is very important. Our studies have shown that potassium antimony tartrate, the active form of Sb (V), kills intracellular *L. donovani* amastigotes by apoptosis characterized by nuclear DNA fragmentation and externalization of phosphatidylserine³⁵. Holzmüller and co-workers³⁶ showed that when intracellular amastigotes were exposed to nitric oxide (NO) donating compounds or lipopolysaccharide (LPS) generated NO, DNA fragmentation occurred. In another study, DNA fragmentation was observed in the infective form of the parasite in response to serum deprivation, heat shock and NO³⁷. Therefore, intracellular amastigotes are competent to undergo apoptosis as is evident from the above studies.

When Sb(V) fails to combat *Leishmania* infection, patients are treated with amphotericin B. Amphotericin B, a pore forming polyene antibiotic, induces caspase-like activity in both axenic amastigotes and promastigotes of *L. donovani* resulting in DNA fragmentation within 2 h *in vitro*³¹ implying that the effect of amphotericin B is faster as compared to antimony. Another drug, the aromatic diamidine pentamidine, initially developed for use against *Trypanosoma brucei*, is considered an alternative to pentavalent antimony in treatment of leishmaniasis³⁸. Concurrent inhibition of respiratory chain complex II with pentamidine administration increases cytotoxicity of the drug leading to death by apoptosis.

Miltefosine, a recently developed oral drug for leishmaniasis induces apoptosis-like death in *L. donovani* that show nuclear DNA condensation, DNA fragmentation with accompanying ladder formation³⁹. Pentacyclic triterpenoid, dihydrobetulinic acid (DHBA), is a novel lead compound for antileishmanial therapy. It acts by targeting DNA topoisomerases and induces apoptosis. Therefore it is a strong candidate for use in designing new antileishmanial drugs⁴⁰. Luteolin and quercetin are effective antileishmanial agents and these compounds arrest cell cycle progression in *L. donovani* promastigotes, leading to apoptosis⁴¹. The above reports indicate that the process of apoptosis is operative when a variety of antileishmanial drugs are given in both the promastigote and amastigote forms.

Other agents causing apoptotic death

Apart from antileishmanial drugs, other modes of inducing cell death have been used to study *Leishmania* apoptosis. For example, heat-shocked parasites present ultrastructural and molecular features characteristic of cells dying by apoptosis. Morphological changes observed only in the presence of calcium are mainly nuclear while cytoplasmic organelles are preserved. Heat shock is also able to induce DNA cleavage into oligonucleosomal fragments^{37,42}. Staurosporine, a protein kinase inhibitor, that induces apoptosis in all mammalian nucleated cells, also induces in *L. major* a death

process with several cytoplasmic and nuclear features of apoptosis, including cell shrinkage, phosphatidyl serine exposure, and maintenance of plasma membrane integrity³. Other than drugs and chemicals, knocking out genes relevant to cell cycle have been studied. For example, centrin, a calcium-binding cytoskeletal protein involved in the duplication of centrosomes in higher eukaryotes has been used as a marker gene. *Leishmania* deficient in the centrin gene shows selective growth arrest at the G(2)/M stage as axenic amastigotes but not as promastigotes. The axenic amastigotes also show failure of basal body duplication and failure of cytokinesis resulting in multinucleated “large” cells. Increased DNA fragmentation is observed in centrin mutant axenic amastigotes compared with wild type cells, suggesting the activation of apoptotic pathway⁴³. The Silent Information Regulator (SIR2) family of genes have been cloned from a variety of species ranging from bacteria to man. *Leishmania major* gene encoding a protein with extensive homology to yeast SIR2p is expressed by different *Leishmania* species and parasite developmental stages and is termed LmSIR2⁴⁴. Using *L. infantum* as a recipient for a plasmid vector which allows overexpression of LmSIR2p led to the accumulation of the protein in the parasite cytoplasm of both promastigote and amastigote forms and a striking increase in the survival of amastigotes, was observed under normal axenic culture conditions. This phenotype was also observed when *L. infantum* parasites were transfected with a cosmid vector (CLHyg), isolated from a *L. infantum* cosmid library, carrying the *L. infantum* SIR2 gene (CLHyg-LiSIR2). In contrast, no effect was observed on survival of the promastigote forms under similar culture conditions. These results demonstrated the existence of SIR2-related proteins encoding genes in different *Leishmania* species and suggest that LmSIR2p could participate among other factors in the control of cell death⁴⁴. Under a variety of stress conditions such as serum deprivation, heat shock and nitric oxide, cell death can be induced leading to genomic DNA fragmentation into oligonucleosomes where no caspase activity increase is found. These data are consistent with the presence of a caspase-independent cell death mechanism in *Leishmania*, induced by stress²⁸. There are not many observations on *Leishmania* apoptosis *in vivo*. One

study found apoptotic death of the parasites after 90 days of initiation of infection in hamsters indicating apoptosis as a mode of death in case of *in vivo* infection⁴⁵. It is necessary to initiate studies that investigate the extent of parasite apoptosis and host cell apoptosis that occur *in vivo* and correlate how these two events contribute to infection.

