Povidone-Iodine Induced Changes in the Cyst of *Acanthamoeba* spp.: Transmission Electron Microscopic Study

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ABSTRACT

Objective: This study was to investigate morphological changes and fine structures of the cyst form of *Acanthamoeba* after treatment with various concentrations of povidone-iodine (Betadine™) using transmission electron microscopy.

Methods: *Acanthamoeba* spp. were isolated from patients with amebic keratitis and obtained from the cultures on 3% non-nutrient agar (NNA) plates seeded with heat killed *Escherichia coli* (NNA-*E. coli*) with incubation at 30°C for 7 days. The cysts were harvested and washed in ameba saline solution and adjusted to a final concentration of 10^4 cysts/ml. Various concentrations of povidone-iodine were put in the microtiter plate wells. The minimum cysticidal concentration was the lowest concentration that there was no excystment after 1 week of incubation. The cysts were prepared for routine transmission electron microscopy to determine the structural and organelle damages.

Results: Structural damages were observed in the cysts treated with povidone-iodine of 0.04% dilution. Many cysts showed shrinkage of amoeba from the cyst wall and there was a slight withdrawal of the cytoplasm from the cyst wall. Many cysts were ruptured and broken into small pieces.

Conclusion: Structural damages were observed in the cysts treated with 0.04% dilution of povidone-iodine solution or more than that. The damage started with pores produced in the cyst wall and the loss of water, shrinkage and loss of the cytoplasm of the inside cell from the cyst wall, followed by breaking of the cyst wall and the inside cell into small pieces.

Keywords: Povidone-Iodine, *Acanthamoeba*, TEM

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Acanthamoeba spp. may be associated with severe eye infections in wearers of contact lenses. They are ubiquitous free-living amoebae[1], and are responsible for an uncommon yet increasingly diagnosed keratitis in humans which may be associated with contact lenses[2-5]. They exist principally in an active trophozoite form that feeds on bacteria and other microorganisms. When challenged by unfavourable conditions, the amoebae are able to transform into a highly resilient cyst form, from which the trophozoite may emerge when conditions are again favourable. This survival strategy has earned these species the rank of the most common free-living amoebae in soil and fresh water.

Acanthamoeba spp. have also been isolated from a variety of habitats, including domestic water supply, bottled mineral water, swimming pools, aquaria, air conditioning units, sewage and dust in the air.[6] In human, they have been found in the nose and throat of patients with respiratory illness as well as in healthy persons.[1] More recently, they have been isolated from contact lens care solution,[7] leading to the sudden upserge of *Acanthamoeba* keratitis in individuals wearing contact lenses.[8-9] This rare condition becomes of great interest because of the nature of the disease and its resistance to treatment. Substantial pain and sight-threatening corneal changes characterize *Acanthamoeba* keratitis. Although treatments have evolved over the past several years, long course of treatment pain, and permanent and severe visual loss are still common features in many cases.

As a number of cases are increasing, the contact lens users clearly warrant attention and cautions because these free-living protozoa are more resistant to biological agents. The electron microscopic studies of the

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ultrastructure of the trophozoites and cysts have been undertaken[10]. It is also imperative that a study on the morphology of this parasite should be pursued after treatment with various concentrations of povidone-iodine (Betadine™) using transmission electron microscopy. It was hoped that Transmission Electron Microscopy (TEM) would provide additional information about the mechanisms of inactivation and relative responses as well as ultrastructural changes of the cyst form.

MATERIALS AND METHODS

Culturing technique: The cystic form of *Acanthamoeba* spp. was isolated from patients with amoebic keratitis which was further obtained from culture plates on 3% non-nutrient agar (NNA) seeded with heat killed *Escherichia coli* (NNA-*E. coli*). The incubation was at 30°C for 7 days. The cysts were harvested, washed in amoeba saline solution, and adjust to a final concentration of 10^4 cyst/ml before treatment with the povidone-iodine concentration range between 0.002-5%. The minimum cysticidal concentration was the lowest concentration that prevented the excystment after being recultured for a week.

