VISUALIZATION ON THE EFFECT OF CHLORHEXIDINE GLUCONATE, A BIOCIDE ON *ACANTHAMOEBA* SP BY ELECTRON MICROSCOPY

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ABSTRACT

Acanthamoeba, an amoeba genus belonging to free-living amoebae has been reported to cause eye keratitis in man both in contact lens and non-contact lens users. For treatment of this disease, chlorhexidine gluconate (CHX) is effective especially at early stage of infection. In this study, this biocide was exposed on *Acanthamoeba* for 72 h to examine its effect on the cell morphology by scanning (SEM) and transmission electron microscopy (TEM). By SEM, control *Acanthamoeba* cells showed numerous acanthapodia structure on their surface. These structures however, were significantly reduced in CHX-treated *Acanthamoeba.* By TEM, the amoeba nucleus and nucleolus were intact and prominent, while the cristae of mitochondria structure and endoplasmic reticulum are distinctive in untreated *Acanthamoeba* compared to CHX-treated *Acanthamoeba.* In the later amoeba, degradation of nucleolar structure, abnormal shape of mitochondria and fragmentation of endoplasmic reticulum were observed. Under SEM and TEM, ultrastructural changes occurred in *Acanthamoeba* cells after exposure to CHX can be visualized in details.

Keywords: *Acanthamoeba* sp., chlorhexidine gluconate (CHX), scanning electron microscopy, transmission electron microscopy.

INTRODUCTION

Acanthamoeba spp are opportunistic protozoan parasites that are distributed in diverse environments including chlorinated swimming pools and sewage. They also have been isolated from vegetables, fish, reptiles, birds and mammals and are known to be one of the most ubiquitous organisms [1]. Although *Acanthamoeba* exist primarily as free-living this amoeba could infect the eye, brain and skin, and can spread haemotogenously to the central nervous system (CNS) and various organs. Eye infection elicited by an *Acanthamoeba* sp. is potential to produce a progressive sight threatening, *Acanthamoeba* keratitis. The adhesion of *Acanthamoeba* trophozoites to the corneal epithelium is a crucial step in pathogenesis [2,3,4] and had caused millions cases of blindness and thus became important ocular pathogens particularly in contact lense wearers [5].

In many *Acanthamoeba* keratitis cases, chlorhexidine gluconate (CHX) has been recommended for treatment [6]. In addition, Noble [7] indicated that CHX is very effective as anti-Acanthamoeba agent when analysed by flow cytometry technique. The objective of the present study was to observe the effect of CHX on *Acanthamoeba* at its ultrastructural level. Details changes on morphological of *Acanthamoeba* were observed by scanning (SEM) and transmission electron microscopy (TEM) resulted from amoeba exposure to CHX. Under SEM, the effect of CHX on the amoeba surface such as acanthapodia structure, cell's shape and size as well as formation of sunken food cups or amoebastomes could be revealed in detail. While by TEM, the ultra structural changes in the cytoplasm and organelles like the mitochondria, nucleus and lipid droplet structures could be observed to facilitate understanding and interpretation on the mechanisms of action of the CHX on the *Acanthamoeba* cell morphology.

MATERIALS AND METHOD

Observations under Scanning Electron Microscopy

Trophozoites of *Acanthamoeba* were treated with its IC_{50} value, 0.97 μM on cover slips in 6-well plates containing 3 mL culture medium with 10^4 cells/ml for 72h at 30 °C. After incubation, the culture medium was replaced and fixed with warm

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4% glutaraldehyde in phosphate buffered saline (PBS) for 30 minutes. The amoebae on cover slips were then post fixed in 1% osmium tetraoxide at room temperature for 90 minutes. After post fixation, the cover slips containing amoeba cells were rinsed with PBS buffer twice and continued with dehydration process using a graded series of ethanol from 10% to 100%. The specimens were rapidly transferred to critical-point drying device (BAL-TEC 030) in liquid carbon dioxide. After critical point drying, the cover slips were attached to 13 mm aluminum stub ducting paint, coated with gold (JEOL JFC-1600 Auto Fine Coater, ion-sputtering device), and examined under scanning electron microscope (JEOL JSM- 6360LA, Analytical SEM).