Involvement of mitochondria in apoptosis of *Leishmania* spp.

Mitochondria are the sites of respiratory chain location and generates adenosine tri phosphate (ATP) to maintain viability of cells. Maintenance of proper mitochondrial transmembrane potential ($\Delta\psi_m$) is essential for the survival of the cell and the study of mitochondrial potential has become a focus of apoptosis regulation as many investigations demonstrate a major functional impact of mitochondrial alterations on apoptosis^{46,47}. The protozoan parasites have a single mitochondrion and the importance of functioning of this organelle in *Leishmania* spp. is very vital as compared to organisms with numerous mitochondria because the presence of multiple mitochondria ensures compensation for the injured ones but for organisms with a single mitochondrion, no such choice exists and survival depends on proper functioning of a single organelle.

Studies from our laboratory demonstrate that *L. donovani* promastigotes undergo an apoptosis-like death in response to a well established model of a biologically active oxygen derived intermediate - H_2O_2 . While the H_2O_2 induced death shares many features common to metazoan apoptosis such as nuclear condensation, DNA fragmentation (Fig. 1) and cell shrinkage⁴⁸, it also leads to $\Delta\psi_m$ disruption (Fig. 2) and areas of high and low $\Delta\psi_m$ within the single mitochondrion of the promastigotes are detectable. This is of obvious importance for the survival of these parasites because metazoan cells with multiple mitochondria have the advantage of containing these organelles in different energy states giving the cell the opportunity to survive based on the ATP generated by the active ones^{49,50}. However, in a single celled organism with a single mitochondrion, total loss of potential would result

in abrupt shutting off of the survival machinery leading to necrosis. Therefore, the single mitochondrion of *L. donovani* adopts a variable energy generating state to sustain ATP generation and viability till the apoptotic process is complete. The maintenance of about 20 per cent of ATP generation till late after H_2O_2 treatment is evidence that a minimal level of ATP is maintained to take the cell through the apoptotic process⁴⁸.

In metazoans, mitochondrial megachannel is involved in the process of apoptosis leading to the release of mitochondrial factors for initiation of the apoptotic process⁵¹. In our studies, we noted a considerable variation in the involvement of this channel which was dependent on the type of apoptotic stimulus given. We used cyclosporin A, an inhibitor of the megachannel during oxidative stress and found that $\Delta\psi_m$ loss was not reversed by cyclosporin A,⁴⁸ ruling out the involvement of the megachannel in oxidative stress induced apoptosis. In contrast, limitation of electron transport by thenoyltrifluoroacetone and antimycin A, inhibitors of complexes II and III respectively, resulted in dissipation of mitochondrial membrane potential that was sensitive to cyclosporin A⁵². The involvement of this channel needs to be probed further to understand how and when this channel is involved in apoptosis.

Inhibitors of respiratory chain complexes I, II and III were able to induce apoptotic death of the blood stream form of *L. donovani*, however, the involvement of the mitochondrial potential was variable. While complex I inhibition resulted in mitochondrial hyperpolarization that was preceded by increased superoxide production, thenoyltrifluoroacetone (complex II inhibitor) showed maximal generation of hydrogen peroxide with a moderate elevation of superoxide levels and a fall in $\Delta\psi_m$ and complex III inhibition provoked superoxide generation only with a fall in $\Delta\psi_m$ ⁵². Studies on the involvement of mitochondrion with stationary phase *Leishmania* culture shows lower $\Delta\psi_m$ than a log phase culture implying that aging *Leishmania* lose $\Delta\psi_m$ ²⁸. The third line of antileishmanial drug, amphotericin B treatment causes a significant fall in $\Delta\psi_m$ that is followed by

