Evidence-based insights on *Leishmania* replication and cytokinesis

Original Article

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ABSTRACT

Background: *Leishmania* parasites are transmitted to humans and mammals by sand fly vectors in the form of flagellated promastigotes that propagate inside tissue macrophages in the form of aflagellate amastigotes. Majority of information on sequential development is derived from that of the kinetoplastids: trypanosomatidae; notably that of *T. brucei*.

Objective: Investigation of ultra-morphologic events involved in *Leishmania* replication to provide a detailed description of the sequential processes by which *Leishmania* replicates its single-copy organelles

Material and Methods: *Leishmania* promastigotes were maintained by *in vitro* culture, and amastigotes were attained by inoculation of laboratory out bred male Syrian hamsters with cultured promastigotes. Concentrated pellets of promastigotes and 1 mm thick tissue biopsies from infected hamsters were optimally processed for electron microscopy. Ultrathin-sections were examined and photographed by a Zeiss EM 952 electron microscope.

Results: In promastigotes and amastigotes, initial amplification of subpellicular microtubules resulted in morphometric increase in size of the dividing forms. Replication of nucleus and kinetoplast was asynchronous. Kinetoplast S phase occurred before commencement of nuclear S phase. Lengthening and merging of the kinetoplast mitochondrion occurred into that of the amastigote cell and coincided with detection of the hidden basal body which started duplication of the flagellum. Separation of basal body, flagellar pocket and flagellum appeared to occur before kinetoplast division which was found to divide and separate before closed mitosis (pleuromitosis). Centrosomes were recognized in the centre of the spindle synchronously with the K-DNA lengthening. Cytokinesis progressed from the posterior pole to the anterior pole of the cell along the longitudinal axis, bisecting the cell symmetrically.

Conclusion: Compiled records provided definite information on the sequential cellular cycle of the *Leishmania* spp. and the chronological proceedings of replication of their nucleus and kinetoplast. Replication by nuclear (karyokinesis) and cytoplasmic (cytokinesis) division machineries, documents its non-conformability with its taxonomically related trypanosomatids.

Keywords: closed mitosis, cytokinesis, DNA replication, karyokinesis, *Leishmania*.

Received: 5 September, 2018, **Accepted:** 1 November, 2018 .

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Print ISSN: 1687-7942, **Online ISSN:** 2090-2646**, Vol. 11, No. 3, December, 2018.**

INTRODUCTION

In humans, the kinetoplastid, sand fly-transmitted *Leishmania* spp. causes infections that range from localized cutaneous lesions to disseminated mucocutaneous and visceral forms. Leishmaniasis is endemic in the tropics and sub-tropics, establishing a major public health concern for residents and travelers to the affected regions. The "World Health Organization (WHO) team"[1] reported that 310 million people were at risk of *Leishmania* infection in 98 countries around the world, with approximately 1.3–2 million new cases per year. Sand fly inoculated promastigotes actively invade circulating bone marrow macrophages, in which they differentiate into amastigotes. After reaching a critical number by active divisions, amastigotes from disrupted host cells invade other macrophages, thus repeating the cycle and causing the disease[2]. Most of the information available on the sequence of nuclear and kinetoplast DNA replication is derived from data on the taxonomically related trypanosomatidae; specifically *T. brucei*[3-8]. While DNA replication presents a target for DNA damage resulting in diverse diseases and infections[9], much is unknown about the definite specific events involved in replication process of *Leishmania*. Thereby, the present study is concerned with the micro-morphological chronological replication of the dimorphic stages of the organism and its single–copy organelles by investigating three different cutaneous and visceral strains. The study focuses on morphogenetic characteristics of replication and cytokinesis of *Leishmania* organisms at an ultrastructure level. The morphogenetic aspects of replication may indicate targets for DNA damage

 DOI: 10.21608/puj.2018.5536.1020

and hence predict new approaches for treatment of leishmaniasis.

