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Review Article

The Role of Metacaspases and Other Proteins Involved in the Apoptosis of *Leishmania:* Review Article

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Abstract

Background: Apoptosis, a determined form of programmed cell death (PCD), occurs in multi-cellular and single-celled organisms. Given that a general understanding of apoptosis in single-cell *Leishmania* is crucial for designing disease control policies, we reviewed the apoptosis mechanism and the proteins involved.

Methods: The information was obtained from articles published in PubMed, SciELO, Science Direct, Scopus, Google Scholar, and Web of Science databases (1998-2021). Search terms used were" apoptosis" or" Leishmaniasis".

Results: The 77 subjects were included in the study that revealed the significance of the apoptosis process for *Leishmania* survival. Although, various stimuli induce *Leishmania* apoptosis, the proteins involved in apoptosis have been poorly understood. Metacaspases in *Leishmania* instead of caspase and death receptors in mammals play the same role in the PCD pathways. Also, other apoptotic proteins in *Leishmania* such as endonuclease G (EndoG), caspases- like cysteine proteases, TSN (Tudor Staphylococcal Nuclease), and Zinnia endonuclease 1 (ZEN1) lead to phenotype similar to mammalian apoptosis. Furthermore, there are differences in these mechanisms between the different species of *Leishmania* and studies to illustrate downstream events related to the serine phosphatidylcholine exposure, cytochrome C secretion, etc. remain an ongoing challenge.

Conclusion: Determining the essential regulatory proteins in the *Leishmania* apoptosis and the specific present of metacaspases in parasite, is effective for designing new therapeutic strategies against leishmaniasis and vaccine development.

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Introduction

Apoptosis or PCD is one of the pathways that the cell goes to remove infection more quickly and is necessary for the proper function of the immune system (1, 2). Conservation of integrity, cell differentiation, and population are controlled by apoptosis (3, 4). It is related to the number of cellular events like reactive oxygen species (ROS) production, Phosphatidylserine expression in the outer membrane of mitochondria, enzymatic breakage of chromosomal DNA, etc. (5, 6). Apoptosis involves external and internal ways. In both apoptosis pathways, there are specialized proteases called caspases and are produced as inactive pro-caspases that activated by other caspases. Caspases are divided into initiators and executioner groups. Initiator types like caspases 2, 8, 9, and 10 are *activated* under apoptotic signals. Then, they activate executioner types like caspases 3, 6, and 7. The mitochondrial pathway of apoptosis is stimulated by responding to stimuli such as ultraviolet rays, hunger, or lack of essential growth factors for survival (7). The external pathway of apoptosis is started by activating death receptors (TNF receptors family members such as TNFR1, Fas (CD95), and extracellular ligands (for example FasL, TNF) (8).

Apoptosis in single-cell organisms

Similar to multicellular organisms, single-celled organisms can also undergo apoptosis (9). Apoptosis in unicellular organisms controls cell population to adjust to environmental pressures, and strictly controls cell cycle and differentiation (10, 11). Apoptosis maintains asexual reproduction within the population (12). In the mid-20th century, apoptosis was reported in *T. Cruci, T. Brucei,* and *L. amazonensis* (13, 14). The mechanisms involved in the single-cell parasites apoptosis are divergent because of evolution, however the involved morphologies and processes are similar to fungi, protists, and animals (15). The apoptosis in the single-cell parasites is identical to para-apoptosis (non-apoptotic programmed cell death) that does not lead to phagocytosis of dead cells in multi-cellular organisms (16).

Apoptosis in Leishmania

Leishmania has a changing two-phase life cycle (17): pro-cyclic promastigotes living in the midgut of sand fly convert to virulent metacyclic promastigotes in the bite of sand fly. While in the mammalian host, promastigotes are swallowed by phagocytic cells and transform into amastigote.

Like other parasites, *Leishmania* requires metabolic pathways for survival and pathogenesis (18). Many *Leishmania* enzymes like sterol biosynthesis, glycolysis, purine salvage, glycosylphosphatidylinositol biosynthesis, and specific enzymes like protein kinases, topoisomerases, metacaspases, and most proteases support the survival and *Leishmania* differentiation to continue infection (19, 20). Many biochemical and morphological events related to mammalians apoptosis have been studied in trypanosomatid parasites like *T. cruzi*, *T. brucei,* and *Leishmania* spp. (21). As, in various species of *Leishmania*, apoptosis occurs under different regulatory signals and in reply to a broad range of stimuli like heat shock, ROS, Nitric oxide (NO) produced by the innate immune response, anti-parasitic drugs such as miltefosine (MLF) and camptothecin (CPT), prostaglandins, silencing cell cycle-related genes like ceramide, antimicrobial peptidases, and etc. (22).