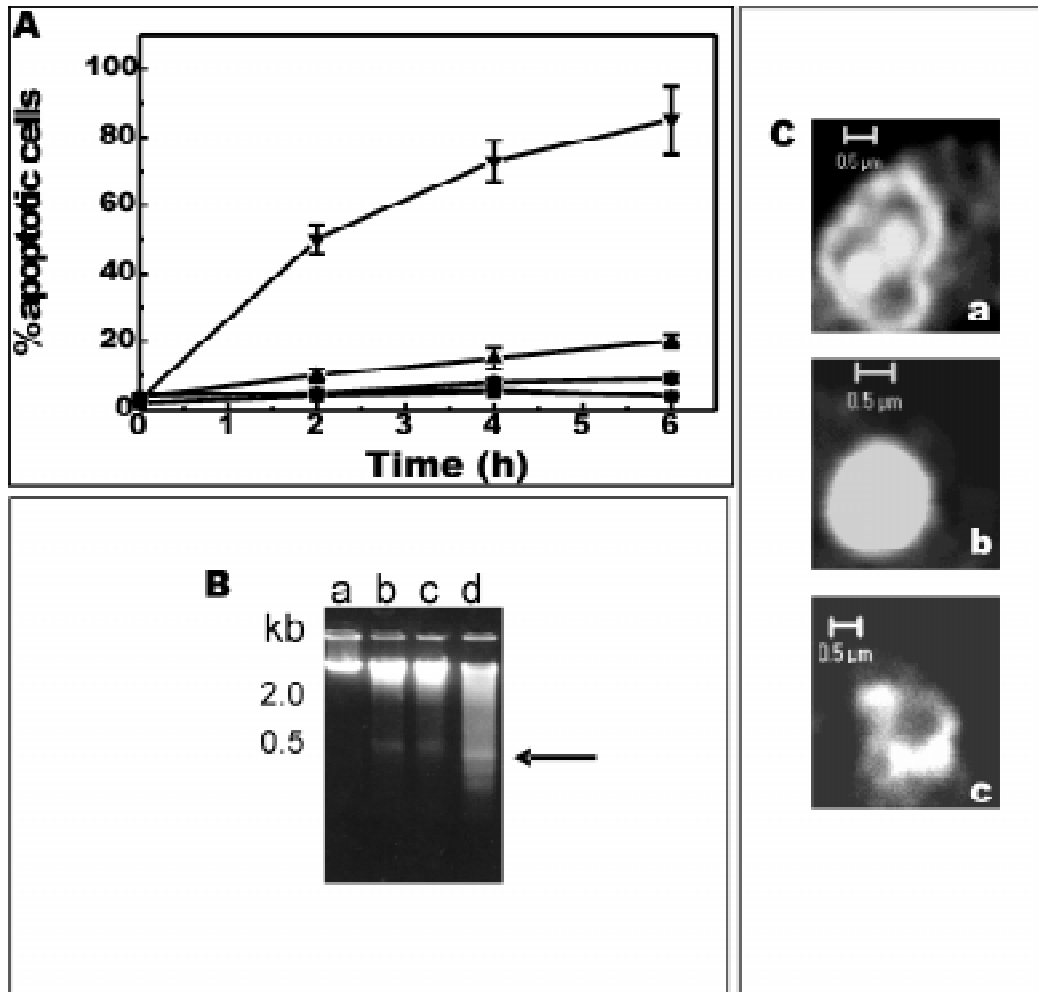


Fig. 1. Effect of H₂O₂ on cell survival and nuclear morphology in *Leishmania donovani*. **A.** Figure shows the effect of oxidative stress on apoptotic death in the parasite. Note the percentage of cells undergoing apoptosis-like death as counted by a combination of Hoechst dye and PI staining. Symbols: ■, control; ▲, 1 mM H₂O₂; ▼, 4 mM H₂O₂; ◻, 4 mM H₂O₂ + catalase (100 IU); **B.** DNA profile in agarose gels from treated and untreated promastigotes. (a) Without H₂O₂ exposure. (b) After 2 h of 4 mM H₂O₂ treatment. (c) After 4 h of 4 mM H₂O₂ exposure. (d) After 6 h of 4 mM H₂O₂ treatment. Arrow indicates the DNA ladder. **C.** Changes in nuclei at different stages of cell death. Close ups of nuclei from a cell not exposed to H₂O₂ (a), a cell exposed to 4 mM H₂O₂ for 4 h showing nuclear condensation (b) and a cell exposed to 4 mM H₂O₂ for 6 h showing breakdown of nuclear material (c).

membrane permeability increase and a rise in caspase-3 activity²⁸. Topoisomerase I poison camptothecin, induces apoptotic death in *L. donovani* through interference with $\Delta\psi_m$ ⁵³. Intracellular amastigotes are very different from promastigotes in their response to drugs. Potassium antimony tartrate induced apoptotic death in intracellular *L. donovani* shows a fall in the amastigote mitochondrial membrane potential prior to expressing features of apoptosis³⁵. Staurosporine induced death is also mediated through interference with $\Delta\psi_m$ ³. Therefore, it is apparent that the single

mitochondrion of *Leishmania* plays a pivotal role in cellular apoptosis.

Role of ions during apoptosis in *L. donovani*

The role of various ions during apoptosis forms a very important component of any study on the apoptotic pathways because many of these ions are intimately related to the process of apoptosis. Ca²⁺ appears to be a vital ion that is involved in *Leishmania* apoptosis. $\Delta\psi_m$ modulation is linked to Ca²⁺ homeostasis and partial inhibition of cytosolic

