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# Ultrastructural Changes of Radioattenuated *Leishmania donovani,* The Causative Organism of Human Kala-azar Disease

**Sanjukta Manna***

**Abstract**

The promastigotes of *Leishmania donovani* which were exposed above 20krad gamma radiation (Gammacell 220, $Co^{60}$ source at $23^0$C) looked like amastigotes by shedding their flagella and became round in shape. With the increasing doses of radiation, the number of amastigote like structures increased. At higher doses, abnormalities included shrinkage, rounding, loss of flagellum, aggregation, vacuolation, progressive loss of motility and these changes occurred producing an almost endless variety of morphological forms. From the electron micrograph it was revealed that the most effected organelles after irradiation were mitochondria, kinetoplast and flagellum. Irradiated cells have different levels of injury; ones with less damage recover quickly and have opportunity to modify antigenic profile while those with more damage repaired later. This result was also supported by the alteration in microtubular structure of plasma membrane. Nucleus showed a larger relative volume in 30 krad irradiated promastigotes. The relative volume of the kinetoplast does not appear to differ so much. It was evident that flagellar pocket was much dilated in case of promastigote irradiated at 20 and 30 krad radiation doses. Although this damage did not always contribute to cell death but the damages were irrepairable. These findings will be crucial for the outcome of modified infection and probable vaccine development.



***Keywords:***

*Leishmania donovani*,
gamma radiation,
irradiated promastigotes,
kinetoplast

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## 1. Introduction

A comparative study and comprehensive understanding of the effect of gamma radiation on *Leishmania donovani* promastigotes *in vitro* and *in vivo* at subsequent morphological, biochemical and immunological level is lacking. Visceral leishmaniasis (VL or kala-azar) caused by *Leishmania donovani* is the most dreaded disease and devastating among various forms of leishmaniasis. The disease has gained significance because of high mortality rate particularly amongst the children and poor people. In India high incidence has been reported from the states of Bihar, Assam, West Bengal and Eastern Uttar Pradesh where resistance and relapse are on increase [1]. According to official figures, in 2010 over 28,000 new cases were reported in Nepal, Bangladesh and India [2]. However, this figure is an underestimation of the real number of cases [3] and falls far from the objective set by the regional governments to eliminate VL from the Indian subcontinent by 2020.

*Leishmania donovani*, an intracellular protozoan parasite causes kala-azar in India. The parasite is transmitted by various species of female sandflies (*Phlebotomus* sp.). It exists in two morphological forms: the promastigotes, residing in the gut of female sandflies, and the amastigote, living in the reticuloendothelial system of the mammalian hosts. The promastigotes possess a full–length, free-flagellum whereas it is rudimentary in amastigotes. Gamma radiation had interesting effects on leishmanial cells allowing both sterilization and immune enhancement, without introduction of new epitopes in the preparation, but parasites

presented unequal radiosensitivity, related to their flagellar disposition and kinetoplast-mitochondrial organization. Although studies on fine structural properties of normal promastigotes and amastigotes and also of some stressed leishmanial cells have been published [4,5,6,7,8], yet structural comparison between normal and irradiated cells have not been analyzed. For that, the study was undertaken to demonstrate the ultrastructural changes, as they occur *in vitro*, produced by gamma radiation exerted upon promastigotes.

## 2. Research Method

Media and Chemicals

Glutaraldehyde, paraformaldehyde were from Mark, cacodylate buffer, epoxy resin, uranyl acetate and lead citrate, bovine serum (FBS, heat inactivated) were from Difco (Detroit, Michigan); M199, penicillin, streptomycin, and gentamycin were from Gibco Laboratories (Grand Island, New York). All other reagents used were of analytical grade.

*Leishmania* Stock

Promastigotes of *Leishmania donovani* (MHOM/IN/1983/AG83) were grown at $22^0$ C in medium 199 (pH 7.4) supplemented with 10% heat inactivated fetal bovine serum, 2mM L-glutamine, 100U of penicillin G sodium and 100µg of streptomycin sulfate per ml and subcultured in the same medium at an average density of $2x10^6$ cells/ml.

Radiation Exposure

The stationary phase cell culture ($3.7x10^6$cells/ml) was taken for radiation exposure and exposed to a $^{60}$Co gamma source for irradiation at $23^0$ C using doses in the range of 10, 20, 30, 40 and 50 krad (Gammacell 220) which delivered a radiation dose at the rate of approximately 12 krads/hr at an exposure distance of 50 cm to the 100% radiation area. The dose rate (12 krad/hr) was calibrated with Fricke dosimeter[9]. The irradiated promastigotes were harvested from culture by centrifugation at 1000 x g at $4^0$ C and taken for the experiment.