Observations under Transmission Electron Microscopy

Both CHX-treated and untreated *Acanthamoeba,* were first collected by centrifugation at 1000 rpm for 10 minutes and the pellets were transferred into 1.5 mL tubes. The samples were resuspended and fixed with Karnovsky fixative [8] for 4-6 hours. After fixation, the samples were recentrifuged and the fixatives were discarded. The pellet was then resuspend in one to two drops of cow serum and allow the serum to clot. Following that, the tubes again were filled with Karnovsky fixatives (5% glutaraldehyde with 4% formaldehyde) and were kept in the refrigerator overnight. On the next day, the fixed clotted serum containing amoeba samples were cut into a number of 1 mm^3 slices and were transferred into glass vials. The samples were fixed again with the fixatives for 2 hours and were washed three times with 0.1 M cacodylate buffer for 10 minutes each. The specimens were then post fixed with 1% buffered osmium tetraoxide for 2 hours at 4 °C and were washed again three times with 0.1 M cacodylate buffer for 10 minutes each. The specimens then were dehydrated in a series of ethanol (20% to 100%), infiltrated with resin finally embedded at 60 °C for 48 hours in a standard manner. Followed by thick sectioning, the specimens were stained with toluedine blue. Silver sections were 50 nm in thickness were obtained and stained with uranyl acetate and lead for 10 minutes each. All specimens sections were examined using TEM (Hitachi H-7100, Japan) operated at 75 kV.

RESULTS AND DISCUSSION

***Scanning Electron Microscopy observation on Chlorhexidine treated Acanthamoeba* sp.**

Scanning electron microscopy images of untreated trophozoites of *Acanthamoeba* sp. used in the this study showed the characteristics of healthy cells with prominent and numerous needle-like structures of acanthapodia. Acanthpodia is important in adhesion to surface, cellular movement or capturing prey (Khan, 2008). This structure is the main criterion for genus *Acanthamoeba* which is only observed during trophozoites stage (Fig. 1).

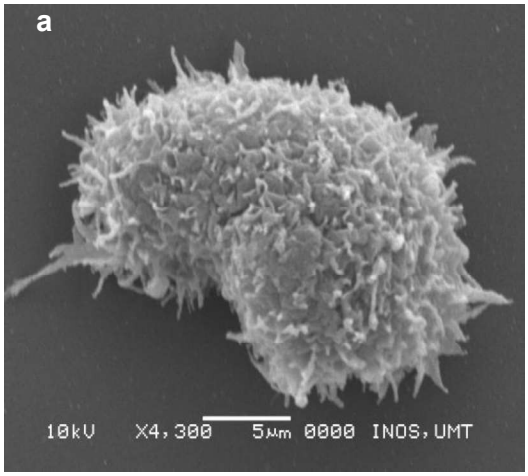

Fig. 1. Scanning electron micrographs of untreated trophozoites of *Acanthamoeba* sp. Irregular cell shape and numerous of acanthapodia structure could be seen on the cell surface of untreated *Acanthamoeba*.

Seventy-two hours treatment with CHX showed significant changes to the *Acanthamoeba* cells. Collectively, the trophozoites became reduced in size as well as cystic cell appearance; sunken food cups including loss of acanthapodia and wrinkle on the cells' surface (Fig.2).

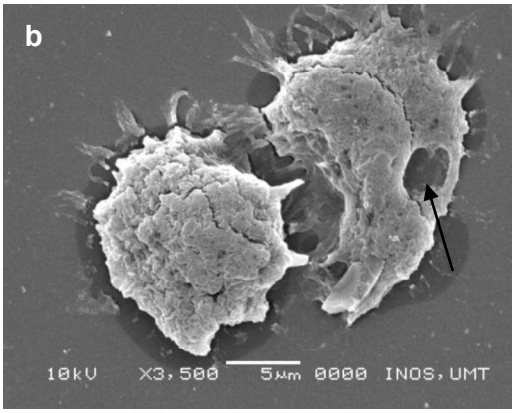

Fig. 2. CHX-treated *Acanthamoeba* sp. showed damage on acanthapodia structure and cell surface and sunken food cup were observed (arrow).