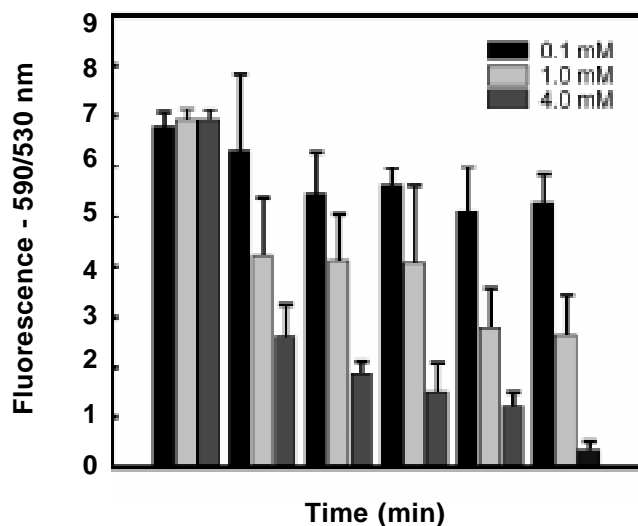


Fig. 2. Dissipation of $\Delta\Psi_m$ occurs in *Leishmania donovani* promastigotes after exposure to H_2O_2 . *L. donovani* promastigotes ($10^7/ml$) were exposed to different doses of H_2O_2 (0.1, 1 and 4 M) *in vitro* for the time periods indicated and were subsequently stained with the potentiometric probe - JC-1 ($10 \mu M$). Dose and time dependent changes of relative mitochondrial potential value is expressed as the ratio of the reading at 590 nm (aggregate) to the reading at 530 nm (monomer). Data are \pm SD of 4 experiments. $**P < 0.001$ in comparison to 0 h values.

Ca^{2+} increase achieved by chelating extracellular or intracellular Ca^{2+} by the use of appropriate agents during oxidative stress results in significant rescue of the fall of the mitochondrial membrane potential and apoptosis-like death. The increase in cytosolic Ca^{2+} during oxidative stress is an additive result of release of Ca^{2+} from intracellular stores as well as by influx of extracellular Ca^{2+} through flufenamic acid sensitive non-selective cation channels, contribution of the latter being larger⁴⁵. The antileishmanial drug potassium antimony tartrate, which induces apoptotic death in intracellular *L. donovani* amastigotes characterized by nuclear DNA fragmentation and externalization of phosphatidylserine, demonstrates elevation of intracellular Ca^{2+} concentrations in both the parasite and the host that is preventable by antioxidants³⁵. Flufenamic acid, a non-selective cation channel blocker decreases the elevation of Ca^{2+} in both the host and the parasite and reduces amastigote death, thus establishing a central role of Ca^{2+} in intracellular parasite clearance³⁵. As mentioned earlier, inhibitors of respiratory chain complexes I, II and III induce apoptosis through

modulation of mitochondrial activity. In such events, interference with complex II but not complexes I and III increased intracellular Ca^{2+} . Chelation of extracellular Ca^{2+} is able to abrogate the early increase of reactive oxygen species (ROS) providing evidence that Ca^{2+} elevation was downstream to ROS generation. Ca^{2+} influx in the above situation occurred through non selective cation and L-type channels and Na^+/Ca^{2+} exchanger like pathways⁵². In camptothecin induced leishmanial apoptotic death, oxidative stress causes impairment of the $Na^+ - K^+ - ATPase$ pump and subsequently decreases the intracellular K^+ levels. A decrease in both intracellular pH and K^+ levels propagates the apoptotic process through activation of caspase 3-like proteases by rapid formation of cytochrome C-mediated apoptotic complex. In addition to caspase-like protease activation, a lower level of intracellular K^+ also enhances the activation of apoptotic nucleases at the late stage of apoptosis⁵⁴. This suggests that the physiological level of pH and K^+ are inhibitory for apoptotic DNA fragmentation and caspase-like protease activation in leishmanial cells. Promastigotes from *Leishmania (Leishmania) amazonensis*, when shifted from their optimal *in vitro* growth temperature ($22^\circ C$) to the temperature of the mammalian host ($37^\circ C$), die by a Ca^{2+} -modulated mechanism showing features of apoptotic death. More parasites die in the presence of this ion than in its absence⁴⁷.

In summary, the data analyzed above clearly indicate that Ca^{2+} is very vital for the survival of the parasites. These parasites differ from the metazoans in the storage of Ca^{2+} and acidocalcisomes represent the bulk Ca^{2+} storage organelle. However, no data are available as to how these organelles respond to apoptotic stimuli.

Caspases in *Leishmania* apoptosis

Although several studies report a caspase-like activity upregulation with apoptotic stimuli^{55,31}, the cysteine proteases that play a role in metazoan apoptosis have not yet been detected in *L. donovani*. One metacaspase-like sequence for *L. donovani* was reported⁵⁶. Recently, five metacaspases (analogs of caspases) were identified in the *T. brucei* genome.

Expression of *T. brucei* metacaspase 4 in yeast resulted in growth inhibition, mitochondrial dysfunction and clonal death of the yeast. Moreover, studies on *T. brucei* have confirmed that, as previously reported for mammalian caspases, metacaspase 4 is also a cysteine proteinase⁵⁷. Therefore, clearly it is a substantial lack of knowledge of the proteases that bring about cell dismantling.