Scanning Electron Microscopic Study of Whole Cell

The technique was followed from standard research methods [10,11]. We use the HITACHI S530 Scanning Electron Microscope to observe the surface structure and the photographs were taken by MAMIYA 6X7 camera using NOVA 120 ASA films.

Transmission Electron Microscopic Study of Whole Cell

The standard protocol was followed [11,12]. The internal structure of *Leishmania donovani* was observed through Philips CM-10 Transmission Electron Microscope at an accelerating voltage of 80 kV with 15,000 to 20,000 magnifications.

Morphometric Analysis

Ultrathin sections (70 nm thick) of promastigote (photographed under 25,000 magnification) were calibrated. The outlines of the whole cell and of the different organelles (nucleus, mitochondrion, pocket flagellum, kinetoplast) were manually contoured using the Leica Quantimed 500IW image analysis program to obtain the area. The volumes of the cell and organelles were obtained by multiplying the total area by the section thickness. The relative volume of each organelle was obtained by dividing the organelle volume by the total cell volume.

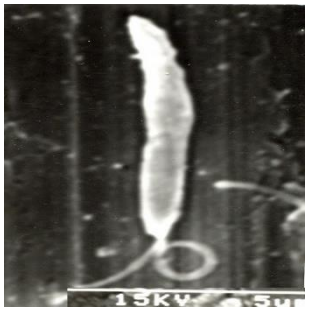
## 3. Results and Analysis

Scanning Electron Microscopic Study

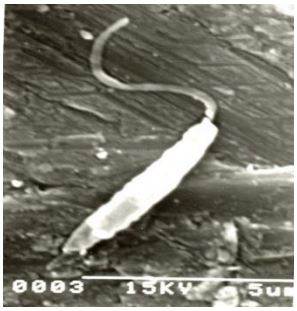
The non-irradiated parasites were spindle-shaped with elongated anteriorly attached distinct flagellum and rough contour. Membrane folds were evident along the same longitudinal axis as the microtubules of the cytoskeleton. In addition, cells contained membrane indentations which resembled pores After exposure to gamma radiation the shape of cells became a more homogeneous mixture of spherical forms as gradually swelling at 10 and 20 krad radiation doses (Figs.1). A reduction in the depth of membrane folds of irradiated parasites paralleled the expansion of cells. At 30, 40 and 50 krad radiation doses, morphological alterations occurred which lead to the formation of amastogote-like organisms (Fig.1). The shortening and almost

complete disappearance of flagellum, as well as a transformation from long, slender, motile cells into nonmotile, ellipsoidal organisms were observed.

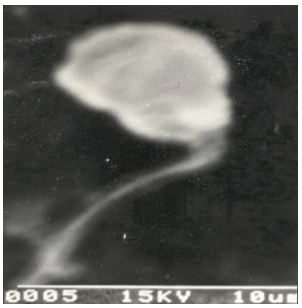
Fig. 1: Scanning Electron Micrograph



Normal promastigote

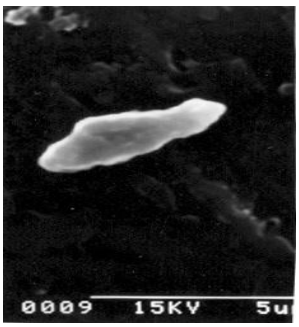



10krad irradiated

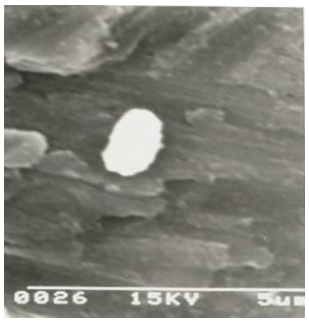



20krad irradiated


30krad irradiated




40krad irradiated




50krad irradiated

Transmission Electron Microscopic Study

A. Non irradiated parasite

The motile cylindrical promastigote was upto 18 µm long with a single flagellum. Flagellum was surrounded by a plasma membrane, arising from a basal body lying close to the kinetoplast was noted at the anterior end of the parasite. The flagellar pocket with desmosome-like plaques was not distended and enclosed the flagellum loosely. The vesicular nucleus was elliptical in shape in cross section. A relatively rod shaped, deeply stained kinetoplast was located at the anterior end of the organism near the point of emergence of the flagellum. The granular cytoplasm with ribosomal particles distributed throughout the cell. Two subcellular organelles, the glycosomes and polyphosphate containing bodies were found. The paraxial rod has the appearance of a cross hatched paracrystalline structure with nine pairs of peripheral axonemal doublets encircling a central pair. The mitochondrion appeared as tubular form. The cytoplasm was granular and homogeneous throughout the cell (Fig .2).