MATERIAL AND METHODS

In this experimental study, *Leishmania* promastigotes of three different local strains were isolated by *in vitro* culture[10] and harvested at the exponential growth phase. The strains comprised a human cutaneous strain (acquired infection in Sinai), a strain from *Rattus norvegicus* (from Ismailia province) and a visceral strain from stray dogs (at Agamy, Alexandria). Amastigotes were experimentally attained by inoculation of laboratory out bred male Syrian hamsters with *in vitro* promastigotes harvests of each strain. For each strain concentrated pellets of both of isolated promastigotes harvests and 1 mm thick tissue biopsies from infected hamsters were optimally fixed in cold 2.4% gluteraldehyde in 2% Cacacodylate buffer (pH 7.2), for one hour at room temperature (23°C), then post-fixed in 2% osmium tetroxide for 24 h and processed for electron microscopy[11]. Ultrathin-sections using LBK Reichard ultra-microtome were post-stained with uranyl acetate in lead citrate solution, examined and photographed by Zeiss EM 952 electron microscope.

Ethical consideration: The experimental animal studies were conducted in accordance with the international valid guidelines. The hamsters were housed in good ventilated filter-top cages at 25°C, relative humidity of 40-60%, and provided sterile rodent chow and water *ad libitum*.

RESULTS

The sequential features recorded for *Leishmania* spp. replication was compiled from ultra-morphologic data of different *in vitro* cultured promastigotes, and amastigotes in tissue of experimentally infected hamsters. Asynchronous non-coinciding replication of the nucleus and the kinetoplast, each one sequentially replicating and segregating in order was noted. With exception of the nucleus located at the middle region of promastigotes and at the posterior pole of amastigotes, other single-copied organelles were located at the anterior region of the organism (Figure 1). Compared with non-dividing forms, an initial amplification of the subpellicular microtubular corset resulting in morphometric increase in size of the dividing forms was observed (Figure 2). The start of kinetoplast S phase was detected before commencement of nuclear S phase, with prior K-DNA lengthening and merging of the kinetoplast mitochondrion into that of the amastigote cell (Figures 3-5); and coinciding with detection of the hidden basal body (Figure 5) which started duplication of the flagellum. The separation of the basal body, flagellar pocket and flagellum appeared to occur before the kinetoplast division (Figures 6 and 7) which was found to divide and separate before mitosis (Figures 6-8). Mitosis appeared as a closed process (pleuromitosis) without nuclear membrane breakdown (Figure 9). The recognition of centrosomes which are organizing centers for the spindle was synchronous with the K-DNA lengthening (Figure 10). Cytokinesis then progressed with furrow ingression along the longitudinal axis, bisecting the cell symmetrically and progressing from the posterior pole to the anterior pole of the cell (Figure 11).

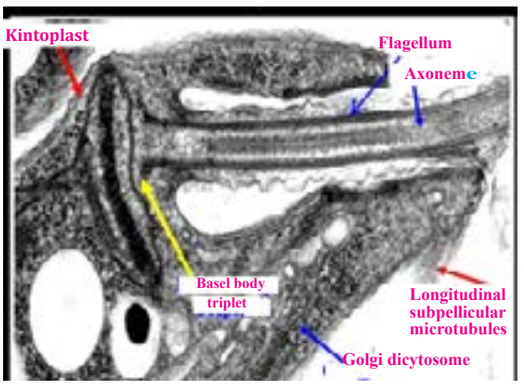

Fig. 1. Electron micrograph illustrating single-copy organelles located in *Leishmania* anterior region: basal body, flagellum, kinetoplast and Golgi apparatus (arrows). The nucleus is not apparent being located at the middle region of promatigote form, and in the posterior pole of the aflagellated (crypto-flagellated) intacellular amastigote form (X 9600).