How apoptotic mimicry may help parasites

Phosphatidylserine (PS) is one of the ligands displayed by apoptotic cells that participates in their noninflammatory removal when recognized by neighbouring phagocytes. Advantageous features of the apoptotic process can operate without death as the necessary outcome is an interesting concept. *Leishmania* spp. are able to evade the killing activity of phagocytes and establish themselves as obligate intracellular parasites by exposing PS and inhibiting macrophage activity. Exposed PS participates in amastigote internalization. Recognition of this moiety by macrophages induces transforming growth factor (TGF- β) secretion and interleukin 10 (IL-10) synthesis, inhibits NO production, and increases susceptibility to intracellular leishmanial growth⁵⁸.

Other types of death in *Leishmania* spp.

Autophagy is a form of death that occurs in *L. donovani* which is independent of caspases and occurs without DNA fragmentation⁵⁹.

Apoptosis in a related parasite group - the *Trypanosoma*

Trypanosoma, the haemoflagellate shares close similarity to *Leishmania* in many biochemical features. *Trypanosoma brucei brucei* responds to ROS by undergoing apoptotic death via a Ca²⁺ mediated mechanism but nuclease activation observed was not a consequence of caspase 3, caspase 1, calpain, serine protease, cysteine protease activation and hence the process is caspase independent⁶⁰. *T. brucei rhodesiense* can be induced to undergo apoptosis after stimulation with Con A and twenty two differentially displayed products have been cloned and sequenced which are

believed to be related to cellular apoptosis in this organism⁶¹. Two upregulated genes late during ConA-induced apoptosis have been characterized. The first represents a homologue of prohibitin, a proto-oncogene originally described in mammals and subsequently in yeast, which is involved in cell cycle control and senescence and the second gene, homologous to a family of regulatory proteins which are receptors for activated protein kinase C, is also shown to be upregulated in terminally differentiated bloodstream form trypanosomes⁶². Treatment of *T. cruzi* parasites with the antibiotic drug geneticin which induces the death of epimastigotes by apoptosis showed selective localization of elongation factor-1 (EF-1) alpha in the nucleus of dying parasites. This observation supports the notion already reported in the case of mammalian cells that EF-1 alpha could participate in the transcription processes and possibly in the case of *T. cruzi*, in the expression of genes involved in the control of cell death⁶³.

Significance of apoptosis in *Leishmania*

Since cell death is the final outcome of apoptosis it is almost intuitive that this process serves only multicellular organisms. If one considers a unicellular organism as a self-sufficient individual, there is no *a priori* reason to believe that such an organism can benefit from a suicidal programme. A corollary of this way of thinking is that programmed cell death has evolved after the onset of multicellularity. However, this notion has been challenged over the past few years. The identification of a regulated cell death programme inducing an apoptotic phenotype in nine different single celled eukaryotes that belong to four diverging branches of the eukaryote phylogenetic tree provides a paradigm for a widespread role for programmed cell death in the control of cell survival, and raises the question of the origin and nature of the genes that may be involved in the execution and regulation of such a process. Evolutionary advantages conferred by such programme of self-destruction could include the constant selection for the survival of the fittest cell in the single-celled eukaryote colony, optimal adaptation of the cell numbers to the environment, and tight regulation of cell cycle and cell differentiation in response to environmental changes.

In such a context, it is important to realize that the frontiers between multicellular organisms and single-celled organisms may not be as stringent as usually believed. Thus, the regulation of programmed cell death in this single celled eukaryote allows a stringent coupling of appropriate cell differentiation with cell survival. In the three kinetoplastid parasites, *T. cruzi*, *T. brucei*, and in the *Leishmania*, programmed cell death may play an important role in the regulation of the complex interactions between unicellular and multicellular organisms that allow the establishment and persistence of stable host/parasite interactions, and the 'apoptosis-like' phenotype of the parasite death process may reduce the onset of inflammation and favour parasite evasion from the host immune system. Therefore, in the context of host immune response and the successful establishment of an infection, apoptosis is very relevant.

Trypanosomatids might be endowed with an apoptosis mechanism that is derived from ancestral death machinery. Apoptosis in trypanosomatids could be a process without a defined function, inherited through eukaryotic cell evolution, which might be triggered in response to diverse stimuli and stress conditions. However, recent observations suggest that apoptosis might be used by trypanosomatids to maximize their biological fitness⁶⁴. Apoptosis could represent an altruistic mechanism for the selection of cells, from the parasite population, that are fit to be transmitted to the next host. Alternatively, apoptotic death could help in controlling the population of parasites in the host, thereby increasing host survival and favouring parasite transmission. Therefore, apoptosis in trypanosomatid parasites may represent a pathway involved both in survival and propagation of the species, also it could represent a potential pharmacological target for protozoan control⁶⁵.

The elucidation of the molecular events which tightly regulated the processes of growth arrest, differentiation and death of *T. cruzi*, *Leishmania* spp. and African trypanosomes, might allow not only to define a more comprehensive view of the cell death machinery in terms of evolutionary origin but may also be useful to identify new target molecules for chemotherapeutic drug development and therapeutic intervention.