B. Irradiated parasite

10 krad

In this case, mostly the irradiated parasites were somewhat stumpy but virtually all had a conspicuous kinetoplast. Flagella remains attached (Fig.2). Other changes were associated with dilation of the flagellar pocket, enlargement of cytoplasmic bodies (multivesiculate bodies and lipid droplets) and a decrease in ribosomal content of the cells. When parasites were exposed with 10 krad gamma radiation, damage of the cell organelles were not so prominent. Flagellar pocket was usually distended and the distance between kinetoplast and this pocket had been increased. Membrane damage was not so significantly visible and structural organization remain more or less unaltered. The distinct kinetoplast had been found to send off long mitochondrial branches deep into the cytoplasm. Nucleus with distinct nuclear membrane and heterochromatin was also observed.

20 krad

On exposure to 20 krad radiation doses the promastigotes tended to be aflagellate, sausage-shaped and mostly immotile (Fig.2). Mitochondria appeared swollen. The double membrane surrounding the kinetoplast became poorly resolved in these treated cells and increased volume fraction occupied by kinetoplast was also evident. Some parasite displayed short flagella that were most often restricted to the flagellar pocket. The flagellar pocket was distended. The cytoplasm was not so dense as in the normal cell. The cytoplasm contained lipid droplets and frequently large electron dense inclusions. Dilated nucleus and scattered chromatin organization were found.

30 krad

Parasites exposed to 30 krad remain large, stunted and except for the appearance of lipoidal droplets in an occasional parasite there was little further evidence of any significant cell activity other than obvious deterioration. More ovoid forms appeared (Fig. 2). Mitochondria broke into two parts, extended throughout the periphery and appeared as elongated bulbous structure which is. related to autolytic processes. The kinetoplast lost its shape and the DNA material acquired less compact appearance. Small vacuoles were visible in the fine granular cytoplasm. The flagellar pocket as well as desmosome-like plaques disappeared. Disorganization of nucleus, nuclear membrane, dispersion of heterochromatin material was evident.

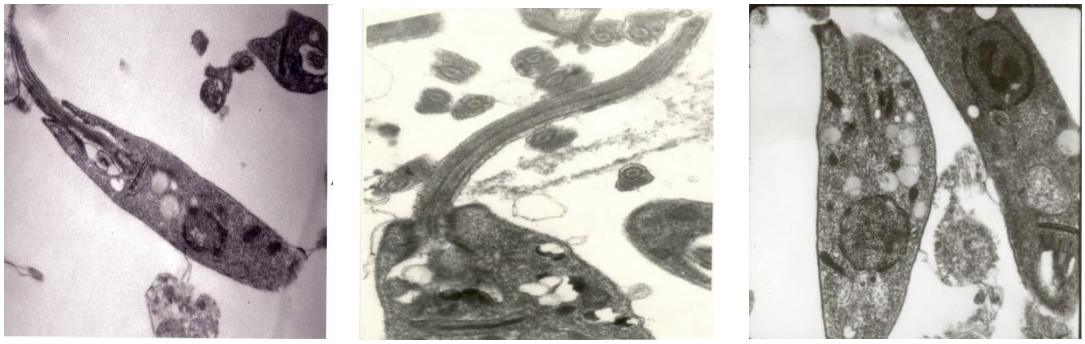
40 krad

Many have to undergo a kind of abortive development, some parasites reached the stage characterized by an opaqueness of protoplasm and the presence of much lipid inclusions before an extensive vacuolization, shrinkage away from the plasma membrane (Fig 2). The parasite was continued to increase greatly in size. Dyskinetoplastic situation occurred, that is kinetoplast was impaired but still present. Definite nuclear structure disappeared. Significantly, some cells contained megasome-like structure which were identified by their electron density. Parasites displayed large vacuoles which appeared to fuse with each other, as well as with the distended flagellar pocket.