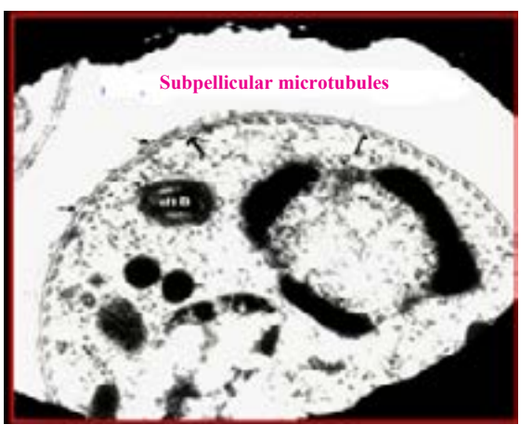

Fig. 2. Electron micrograph showing increased number of subpellicular microtubules in a dividing form (arrows), proved by morphometric comparison with non-dividing forms (X 9600).

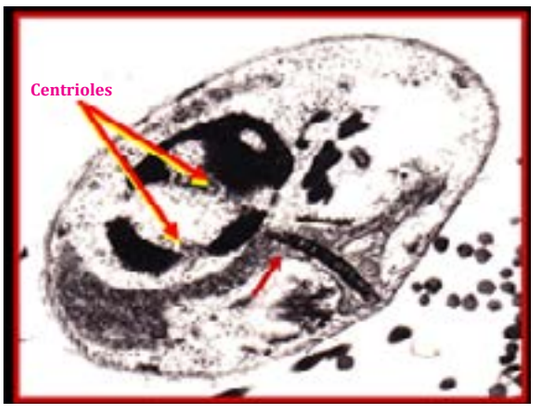

Fig. 3. Electron micrograph showing merging of kinetoplast capsule into the mitochondrion (arrow) and apparent centrosome (centriole) within the nucleus near the peripheral chromatin (double arrows) (X 9600).

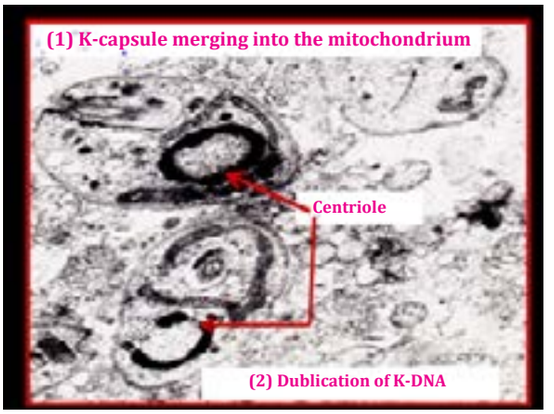

Fig. 4. Electron micrograph showing merging of kinetoplast capsule into the mitochondrion and duplication of K-DNA, and apparent centrosome (centriole) near the peripheral chromatin of the nucleus (X 6400).

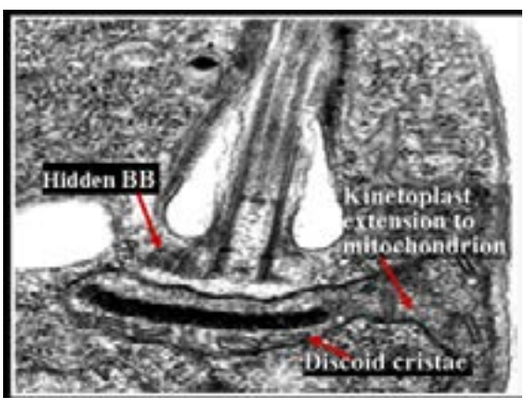

Fig. 5. Electron micrograph revealing the hidden basal body (BB), rooting for duplication of the flagellum, merging of the kinetoplast into the mitochondrion, and discoid cristae (arrows) (X 12400).