The onset time of apoptosis is essential, as food sensory pathways can provide signals in response to starvation to initiate PCD (15). The parasite population is constant over the life of the insect, and parasite and insect compete for proline (as an energy source) (23). Also, *Leishmania* utilizes PCD either in the sand-fly vector to protect the survival of infectious metacyclic forms in response to the limited resources or in the mammal host to evade hyper-parasitism that would prematurely kill the host. Furthermore, the late studies propose *Leishmania* uses PCD features to simplify

its silent entrance into the mammal host and institute a prosperous infection (21). Only 1% of promastigotes differentiate into metacyclic form and the remaining pro-cyclic features will endure PCD to restrict the use of definite nutrients in the insect gut and in that way, allow the more consistent forms to transfer the disease (11). Moreover, unlike necrosis, cells that are deleted through an apoptosis-like process are not lysed but eliminated by phagocytosis, and this will prevent the inflammatory response from spreading. Thus, the sudden death of non-infectious promastigotes through the apoptosis-like process can guarantee avoidance of immune responses and therefore plays a significant role in establishing the early infection and progressing to the chronic phase of the disease (24). However, studies have revealed the apoptotic factors in various parasites like Trypanosomes, *Leishmania* (25), and Plasmodium, information on the mechanisms involved in parasite apoptosis is limited (11, 26). The complexity of apoptosis stimulation and implementation mechanisms propose that several pathways are involved in *Leishmania* apoptosis (27), and it is hypothesized that protozoan parasites apoptosis is more like the apoptosis process in plants than animals (15). Mitochondria has a prominent role in the *Leishmania* apoptosis and show some similarities to the path of internal apoptosis in mammal cells. The release of cytochrome C in response to many apoptotic stimuli has been reported in *Leishmania*, but downstream events of cytochrome c secretion like binding to Apaf-1 and caspase-9 homologous activation in *Leishmania* have not been described (21).

Effective proteins in the Leishmania apoptosis

Although, late research on the *Leishmania* genome has suggested the weak homologous of mammalian AIF in the *Leishmania*, apoptosis proteins in mammalian, mainly Bcl-2 / Bax do not express in the *Leishmania* or Protozoa genomes. Among the effective molecules of PCD in *Leishmania*, nucleases and proteases have been better characterized (28) (Table 1). In *Leishmania* subspecies, no caspase gene has been found (29-31). Moreover, metacaspases have a main role in inducing apoptosis in response to various stressors, aging, and biological abnormalities in trypanosomes (32).

A) Metacaspases

In the late nineteenth century, Aravind described orthologues of caspase, metacaspase and para-caspase (42). It is suggested para-caspases, metacaspases, and caspases are members of the C14 family, clan CD. Then, Uren identified para-caspases in caspase-containing eukaryotes (animals), slime mold and caspase-free organisms such as *Dictyostelium discolideum*. Para-caspases are involved in the development of MALT lymphoma but not in the performance of cell death (43). cytosolic Metacaspases (MCs) are cysteine-dependent proteases (cysteine peptidases) that based on the structural similarity to the catalytic domain of caspases, their sequence has been identified in caspase-free eukaryotes such as plants, protozoa, fungi, Plantae, and Chromista (44).

The restriction of caspases to multicellular cells and the lack of metacaspases in them propose that there is no functional overlap between metacaspases, and caspases. As, metacaspases are similar to ancestral proteases and caspases have diverged via evolution under environmental stresses (45). Para-caspases and metacaspases exhibit different substrate specificity from caspases (46, 47). Caspases possess substrate specificity guided by aspartic acid at position P1. Metacaspases contain a pair of catalytic histidine/cysteine detection sites. They have the lysine/arginine sequence instead of aspartate (ASP) at the P1 position (29, 48). Also, a specificity driven towards arginine has been displayed for *L. major* metacaspase expressed in yeast (47).