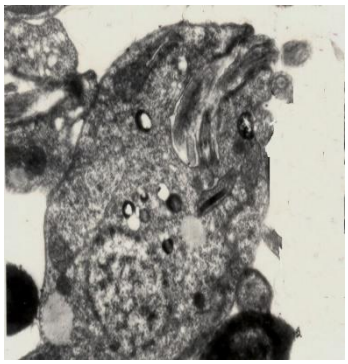
50 krad

The promastigotes rounded up and lost their motility because the flagellum with flagellar pocket had been desolated. The cells were scarcely larger than the normal, while lipoidal granules continued to persist in a deteriorated and patchy protoplasmic mass. The kinetoplast was changed almost beyond recognition (Fig. 2). Numerous large lipid like volutin bodies were evident throughout the cell. The assembly of mitochondria seemed to involve changes linked to the development of membranous whorl formation, as well as the transformation of such structures into lysosomal bodies which eventually could be converted in myelin like figures and undergone autodigestion. Some flattened sacs with rough surfaced membrane filled with unidentified material were also evident. The plasma membrane of many of the parasites exposed at this dose developed a kind of bleb.

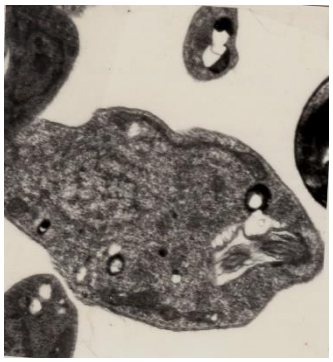
Fig.2: Transmission Electron Micrograph



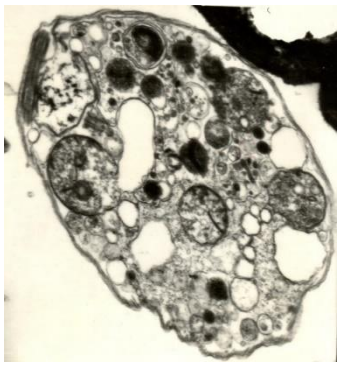
Normal promastigote 10krad irradiated 20krad irradiated



30krad irradiated



40krad irradiated



50krad irradiated

Morphometric Analysis

After exposed to gamma radiation at different doses, the normal promastigote transformed to amastigote-like form and there was an increase in the absolute cellular volume from 30.43 $\mu m^3$ in the nonirradiated promastigote to 33.23 $\mu m^3$ (average) in the irradiated promastigote. The relative volumes occupied by the nucleus were 8.72%, 8.8%, 6.32%, 10.5%, 9.5% and 3.8% in nonirradiated and irradiated promastigotes at 10, 20, 30, 40, 50 krad radiation doses respectively (Table 1). Nucleus showed a larger relative volume in 30 krad irradiated promastigotes. The relative volumes of kinetoplast were 3.13%, 3.8%, 3.8%, 4.23%, 2.02%, 1.02% in nonirradiated and irradiated promastigotes at 10, 20, 30, 40, 50 krad radiation doses respectively. In contrast, the relative volume of the kinetoplast does not appear to differ so much. Most other organelles, including flagellar pocket, mitochondria to varying degrees, showed not much larger relative volume in irradiated promastigotes when compared to nonirradiated promastigotes. The relative volumes of flagellar pocket occupied were 5%, 6.5%, 7.92%, 8.1%, 5.37% and 1.7% in normal and irradiated promastigote at 10, 20, 30, 40 and 50 krad radiation doses respectively. It was evident that flagellar pocket was much dilated in case of promastigote irradiated at 20 and 30 krad radiation doses.

**Table 1 :** Morphometric Analysis (Relative volume percentages)

| Radiation doses | Nucleus | Kinetoplast | Mitochondria | Flagellar pocket |
|---|---|---|---|---|
| Non irradiated | 8.72 ± 1.98 | 3.13 ± 0.5 | 7.09 ± 0.5 | 5 ± 0.25 |
| 10 krad | 8.8 ± 0.11 | 3.8 ± 1.1 | 6.85 ± 0.3 | 6.5 ± 0.3 |
| 20 krad | 6.32 ± 0.13 | 3.8 ± 0.5 | 5.75 ± 1 | 7.92 ± 0.5 |
| 30 krad | 10.5 ± 1.5 | 4.23 ± 1.2 | 5.06 ± 0.4 | 8.1 ± 0.9 |
| 40 krad | 9.543±0.89 | 2.02 ± 0.5 | 5.2 ± 0.1 | 5.37 ± 1.1 |
| 50 krad | 3.81 ± 0.85 | 1.02 ± 0.2 | 5.73 ± 1.2 | 1.7 ± 1.1 |

**4. Conclusion**

Axenic, amastigote-like cells are advantageous in that they can be used as an in vitro model to study parasite development that occurs *in vivo*.