References

1. Vaux DL, Strasser A. The molecular biology of apoptosis. *Proc Natl Acad Sci USA* 1996; 93 : 2239-44.
2. Raff MC. Social controls on cell survival and cell death. *Nature* 1992; 356 : 397-400.
3. Arnoult D, Akarid K, Grodet A, Petit PX, Estaquier J, Ameisen JC. On the evolution of programmed cell death: apoptosis of the unicellular eukaryote *Leishmania major* involves cysteine proteinase activation and mitochondrion permeabilization. *Cell Death Differ* 2002; 9 : 65-81.
4. Ameisen JC. The origin of programmed cell death in the flow of evolution and its role in host-pathogen interactions. *C R Seances Soc Biol Fil* 1998; 192 : 1095-8.
5. Olie RA, Durrieu F, Cornillon S, Loughran G, Gross J, Earnshaw SG, et al. Apparent caspase independence of programmed cell death in *Dictyostelium*. *Curr Biol* 1998; 8 : 955-8.
6. Welburn SC, Lillico S, Murphy NB. Programmed cell death in procyclic form *Trypanosoma brucei rhodesiense* identification of differentially expressed genes during con A induced death. *Mem Inst Oswaldo Cruz* 1999; 94 : 229-34.
7. Moreira ME, Del Portillo HA, Milder RV, Bananco JM, Barcinski MA. Heat shock induction of apoptosis in promastigotes of the unicellular organism *Leishmania (Leishmania) amazonensis*. *J Cell Physiol* 1996; 167 : 305-13.
8. Straarup EM, Schousboe P, Hansen HQ, Kristiansen K, Hoffmann EK, Rasmussen L. Effects of protein kinase C activators and staurosporine on protein kinase activity, cell survival, and proliferation in *Tetrahymena thermophila*. *Microbios* 1997; 91 : 181-90.
9. Vardi A, Berman-Frank I, Rozenberg T, Hadas O, Kaplan A, Levine A. Programmed cell death of the dinoflagellate *Peridinium gatunense* is mediated by CO₂ limitation and oxidative stress. *Curr Biol* 1999; 9 : 1061-4.
10. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972; 26 : 239-57.
11. Hengartner MO. The biochemistry of apoptosis. *Nature* 2000; 407 : 770-6.
12. Grassme H, Jendrosseck V, Gulbins E. Molecular mechanisms of bacteria induced apoptosis. *Apoptosis* 2001; 6 : 441-5.
13. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science* 1998; 281 : 1322-6.
14. Putcha GV, Johnson EM Jr. Men are but worms: neuronal cell death in *C elegans* and vertebrates. *Cell Death Differ* 2004; 11 : 38-48.
15. McDonnell MA, Wang D, Khan SM, Vander Heiden MG, Kelekar A. Caspase-9 is activated in a cytochrome