Pharmaceutical agent: Povidone-iodine (PVP-I) or Betadine is a solution of polyvinyl pyrolidone (PVP) and 10% iodine. PVP-I is an antiseptic agent that has been proved to be effective against a wide spectrum of bacteria, yeasts, molds, and some viruses. It was recently used as a prophylaxis against neonatal conjunctivitis in prospective trials in developing countries instead of treatment with silver nitrate or erythromycin because it is less toxic and less expensive.[12]

Equal volumes (5 ml) of treated cells suspension were centrifuged at 500 g for 2 min. and washed with the amoeba saline solution 2 times. The pellets were fixed with 2% glutaraldehyde in 0.1 mol sodium cacodylate buffer pH 7.4 at room temperature for 1 hour. Then they were post-fixed with osmium tetroxide solution for one hour at room temperature. They were dehydrated with a graded series of ethanol and finally with three changes of absolute ethanol. The dehydrated cell pellets were transferred to propyrene oxide and infiltrated in a mixture of araldite resin and propylene oxide (1:1) for 24 hours at room temperature. The cells were then transferred into polystyrene capsules containing freshly prepared araldite resin. The capsules were incubated at 60°C for 48 hours, after which the resin become polymerized. The blocks were cut into 90 nm thin sections which were transferred on to the copper grid and were stained with uranyl acetate and lead citrate[11]. Then they were observed by transmission electron microscopy with a magnification range from 1,300 to 8,000 x.

RESULTS

Changes in the sizes of control (untreated) and ≥ 0.04% PVP-I treated cells which was the minimum cysticidal concentration were measured. The cysts showed no change in diameter and did not increase in size after treatment at the range of PVP-I of 0.002 – 0.035%. The untreated cyst (Fig 1, 2) had thick ridges over their entire surface, these might be interconnected to form a network of asymmetric patterns that appeared

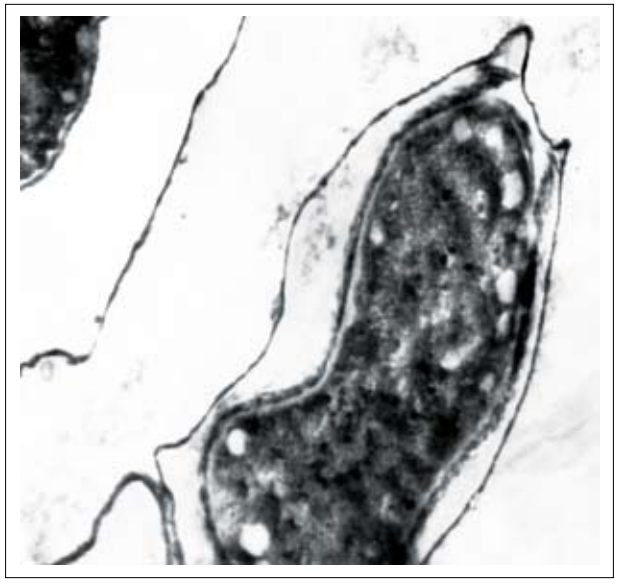

Fig 1. Transmission electron micrograph of the untreated cyst of *Acanthamoeba*.
O = Outer cyst wall I = Inner cyst wall
Op = Operculum

as craters. The cyst wall was wrinkled, giving a stellate appearance in some cysts. The cysts mostly are elongated and oval shaped about 5x12 µm in size and comp rise a double layered wall separated by about 1 µm space. At one end of the double wall, the inner layer disappears forming the operculum because the trophozoite is excysted. The outer layer is more electrondense and thinner than the inner layer and looks like the cellulose of the plant cell wall. The inner layer is thicker, more irregular and comprises numerous densely packed fibers. In the untreated cyst, the cyst wall is continuous throughout without any pore or opening. The weakest point appears to be the operculum because there is no inner layer where the excystment takes place. The inside cell is surrounded by the plasma membrane, the cytoplasm which comprises of many cytoorganelles such as the nucleus, mitochondria and vacuoles. The vacuolated organelles situate around the peripheral of the cell and are reported to be the contractile vacuoles.

PVP-I was tested at various concentrations to determine whether any changes occurred in the structures of

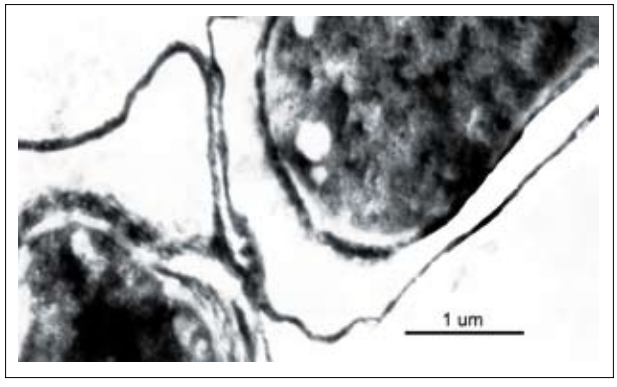

Fig 2. Transmission electron micrograph of the untreated cyst of *Acanthamoeba*.
O = Outer cyst wall I = Inner cyst wall
Op = Operculum