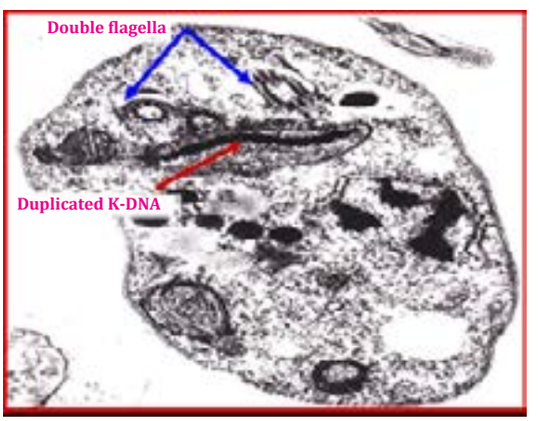

Fig. 6. Electron micrograph of a sliding ultrathin section of a replicating organism showing duplicated K-DNA of the kinetoplast and double flagella, each in a separate flagellar pocket (X 12400).

Fig. 7. Electron micrograph of a sliding ultrathin-section of a replicating promastigote showing symmetrical bisection of the replicated flagellum and intact non-divided nucleus (X 4600).

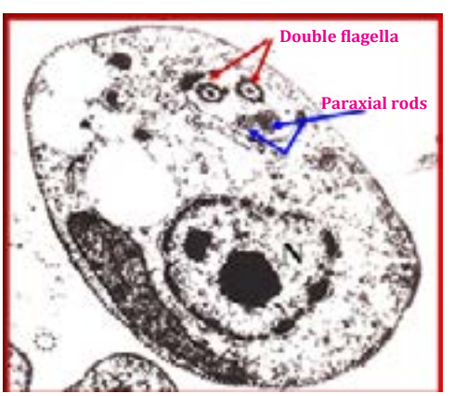

Fig. 8. Electron micrograph showing double flagella associated with double paraxial rods within the parent flagellar pocket and intact non-divided nucleus (N) (X 4200).

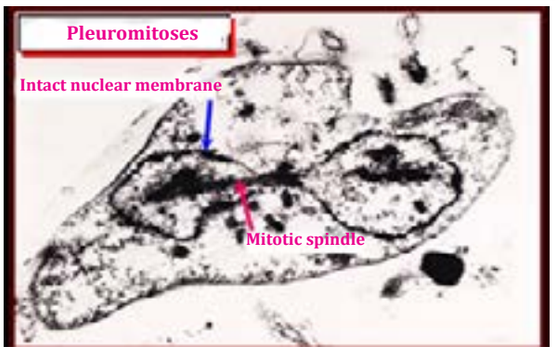

Fig. 9. Electron micrograph revealing mitotic spindle pre-karyokinesis of replicated nucleus with intact nuclear envelope "closed mitosis" (X 3600).

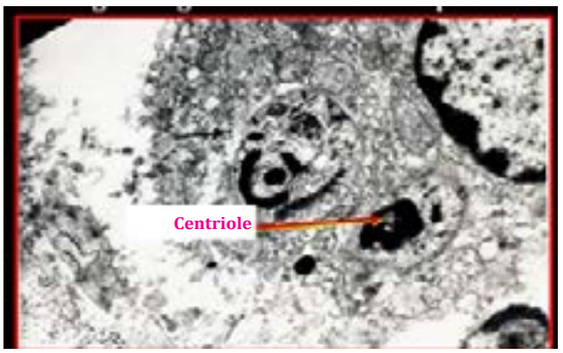

Fig. 10. Electron micrograph revealing intra-nuclear centrosome (centriole) in dividing amastigotes (arrow) (X 2300).

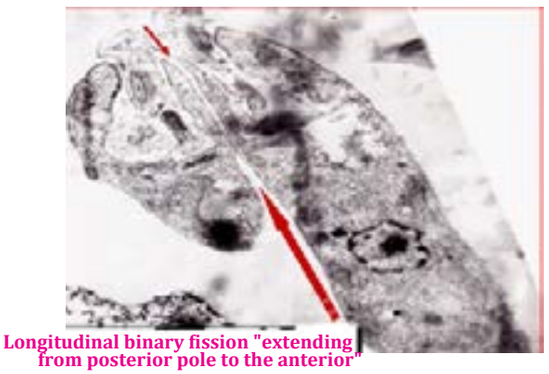

Fig. 11. Electron micrograph revealing symmetrical cytokinesis along the longitudinal axis of the replicated organism, with tip of both segregated flagellar pockets connected by a trans-membrane junction (connector) indicating cytokinesis progression from the posterior pole to the anterior pole of the organism (arrows) (X 2300).