Table 1: Proteins involved in *Leishmania* apoptosis

Protein	Subspecies	Specifications	Mechanism	Ref
Endonuclease G (Endo G)	*L. donovani* *L.infantum*	It is a pro-apoptotic protein of mitochondria	The upregulation of Endo G in Leishmania significantly enhances a caspase-independent apoptotic pathway in response to oxidative or differentiation-induced stress. It fragmentizes DNA during apoptosis. The excessive expression of Endo G in Leishmania leads to spontaneous DNA fragmentation in amastigotes rather than promastigotes and does not need cofactors.	Gannavaram S,2008 (33) BoseDasgupta S,2008 (34)
TatD-related nuclease	*L. donovani*	It is a new class of TIM-barrel 3'–5' exonuclease	It moves to the nucleus to react with endonuclease G during apoptosis.	Gannavaram S,2012 (35) Singh D,2019 (36)
Flap endonuclease-1 (FEN-1)	*L. donovani*	It is a class of nucleolytic enzymes that act as both 5'-3' exonucleases and structure-specific endonucleases on specialised DNA structures that occur during the biological processes of DNA replication, DNA repair, and DNA recombination.	It exhibits inner DNase activity and degrades DNA during parasite apoptosis.	BoseDasgupta S,2008 (34)
Zinnia endonuclease-1 (ZEN1)	*L.infantum*	It is a mitochondrial nuclease.	This DNase enzyme degrades DNA during apoptosis.	Kaczanowski S,2011 (15)
Tudor Staphylococcal Nuclease (TSN)	*L.major*	It is a apoptosis nuclease that has a tandem repeat of staphylococcal nuclease domains and a Tudor domain	Alongside transcriptional role, TSN act in post-transcriptional regulation, like stabilization and degradation of mRNA, RNA interference (RNAi), and prohibiting mRNA splicing. Ribonuclease activities of TSN is essential for the performance of apoptosis	Sundström JF,2009 (37) Broadhurst M,2001(38) Tuteja R,2016 (39)
Cysteine proteases calpains (cathepsin B-like enzyme LmjCPC)	*L.major*	It is a lysosomal cysteine proteinase	It is a potential additional executioner protease in the cell death cascade of Leishmania It hydrolyzes proteins with a broad specificity for peptide bonds and is the Z-VAD-FMK binding enzyme	Klemba M,2002 (40) Arambage SC,2009 (41)

Two types of metacaspases have so far been identified: metacaspases Type I have an N-terminal pro-domain include a proline-rich repeat motif, and the metacaspases type II does not have such a pre-domain, but there is a junction between their small (P10) and large (P20) subunits (49). Most living organisms possess a minimum of two metacaspase genes. The presence of Metacaspases Type I is exclusive to protozoa and fungi, while both types with a higher prevalence of type I over type II, are encoded in the genome of higher plants (50).

During multi-cell apoptosis, caspases cleave the PARP (Poly (ADP-ribose) Polymerase) of DNA repair enzymes (51). Treatment of *Leishmania* with hydrogen peroxide results in the same process involved in PARP-like protein cleavage (52). Parasitic metacaspases be regulated through phosphorylation at specific steps of the parasite's life cycle (45). Calcium activates metacaspases and for most metacaspases, the maturation of enzyme involves an autocatalytic procession of the zymogen. Although, this stage in some cases is not required for proteolytic activation (53). The optimal activity of metacaspases is at *pH 7.0*–8.5 (54), and they can control other biological pathways related or unrelated to cell death (15), such as the regulation of the cell cycle and protein clearance (55). Metacaspases with the caspase-like catalytic domain can act as hangman proteases like mammalian caspases in the apoptosis pathway. However, due to the specificity of their substrate, the path of proteolytic degradation of cell death may be different in *Leishmania* (56). *Leishmania* can tolerate caspase- independent and dependent apoptotic pathways throughout mitochondrial oxidative stress (57). Cell death in *Leishmania* is divided into two primitive types: Necrosis and limited apoptosis that play a considerable role in causing the disease (3, 27, 45). In these process, mitochondrial toxicity due to cytosolic calcium, activation of caspase-like protease 7 /3, and secretion of apoptotic factors of mitochondria was detected (30). However, metacaspases in Trypanosomes, *Leishmania*, and *Arabidopsis* showed resistance to specific caspase inhibitors like Z-VAD-fmk, they exhibited high sensitivity towards serine protease inhibitors such as leupeptin and antipain (58).