In this study, acanthapodia that arise from the surface of untreated *Acanthamoeba* trophozoites are mainly for cellular movement since all isolates of *Acanthamoeba* were grown axenically or in the absence of external live food organisms. Crawling-like movement or amoeboid movement as being described by Khan [9] accompanied by protrusions of the acanthapodia. The extension of the acanthapodia is accomplished by endoplasmic fluid forward streaming in the direction of the extension which coagulate into the ectoplasm at the tip of the acanthapodia structure.

Observation under SEM for CHX-treated amoeba collectively showed that the trophozoites became reduce in size, cystic cell appearance, and sunken food cups, loss of acanthapodia structure as well as wrinkle on the upper side of cells surface. In addition, thickened, broaden and elongated acanthapodia, attached to the surface of the substratum tightly were also observed. This observation provides convincing evidence that CHX generates alterations on the *Acanthamoeba* morphology thus initiates the encystment process in *Acanthamoeba* cells.

TEM observation for changes in Chlorhexidine - treated Acanthamoeba spp.

In order to understand the mechanism of action of CHX on *Acanthamoeba* sp. a study was also carried out to determine the amoeba changes at ultrastructure level by transmission electron microscopy (TEM). Results from this study revealed distinct changes occurred mainly on the nuclear, mitochondrial and lipid droplets structures of the trophozoites following treatments with the CHX. Untreated trophozoites seem to possess single nucleus with one large nucleolus, numerous of mitochondria structure with distinctive cristae, spherical lipid droplets and extensive network of endoplasmic reticulum with ribosomes bound on the cisternae surface forming stacks. Several contractile vacuoles were also observed (Fig. 3.).

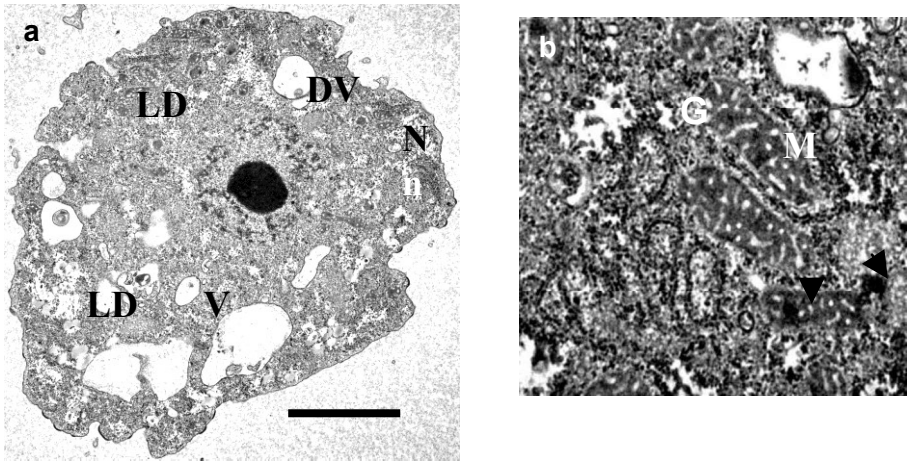

Fig. 3. Transmission electron micrographs of untreated *Acanthamoeba* sp. Cross section of healthy *Acanthamoeba* trophozoite displayed single nucleus (N) with intact and prominent nucleolus (n). Numerous lipid droplets (LD), mitochondria (M) structure and several contractile vacuoles (V) were also observed (a). Bar: 2 µm. Mitochondria (M) of trophozoite comprising dense matrix and distinctive cristae; partly surrounded largely aggregates of cisternae stacks of rough endoplasmic reticulum (ER). Electron-dense material indicated by arrowheads (b). Bar: 0.5 µm. (V: contractile vacuole, DV: digestive vacuole).