DISCUSSION

After transmission by the sand fly vector, metamorphosed *Leishmania* amastigotes in host cells undergo multiple replications. Infection is established on reaching a maximal number by active replicates resulting in a variety of clinical syndromes, depending on the *Leishmania* strain and the immunological status of the infected patient. The recorded ultrastructure knowledge on sequence of *Leishmania* spp. replication is related to that of *T. brucei*[3-8]. Generally, studies on *Leishmania* replication focus on quantitative microscopy, timings of cell cycle phases, phase-contrast microscopy, fluorescence, and biophysical studies of cultured promastigotes[12-14]. Recent studies in the genus correlate genomic alteration to *Leishmania* replication and molecular mechanisms and factors involved in the regulation of DNA replication and repair[15-18].

Expanding on our previous detailed studies on ultra-morphogenesis, differentiation and morpho-transformation, and posterior polar endocytic phagocytosis of *Leishmania* spp.[19-22], the present study dealing with the sequential replicative events of *Leishmania* cell cycle at an ultrastructure level, might be so far unique among recorded literature on the genus. The study adds and documents facts clarifying views and understandings about the kinetoplastid, *Leishmania*. Though both *Trypanosoma* and *Leishmania* are trypanosomatids, remarkable differences between the two genera are confirmed in the present study.

It is known that *Leishmania* is a highly polarized organism, possessing in its two stages single-copy organelles[13] that have defined subcellular locations. These include the nucleus, the Golgi apparatus, the basal body, the mitochondrion which incorporates the kinetoplast, and in promastigotes the flagellum which protrudes from the cell body *via* the flagellar pocket (Figure 1). A key preparatory step appears to involve remodeling of the underlying cortex by increasing the number of subpellicular microtubules (Figure 2), as revealed by morphometric comparison with non-dividing cells. This is followed by alteration of cellular architecture involving replication and separation of duplicated organelles.

The literature reveals disagreement concerning *Leishmania* replication and cytokinesis. By microscopy of exponentially growing *L. donovani* in culture[23] the kinetoplast morphology and pattern of segregation were considered to be a marker for cell cycle progression. It was assumed that nuclear division precedes kinetoplast segregation in 80% of *L. donovani* cells and is preceded by kinetoplast segregation in the other 20%. Also, in *L. major* it was supposed that although nuclear DNA replication precedes kinetoplast segregation its complete division does not occur until

the kinetoplast segregation is completed[13]. On the other hand, by microscopy timing of cell cycle phases[14], large changes in morphology of *L. mexicana* were reported assuming that nuclear and kinetoplast S phase occur simultaneously occupying a large proportion (40%) of the cell cycle. Likewise, it was hypothized that coincident replication of the nucleus and kinetoplast is the case in *Leishmania*[24]. In contradiction, the present study demonstrated sequential ultra-structural features that revealed that *Leishmania* spp. replication process is complex involving asynchronous non-coinciding replication of the nucleus and the kinetoplast, and that each sequentially replicated and segregated in order. Study of the ultra-micrographs showed that karyokinesis is accomplished after kinetoplast separation, and that appearance of centrioles (Figures 3, and 4) coincides with emerging of kinetoplast into the organism mitochondrion and lengthening of the K-DNA. These differences suggest distinct molecular and signaling mechanisms of both organelles. A similar order of kinetoplast segregation before karyokinesis was described in *T. brucei* and *T. cruzi*[25].