Metacaspases in Leishmania parasites

A) Leishmania major

Caspase genes have not been detected in the *L. major* genome so far. *L. major* has a single multifunctional metacaspase (LmjMCA) that codes a polypeptide of 435 amino acids. It has three *major* domains: a mitochondrial localization sequence in N-terminus domain, a C-terminus segment affluent in proline, and a catalytic domain include the histidine/cysteine dyad (11). Hence, LmjMCA is an arginine cysteine protease targeted to the mitochondrion that maybe needs to process to activity as described in yeast (47). Although LmjMCA polypeptides are predominantly located in the cytoplasm, during interphase it is partly diffuse throughout the cell. It tends to colocalize with the kinetoplast within mitochondria segregation, and also it translocates to the nucleus and mitotic spindle of the parasite within mitosis (55). The involvement of LmjMCA is crucial in the process of cell division, particularly when its expression is upregulated in promastigotes. This high expression leads to a notable slowdown in growth and alterations in ploidy. These effects stem from abnormalities in kinetoplast segregation, nuclear division, and impaired cytokinesis (11). LmjMCA possesses the same activities towards arginine and lysine. It be activated by auto processing, as the purified catalytic domain of LmjMCA is active 300 times more than the non-purified LmjMCA (47). LmjMCA overexpression increases the susceptibility of *L.major* to oxidative stress. Under heat shock, H2O2, or anti-leishmaniasis drugs like miltefosine, LmjMCA precursor forms are widely processed to soluble forms containing the catalytic domain, and it aggregates in the cytosolic fragment. This domain is enough to increase the sensitivity of parasites to hydrogen peroxide through destroying the mitochondrion and cell death (56).

The reaction of LmjMCA with proteins involved in other physiological processes like vesicle transfer suggests that LmjMCA may play additional roles in different stages of the parasite life cycle (59). The LmjMCA is expressed in procyclic promastigotes and actively replicating amastigotes, but at a more minor level in metacyclic promastigotes (45). In 2011, Zalila et al. (56) showed that in *L. major*, the active LmjMCA enzymatically does not depend on the interaction of two heterologous subunits as in higher eukaryote caspases. Enzymatically, the secreted catalytic domain is dynamic and directly has a role in the proteolytic destruction of dying parasites.

B) Leishmania donovani

The apoptosis of this parasite begins with the potential depolarization of the mitochondrial membrane followed by stimulating caspase-like activity of metacaspases. *L. donovani* has two metacaspases: LdMc1 and LdMc2 that are encoded by the DB gene, and play the central role in events such as cell cycle and death. The two proteins exhibit a 98% similarity in their sequence and possess a distinctive C-terminal proline-rich region. Both proteins are detected in *L. donovani* axenic promastigotes and amastigotes, with LdMC1 displaying heightened mRNA expression in axenic amastigotes in comparison to promastigotes. These proteins have the ability to cleave substrates containing arginine/lysine residues without necessitating proteolytic activation, whether under normal conditions or during PCD induced by oxidative stress (29).

Immunofluorescence findings suggest a connection between LdMCs and the acidocalcisome portions of *L. donovani*. Specifically, upon the induction of apoptosis in cells through H2O2, LdMCs are released as enzymatically inactive molecules from these vesicles. Analysis of immunoprecipitated LdMCs through enzymatic assays reveals that the native LdMCs exhibit proficient trypsin substrate cleavage, while they lack the ability to cleave caspase-specific substrates. Also, LdMC function is not sensitive to caspase inhibitors but is prohibited by trypsin inhibitors like Nα-tosyl-l-lysine-chloromethyl ketone (TLCK), leupeptin, and antipain *(29)*. Studies propose an essential function of heat shock protein (Hsp70) and LdMC1 in *Leishmania* cell cycle of. As, Raina caused phenotypic and molecular characteristics of PCD such as delay in the S-phase progressing, DNA fragmentation, and a reduction in the potential of the mitochondrial membrane through knockdown of metacaspase transient gene, LdMC1 and Hsp70, in *L. donovani* by using antisense oligonucleotides (ASOs) within MG132-induced PCD (60).

Metacaspases in other organisms:

Some studies have demonstrated genes similarities to metacaspases in organisms such as Trypanosomes (61, 62), yeasts (63) and plants (64) that are associated with the caspases and play a role in PCD (Table 2).

Research on the apoptosis of Leishmania and other organisms using different medications and methods

Some research showed various drugs stimulate apoptosis-like death of *Leishmania* spp. (34). Anti-parasitic drugs destroy parasites through three defined mechanisms: necrosis, autophagy, and apoptosis *(35)*. Based on the employed strain of *Leishmania* for inoculation, suggesting that the host genetic background and the type of *Leishmania* could impact the interaction of death receptors in the specific immune responses to *Leishmania* (Table 3).