TEM observation in the present study showed that nucleoplasm of untreated *Acanthamoeba* sp. contained random distribution of electron-dense material bound by bilayer nuclear envelope which separates it from the cytoplasm. This electron-dense material might be corresponding to the interphase stage and dividing cells. Similar observations indicating interphase stage of *Acanthamoeba* cells also could be seen in trophozoite of *A. palestinensis*, *A. castellanii* (Neff strain) [10] and *Paradermamoeba levis* [11]. Mitochondrial profiles of untreated trophozoites of *Acanthamoeba* observed were abundant and appeared in various shapes; spherical (~ 0.25 µm in diameter), ovoid (~ 0.5 x 0.7 µm) or elongated (~ 0.5 x 1.5 µm). The mitochondrial matrix was dense with distinctive cristae. In the cytoplasm of eukaryotic cells, electron transport chain and mitochondria is responsible for Krebs cycle, therefore the cell's energy production required for metabolic activities involved in the feeding, movement, reproduction as well as regulation of cell death. Untreated *Acanthamoeba* trophozoites exhibited several contractile vacuoles, membrane-surrounded cavity that periodically expanded, filled with water and contracted to expel its contents to the cell's exterior. Contractile vacuole is important for osmoregulation and excretion and usually no precipitates observed.

In CHX-treated *Acanthamoeba* collectively showed the ultrastructural changes occurred mainly on the mitochondria cristae structure and their shapes, degradation of nucleus, leakage of lipid droplets as well as ruptured endoplasmic reticulum (Fig. 4).

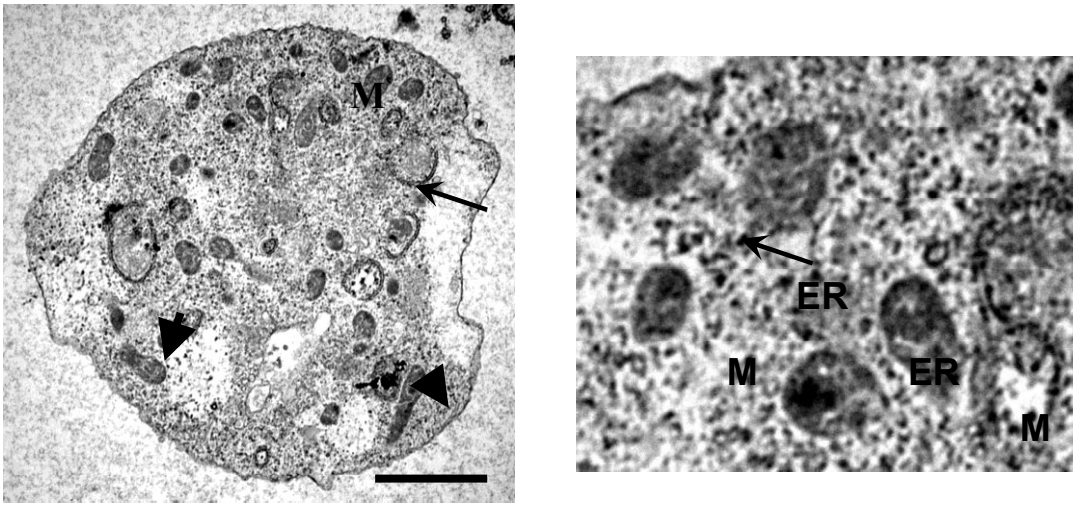

Fig.4. Transmission electron micrographs of *Acanthamoeba* sp. treated with CHX. Rounded-shaped of cell with several mitochondria structure. Mitochondria surrounded by endoplasmic reticulum network might involve in autophagy process indicated by arrow. Bar: 2 µm (a). Smaller shaped of mitochondria (M) with aggregation of mitochondrial cristae matrix while electron- dense granules indicated by arrowheads Bar: 500 nm (b). Ruptured endoplasmic reticulum (ER) surrounding mitochondria. Bar: 500 nm.