In karyokinesis, synthesis of the two DNA strands occurs by two different mechanisms. One is through enhancement of replicative DNA polymerases by the DNA polymerase cofactor PCNA (proliferating cell nuclear antigen)[26]. In the other the DNA elongation, in a PCNA cofactor-dependent manner[27] is controlled by PI3KCB which is a member of class IA phosphatidylinositol 3-kinases (PI3KCs). The PCNA cofactor has been detected throughout the cell cycle in *L. donovani*, *T. brucei* and *T. cruzi*[28,29]. Its subnuclear expression pattern varies during the cell cycle, being localized in sub-nuclear foci at the nucleus periphery during the S phase, while it is more widely distributed in G2/M phase which indicates the presence of replication factories in specific sub-locations in *Leishmania* and related species; a feature which is conserved also in higher eukaryotes[30]. The replicative DNA polymerases belonging to the B family are conserved in *Leishmania* spp.[18]. They are concerned with synthesis of DNA during elongation[31].

By electron microscopy, we were able to determine an order of events, in which the basal body duplication is an early stage of new flagellum growth. Separation of the basal body and flagellum duplication appear to occur before the kinetoplast division, while segregation of the kinetoplast and the flagellar pocket precedes flagellar separation. Similarly, the kinetoplast division in *T. brucei* was reported to be dependent on initial duplication of the basal body complex and flagellum[3].

In culture *L. tarentolae* long slender non-dividing stationary phase forms were found to have an asymmetric mitochondrion, consisting of a single tubule extending from one edge of the kinetoplast portion. This finding was assumed to present a problem for cell division, in that one daughter cell will receive significantly less mitochondrial membranes than the other cell[32]. It was suggested that dividing forms, which are normally shorter and rounder than those of stationary phase, possess a symmetric circular mitochondrion that has mitochondrial tubules extending from both edges of the kinetoplast to join in the posterior region of the cell. The researchers went on to imply that mitochondrion growth occurs after cell division, either from elongation of the longitudinal tubule towards the anterior pole, or from elongation of the kinetoplast portion of the mitochondrion towards the posterior pole and fusion of the tubules. In view of our present findings, such an assumption proved invalid since division of *Leishmania* spp. amastigotes and promastigotes was documented despite the extension of kinetoplast mitochondrion capsule at one of its poles to fuse with cell mitochondrion (Figures 3-5).

Recognition of centrosomes (centrioles), which are essential for mitotic initiation and are organizing centers of the spindle[33], was synchronous with the K-DNA lengthening and merging of kinetoplast capsule into the mitochondrion (Figures 3, 4, and 10). While the recorded pleuromitosis (Figure 9) was also reported to occur in *T. brucei*[34,35], yet, our present demonstrations document that cytokinesis of *Leishmania* is unlike that of *T. brucei*. In the latter trypanosome, furrow ingression was found to be progressing from the anterior end to the posterior end of the cell[3,4], presuming that progression of the cleavage furrow in *T. brucei* occurs in a helical fashion to adapt to the presence of the attached flagella. In our study, cytokinesis process in *Leishmania* promastigotes proved to be *via* an uncharacterized mechanism preceded by cell rounding and progressing by the cleavage furrow bisecting the cell symmetrically along the longitudinal axis from the posterior pole to the anterior pole of the organism (Figure 11).

In conclusion, the described specific characteristics of replication and cytokinesis machineries detected in *Leishmania* spp. are not conformable with that of other trypanosomatids, and therefore fundamental biological knowledge on *Leishmania* should not be based on that of its taxonomically relative trypanosomes. *Leishmania* sequential replication appears to be a complex process involving asynchronous, non-coinciding replication of its single copy organelles; a feature that presents multi-checkpoints for comparison with its taxonomically relative trypanosomes. Differences in sequential replication process of the single-copy *Leishmania* organelles are indicative of distinct molecular and signaling mechanisms of organelles as promising targets for researching therapeutic approaches.

Not only is the cell cycle replication central to understanding fundamental biology of *Leishmania*, but in fact, the specific characteristics of the replication and machineries could be used as possible targets for the development of new treatment.

Author contribution: MM Abdel Mawla performed the electron microscopy. ME Azab maintained and provided the *Leishmania* isolates. Both authors designed the experimental study, analyzed the data and prepared the manuscript.

Conflict of interest: There is no conflict of interest.

Financial support and sponsorship: Nil.

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