The ultrastructure of nonirradiated *L. donovani* in the present study is similar with the earlier observations [13,14]. It included several features such as trilaminate cell membrane, pellicular microtubules, structure of the flagellum with 9+2 arrangement of axoneme, desmosome, kinetoplast in a dilation of the mitochondrial tube. The reduction in the depth of membrane folds paralleled with the formation of amastigote-like form was evident from the scanning electron micrograph. In irradiated promastigote, membrane became more pronounced as cells shriveled along the longitudinal axis of their microtubule cytoskeleton. Since normal promastigotes are motile due to the active motion of a long flagellum, the paraxial rod along the axoneme may be important for the vigorous flagellar motility of the organisms which are in agreement with the observation of Pan and Pan (1986). The desmosome-like plaques acted as a sphincter for closing off the

flagellar pocket [15]. Their formation was believed to maintain the integrity of the flagellum and parasite body as a motile unit.

Higher radiation doses caused condensation of the *L. donovani* and morphologic changes such as shrinkage of cytoplasm, dispersion of nuclear and kinetoplast material into an amorphous mass, ballooning of the membrane, loss of flagellum, aggregation, vacuolation and progressive loss of motility leading to eventual death and degradation. These changes were observed in a large percentage of the parasites and appeared earlier when high doses (30-50 krad) were applied. Above this dose, the paraxial rod of flagellum had been destroyed and the organisms remained static. The physiological regeneration with the condensation of the nucleus and reorganization of some of the organelles was induced by 20 krad doses and these irradiated promastigotes regenerated seemingly at the same rate as nonirradiated. In the groups exposed above 30-50 krad particularly the changes occurred with nearly explosive rapidity and produce an almost endless variety of morphological effects such that it was virtually impossible to find any consistent pattern or sequence to the changes which occurred. Mitochondria broke into two parts which was related to autolytic processes [16]. Numerous small vacuoles were distributed at random throughout the cell, flagella detached and desmosome-like plaques disappeared. The promostigotes irradiated at 30 krad, resembled true amastigotes in morphology and ultrastructure. The dyskinetoplastic situation occurred above 40 krad radiation doses. It had been postulated that kinetoplast was a self dependent DNA containing orgenelle, found in flagellates of the families Trypanosomatidae, was responsible in the morphogenesis of mitochondria and essential for the transformation occurring from one stage to another in the complex life cycles of these parasites[17]. Support for the latter statement comes from the present study that dyskinetoplastic *Leishmania donovani*, though able to live, cannot be propagated *in vitro*. It is obvious, therefore, that above 40 krad radiation doses practically all the developmental processes of the parasite had been effectively arrested. Above 40 krad irradiated parasite the cytoplasm became altered (as shown in electron micrograph), then visibly vacuolated and finally became myelinated. As the myelination occurred the entire cell disintegrated. Doses much less than those immediately lethal stopped cell division or retarded it and caused loss of kinetoplast. It was evident that much lower doses were needed to suppress division (to transform or kill ultimately) than for immediate death. The results were consistent with the observation on sporogenous cycle of *Plasmodium gallinaceum* [18].Terzian demonstrated that parasites exposed to 5, 10, 20 and 30 krad showed the characteristic vacuole formation. The surface membrane of parasites exposed to 30 krad developed a kind of bleb and remained stunted. Parasites exposed above 20 krad never reached maturity and characteristic lipoidal droplets appreared in large numbers. Some observer observed the agglutination of trypanosomes exposed to gamma radiation and morphologic changes such as shrinkage of cytoplasm, condensation of nuclear and kinetoplast material into an amrophous mass, ballooning of the membrane and loss of flagellum [19].

After exposed to gamma radiation, the absolute cellular volume of normal promastigotes increased from 30.43 $\mu m^3$ to 33.23 $\mu m^3$ in the irradiated promastigotes. It was evident in the present study, that the volume percentage of flagellar pocket of 30 krad irradiated promastigote occupied was increased in comparison to other radiation doses. Therefore, the flagellar distension was proved with respective higher radiation doses. Similar previous observation is lacking. From this experiment it was successful to bring the radio attenuated condition of *Leishmania donovani* which could be developed into vaccine candidates and useful as immunological markers of protection in both animal models and humans and to draw up a general framework for development of live attenuated *Leishmania* vaccines. This study would give some valuable information of attenuation dose at 10-20 krad which can be utilised in future study as a vaccine tool.

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