Observation by TEM displayed electron-dense concretion area that were frequently developed in the centre of untreated and treated *Acanthamoeba* mitochondria observed did not indicate the respond of the mitochondria due to the biocide. Bowers and Korn [12] described the formation as an inorganic material in cyst of *A. castellanii* (Neff strain) after they had earlier identified the same structure in *A. castellanii* (Neff strain) trophozoite mitochondria as paracrystalline structure [13]. The structure occurred when the embedding medium failed to penetrate the concretion and therefore drops out of the section. This electron-dense area also has been reported in wild-strain *A. castellanii* [14].

Alterations and disintegration of the mitochondrial cristae into complex cristae, as seen in this study suggested that the alterations were likely to be a direct result a pathway of mitochondrial damage, and perhaps mitochondrion is one of the targeted organelles.

The arrangements may be an adaptive attempt to maintain the energy-generating mechanism of the *Acanthamoeba* cell in response to mitochondrial stress caused by treatment with CHX. Disintegration of mitochondrial cristae into complex arrangements were also observed in the *Acanthamoeba* sp. Similar disintegration of mitochondrial cristae also has been reported in macrolide antibiotics-treated *A. castellanii* [13] and vegetative cells of ciliates, *Colpoda stenii* and *Cyrtolophosis elongate* after exposure to cadmium and zinc [14].

Endoplasmic reticulum (ER) is an important site for synthesis, folding, modification and trafficking of secretory and cell-surface proteins. The CHX-treated trophozoites displayed changes in ER distribution and structure into smaller and ruptured of the cisternae into smaller aggregates within the cytoplasm. Bower and Korns [15] also showed similar observations of ruptured ER in encysting amoeba. As a major intracellular calcium storage compartment, ER played a critical role towards maintenance of cellular calcium homeostasis [16]. This structure however is not often observed in treated *Acanthamoeba* due to the difficulty in distinguishing it among the aggregated small vesicular components in the cytoplasm. Ruptured ER stacks however were clearly observed in *Acanthamoeba* sp. after treatment with CHX. Other important changes observed after treatment with CHX were destruction of the amoeba cytoplasm and deformation of organelles. The destruction or degradation of cytoplasm resulting from the precipitation and depositions occurred randomly throughout the cells cytoplasm. According to Russel [17], CHX is able to cross the cell wall or outer membrane by passive diffusion, presumably, and subsequently charge the bacterial cytoplasmic or inner membrane or the yeast plasma membrane. Later, delicate semi permeable membrane leaked and released the intracellular constituents. Leaking of the membrane resulted in the death of the cell.

Membrane changes related to Ca^{2+} influx appeared among the many possible "critical changes" that compromised cell integrity. CHX is 'membrane-acting' cationic biocides. Trophozoites treated with CHX showed the disruption of plasma membranes followed by rapid release of cytoplasmic constituents, which leads to the death of the *Acanthamoeba*. At alkaline pH, negatively charged surface proteins of *Acanthamoeba* interacting rapidly with those cationic biocides inducing structural and permeability changes, cell membrane leading to the leakage of ions, water and other cytoplasmic components resulting in cellular damage [18]. These findings indicated that CHX affected mainly on plasma membrane but lesser also towards organellar membrane such as nucleus, mitochondria, endoplasmic reticulum and lipid droplets (Fig. 4). Leakage of cytoplasmic membrane was observed after treatment with CHX. The cytoplasmic membrane of *Acanthamoeba* is unusual in the presence of lipophosphonoglycan, which is absent in mammalian cells [15], with sugar exposed on both side of the membrane [19]. According to Xu *et al.* [20], changes on function or structure change of plasma membrane might also lead to the cell death.

CONCLUSION

Results in the present study demonstrated that investigation by scanning and transmission electron microscopy revealed distinctive changes occurred at the outer part of *Acanthamoeba* cells after treatment with CHX. By SEM, the alterations and changes observed were mainly on the surface morphology involving surface of cell membrane and acanthapodia structure. However, observations by TEM revealed the changes occurred at the inner part of *Acanthamoeba* cells at ultrastructural level. The noticeable changes were on the nuclear structure, mitochondria, lipid droplets and endoplasmic reticulum complex.

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