Table 2: Metacaspases in other organisms

Organism	Metacaspases Name	Specifications	Mechanism	Ref
Trypanosoma cruzi	TcMCA3	TcMCA3 exists within the CL Brener clone at a rate of 16 copies per haploid genome	They play a role in PCD similar to caspases in higher eukaryotic organisms.	Kosec G,2006 (61).
	TcMCA5	TcMCA5 is identified as a solitary gene copy.		
Trypanosoma brucei	TbMCA1-5	They do not need autocatalytic processing for their enzymatic activity.	The only form of PCD characterized so far in T. brucei bloodstream forms is the prostaglandin D2-induced cell death	McLuskey K,2012(62) Helms MJ, 2006 (65) Figarella K,2005(66)
Saccharomyces cerevisiae **(Yeast)**	Yac1 (Mca1)	It is a positive regulator of oxidative stress or aging-related cell death	It regulates cell death and controls cell cycle and protein aggregates clearance	Tsiatsiani L,2011(55)
Schizosaccharomyces pombe **(Yeast)**	Pca1	It contains a conserved cysteine 270-histidine 271 catalytic diad in its putative caspase domain	It has increased caspase 3-like peptide cleavage activity.	Madeo F,2002 (63)
Arabidopsis thaliana **(Plant)**	AtMC1-9	They are pro-death caspase-like proteins	They show a apoptotic activity similar to caspases.	Reape TJ, 2010 (64) Coll NS, 2010 (67) Atkinson HJ, 2009 (68)
Populus trichocarpa **(Plant)**	MC1-20	They have specific substrate in comparison with mammalian caspases.	They don't have sensitivity to caspase-specific inhibitors.	Watanabe N, 2005 (69) Vercammen D,2004 (70)

Table 3: The apoptosis of *Leishmania* and other organisms using different medications and methods

Medications& methods	Organism	Mechanism	Ref
Miltefosine (HePc)	*L. infantum*	1) It depolarizes the mitochondria 2)It through targeting DNA topoisomerases resulted in dose-dependent death of L. infantum	Khademvatan, 2011 (71, 72)
Staurosporine	*L. major*	It induces cell shrinkage, phosphatidylserine exposure, etc.	Arnoult,2002 (73)
Cysteine proteinases	*L. major*	It has nuclear pro-apoptotic activity	Arnoult, 2002 (73)
Tunicamycin	*L. major*	It induces mitochondrial depolarization independent of caspase-like proteases.	Dolai ,2011 (74)
Oleuropein	*L. major*	It has a drastic leishmanicidal effect through the apoptosis process	Elamin,2014 (75)
Chronic heat shock	*L. donovani*	It modulates Ca2+ during differentiation and stimu-	Raina,2006 (76)

		lates PCD	
Replacement of Yca1 Gene with Lmj MCA	*S. cerevisiae*	LmjMCA possesses catalytic activities towards arginine in the P1 position and acts on yeast cell death similar to Yca1.	Gonzalez,2007 (47)
MCA mutant (Δmca)	*L. mexicana*	The MCA acts as a negative regulator of amastigote duplication, thereby acting to balance cell growth and cell death.	Munoz, 2012 (9)
Curcumin	*L. major*	It delays the cell cycle in the S-phase, inhibits cell proliferation and induces apoptosis	Elamin, 2021 (77)
Q. velutina ***C. procera*** ***N. tabacum***	*L. tropica*	Herbal extracts with an effective antileishmanial activity induce promastigotes apoptosis.	Ilaghi, 2021 (78)
Total Phenolic Fraction (TPF) from Extra Virgin Olive Oil	*L. major*	TPF acts as immune-stimulator of host's immune system and with chemotherapeutic effect eliminates parasite alone.	Karampetsou,2021 (79)
Sugiol (solated from the bark of Cupressus lusitanica)	*L. infantum*	Particles encompass sugiol and show a response against the intracellular L. infantum amastigotes while preserving the integrity of the host cell.	Scariot, 2019 (80)
***G. hirsutum* bulb fractions**	*L. major*	Both the crude extract and fractions show excellent apoptotic index.	Sharifi, 2019 (81)
Ruthenium (II) complex	*L. amazonensis*	hmxbato and precursor enhance ROS production, mitochondrial membrane depolarization, DNA fragmentation, the emergence of a pre-apoptotic peak, alterations in parasite morphology, and the development of autophagic vacuoles.	Costa, 2019 (82)

Conclusion

Although, apoptosis stimulation of parasites to enhance the effectiveness of anti-parasitic drugs promote human cells degradation in the exact mechanism, considering the specific metacaspases in parasites and their lack in humans, opens a new insight to considering potential drug targets to combat parasitic diseases in the future. Furthermore, inhibition of parasite-stimulated human cell apoptosis along with induction of parasite apoptosis (in the early stages of infection) is a research area for treatment that still requires to be more studied.

Journalism Ethics approval

This study has been approved by the Ethical Committee of Isfahan University of Medical Sciences with code number of IR.ARI.MUI.REC.1403.148.

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Conflict of Interest

The authors declare that there is no conflict of interests.

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