- c-independent manner early during TNF α -induced apoptosis in murine cells. *Cell Death Differ* 2003; 10 : 1005-15.
16. Hill MM, Adrain C, Martin SJ. Portrait of a killer: the mitochondrial apoptosome emerges from the shadows. *Mol Interv* 2003; 3 : 19-26.
 17. Welburn SC, Maudlin I. Tsetse-*Trypanosome* interactions: rites of passage. *Parasitol Today* 1999; 15 : 399-403.
 18. Lainson R, Ryan L, Shaw JJ. Infective stages of *Leishmania* in the sandfly vector and some observations on the mechanism of transmission. *Mem Inst Oswaldo Cruz* 1987; 82 : 421-4.
 19. Chang KP, Chaudhuri G, Fong D. Molecular determinants of *Leishmania* virulence. *Annu Rev Microbiol* 1990; 44 : 499-529.
 20. Nieves E, Pimenta PF. Development of *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis* in the sand fly *Lutzomyia migonei* (Diptera: Psychodidae). *J Med Entomol* 2000; 37 : 134-40.
 21. Killick-Kendrick R. The life-cycle of *Leishmania* in the sandfly with special reference to the form infective to the vertebrate host. *Ann Parasitol Hum Comp* 1990; 65 (Suppl 1) : 37-42.
 22. Puentes SM, Sacks DL, da Silva RP, Joiner KA. Complement binding by two developmental stages of *Leishmania major* promastigotes varying in expression of a surface lipophosphoglycan. *J Exp Med* 1988; 167 : 887-902.
 23. Mahoney AB, Sacks DL, Saraiva E, Modi G, Turco SJ. Intra-species and stage-specific polymorphisms in lipophosphoglycan structure control *Leishmania donovani*-sand fly interactions. *Biochemistry* 1999; 38 : 9813-23.
 24. Sogin ML. Early evolution and the origin of eukaryotes. *Curr Opin Genet Dev* 1991; 1 : 457-63.
 25. Berman J. Current treatment approaches to leishmaniasis. *Curr Opin Infect Dis* 2003; 16 : 397-401.
 26. Sereno D, Cavaleyra M, Zemzoumi K, Maquaire S, Ouassii A, Lenesre JL. Axenically grown amastigotes of *Leishmania infantum* used as an *in vitro* model to investigate the pentavalent antimony mode of action. *Antimicrob Agents Chemother* 1998; 42 : 3097-102.
 27. Shaked-Mishan P, Ulrich N, Ephros M, Zilberstein D. Novel intracellular SbV reducing activity correlates with antimony susceptibility in *Leishmania donovani*. *J Biol Chem* 2001; 276 : 3971-6.
 28. Rosenthal E, Marty P. Recent understanding in the treatment of visceral leishmaniasis. *J Postgrad Med* 2003; 49 : 61-8.
 29. Sundar S, More DK, Singh MK, Singh VP, Sharma S, Makharia A, et al. Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. *Clin Infect Dis* 2000; 31 : 1104-7.
 30. Laguna F. Treatment of leishmaniasis in HIV-positive patients. *Ann Trop Med Parasitol* 2003; 97 (Suppl 1) : 135-42.
 31. Lee N, Bertholet S, Debrabant A, Muller J, Duncan R, Nakhasi HL, et al. Programmed cell death in the unicellular protozoan parasite *Leishmania*. *Cell Death Differ* 2002; 9 : 53-64.
 32. Sereno D, Holzmuller P, Mangot I, Cuny G, Quaiissi A, Lemesre JL. Antimonial-mediated DNA fragmentation in *Leishmania infantum* amastigotes. *Antimicrob Agents Chemother* 2001; 45 : 2064-9.
 33. Matsuyama S, Llopis J, Deveraux QL, Tsisen RY, Reed JC. Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. *Nat Cell Biol* 2000; 2 : 318-25.
 34. Sperandio S, de Belle I, Bredesen DE. An alternative, nonapoptotic form of programmed cell death. *Proc Natl Acad Sci USA* 2000; 97 : 14376-81.
 35. Sudhandiran G, Shaha C. Antimonial induced increase in intracellular Ca²⁺ through non-selective cation channels in the host and the parasite is responsible for apoptosis of intracellular *Leishmania donovani* amastigotes. *J Biol Chem* 2003; 278 : 25120-32.
 36. Holzmuller P, Sereno D, Cavaleyra M, Mangot I, Daulovede S, Vincendeau P, et al. Nitric oxide-mediated proteasome-dependent oligonucleosomal DNA fragmentation in *Leishmania amazonensis* amastigotes. *Infect Immun* 2002; 70 : 3727-35.
 37. Zangger H, Mottram JC, Fasel N. Cell death in *Leishmania* induced by stress and differentiation: programmed cell death or necrosis? *Cell Death Differ* 2002; 9 : 1126-39.
 38. Khalil EA, El Hassan AM, Zijlstra EE, Hashim FA, Ibrahim ME, Ghalib HW, et al. Treatment of visceral leishmaniasis with sodium stibogluconate in Sudan: management of those who do not respond. *Ann Trop Med Parasitol* 1998; 92 : 151-8.
 39. Verma NK, Dey CS. Possible mechanism of miltefosine-mediated death of *Leishmania donovani*. *Antimicrob Agents Chemother* 2004; 48 : 3010-5.
 40. Chowdhury AR, Mandal S, Goswami A, Ghosh M, Mandal L, Chakraborty D, et al. Dihydrobetulinic acid induces apoptosis in *Leishmania donovani* by targeting DNA topoisomerase I and II: implications in anti-leishmanial therapy. *Mol Med* 2003; 9 : 26-36.
 41. Mitra B, Saha A, Chowdhury AR, Pal C, Mandal S, Mukhopadhyay, et al. Luteolin, an abundant dietary component is a potent anti-leishmanial agent that acts by inducing topoisomerase II-mediated kinetoplast DNA cleavage leading to apoptosis. *Mol Med* 2000; 6 : 527-41.
 42. Moreira ME, Del Portillo HA, Milder RV, Balanco JM, Barcinski MA. Heat shock induction of apoptosis in promastigotes of the unicellular organism *Leishmania (Leishmania) amazonensis*. *J Cell Physiol* 1996; 167 : 305-13.

43. Selvapandian A, Debrabant A, Duncan R, Muller J, Salotra P, Sreenivas G, *et al.* Centrin gene disruption impairs stage-specific basal body duplication and cell cycle progression in *Leishmania*. *J Biol Chem* 2004; 279 : 25703-10.
44. Vergnes B, Sereno D, Madjidian-Sereno N, Lemesre JL, Quaissi A. Cytoplasmic SIR2 homologue overexpression promotes survival of *Leishmania* parasites by preventing programmed cell death. *Gene* 2002; 296 : 139-50.
45. Lindoso JA, Cotrim PC, Goto H. Apoptosis of *Leishmania (Leishmania) chagasi* amastigotes in hamsters infected with visceral leishmaniasis. *Int J Parasitol* 2004; 34 : 1-4.
46. Gottlieb RA. Mitochondrial signaling in apoptosis: mitochondrial daggers to the breaking heart. *Basic Res Cardiol* 2003; 98 : 242-9.
47. Gottlieb RA. Role of mitochondria in apoptosis. *Crit Rev Eukaryot Gene Expr* 2000; 10 : 231-9.
48. Mukherjee SB, Das M, Sudhandiran G. Increase in cytosolic Ca²⁺ levels through the activation of non-selective cation channels induced by oxidative stress causes mitochondrial depolarization leading to apoptosis-like death in *Leishmania donovani* promastigotes. *J Biol Chem* 2002; 277 : 24717-27.
49. Diaz G, Setzu MD, Zucca A, Isola R, Diana A, Murru R, *et al.* Subcellular heterogeneity of mitochondrial membrane potential: relationship with organelle distribution and intercellular contacts in normal, hypoxic and apoptotic cells. *J Cell Sci* 1999; 112 : 1077-84.
50. Overly CC, Rieff HI, Hollenbeck PJ. Organelle motility and metabolism in axons vs dendrites of cultured hippocampal neurons. *J Cell Sci* 1996; 109 : 971-80.
51. Kroemer G. Mitochondrial control of apoptosis: an introduction. *Biochem Biophys Res Commun* 2003; 304 : 433-5.
52. Mehta A, Shaha C. Apoptotic death in *Leishmania donovani* promastigotes in response to respiratory chain inhibition: complex II inhibition results in increased pentamidine cytotoxicity. *J Biol Chem* 2004; 279 : 11798-813.
53. Sen N, Das BB, Ganguly A, Tripathi G, Bandyopadhyay S, Rakshit S, *et al.* Camptothecin induced mitochondrial dysfunction leading to programmed cell death in unicellular hemoflagellate *Leishmania donovani*. *Cell Death Differ* 2004; 11 : 924-36.
54. Sen N, Das BB, Ganguly A, Mukherjee T, Bandyopadhyay S, Majumder HK. Camptothecin-induced imbalance in intracellular cation homeostasis regulates programmed cell death in unicellular hemoflagellate *Leishmania donovani*. *J Biol Chem* 2004; 279 : 52366-75.
55. Das M, Mukherjee SB, Shaha C. Hydrogen peroxide induces apoptosis-like death in *Leishmania donovani* promastigotes. *J Cell Sci* 2001; 114 : 2461-9.
56. Aravind L, Dixit VM, Koonin EV. The domains of death: evolution of the apoptosis machinery. *Trends Biochem Sci* 1999; 24 : 47-53.
57. Szallies A, Kubata BK, Duszenko M. A metacaspase of *Trypanosoma brucei* causes loss of respiration competence and clonal death in the yeast *Saccharomyces cerevisiae*. *FEBS Lett* 2002; 517 : 144-50.
58. Freitas Balanco JM, Moreira ME, Bonomo A, Bonomo A, Bozza PT, Amarante-Mendes G, *et al.* Apoptotic mimicry by an obligate intracellular parasite downregulates macrophage microbicidal activity. *Curr Biol* 2001; 11 : 1870-3.
59. Bera A, Singh S, Nagaraj R, Vaidya T. Induction of autophagic cell death in *Leishmania donovani* by antimicrobial peptides. *Mol Biochem Parasitol* 2003; 127 : 23-35.
60. Ridgley EL, Xiong ZH, Ruben L. Reactive oxygen species activate a Ca²⁺-dependent cell death pathway in the unicellular organism *Trypanosoma brucei*. *Biochem J* 1999; 340 : 33-40.
61. Welburn SC, Lillico S, Murphy NB. Programmed cell death in procyclic form *Trypanosoma brucei rhodesiense* identification of differentially expressed genes during con A induced death. *Mem Inst Oswaldo Cruz* 1999; 94 : 229-34.
62. Welburn SC, Murphy NB. Prohibitin and RACK homologues are up-regulated in trypanosomes induced to undergo apoptosis and in naturally occurring terminally differentiated forms. *Cell Death Differ* 1998; 5 : 615-22.
63. Billaut-Mulot O, Fernandez-Gomez R, Loyens M, Loyens M, Quaissi A. *Trypanosoma cruzi* elongation factor 1-alpha: nuclear localization in parasites undergoing apoptosis. *Gene* 1996; 174 : 19-26.
64. Nguewa PA, Fuertes MA, Valladares B, Alonso C, Perez JM. Programmed cell death in trypanosomatids: a way to maximize their biological fitness? *Trends Parasitol* 2004; 20 : 375-80.
65. Debrabant A, Lee N, Bertholet S, Duncan R, Nakhasi HL. Programmed cell death in trypanosomatids and other unicellular organisms. *Int J Parasitol* 2003; 33 : 257-67.