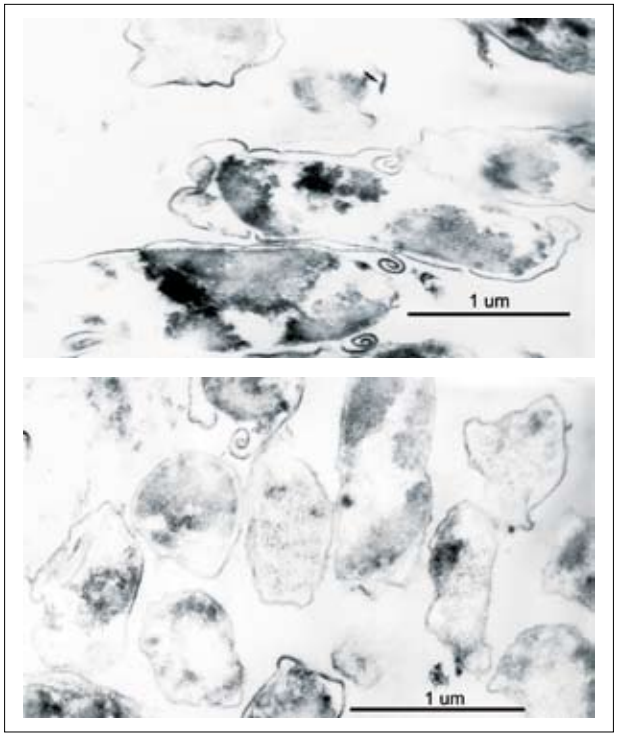

Fig 3,4. Transmission electron micrograph of the treated cyst of *Acanthamoeba* with PVP-I 0.04%. The cysts were broken into small pieces of 0.5−1 μm. The membrane-like structure covers the broken elements.

the cysts. Cysts treated with lower concentration than 0.04% showed no change and appeared normal. Near the concentration of 0.04% in some cells, shrinkage of amoeba from the cyst wall was apparent. This appearance occurred in all cysts at high PVP-I concentration (≥ 0.04%). Some cysts had lost electron-dense material. There was a slight withdrawal of the cytoplasm from the cyst wall in a majority of the cysts at the beginning. This may be because of the pores produced in the cyst wall. Some globules and dense material still remained within the cells. A few cysts treated with less than 0.04% PVP-I showed little or no structural damage. The treated cysts of more than 0.04% showed severe damage of the cyst wall and the inside cell. The cysts were ruptured and broken into small pieces. The structures of the broken cysts are very small elements of about 0.5 x 1 μm. Each element com prises a thin discontinuous wall which is the membrane like structure and the unclassified cytoplasmic mass remains inside.

DISCUSSION

Acanthamoeba spp. are frequently implicated in eye infections in contact lens users. Of the two cell forms, cysts are more resistant to antimicrobial agents used in contact lens solutions. The mechanisms of this resistance are poorly understood and the present study was undertaken to explain the mechanism of destroying the cyst by PVP-I by TEM.

Lower concentration or inadequate concentration of the PVP-I caused less structural and membrane damage because the cyst wall is an excellent barrier to the drug to protect the inside cell. A higher concentration of PVP-I effects the integrity and intercellular coagulation and the coagulation of proteins and nucleic acids of the cyst. The PVP-I solution of high concentration passes through the pores on the cyst wall and induces membrane phospholipid phase separation of blisters with clusters of dense precipitates on cell surface and cytoplasmic precipitation and rupture of the cell membrane of the inside cell.

Swelling of the cyst wall occured after exposure of cysts to a high concentration of more than 0.04% of PVP-I. It is possible that the inner cyst wall, which contains cellulose, may be affected by PVP-I. The shrinkage of the cytoplasmic membrane of the inside cell from the cyst wall after cell treatment with PVP-I was similar to the effect on yeast cells.[13] The treated cyst wall may be discontinuous and the inner cell uptakes more and more PVP-I. This makes the plasma membrane of the inner cell rupture which is followed by the rupture of the cyst wall into small pieces.

CONCLUSION

In conclusion, the primary target site of PVP-I appears to be the plasma membrane after treatment with a high concentration of PVP-I. The concentration of PVP-I more than 0.04% affected on the viability, and leakage of intracellular constituents of the cyst. The cyst wall must act as a major barrier in limiting the uptake of the biocides to their underlying target site. The cyst wall was wrinkled, giving the cyst a stellate appearance in sections. There are 2 layers of cyst wall which look like the cellulose of plant cell wall, the inner layer lacked the operculum through which excystation takes place which are clearly discontinuous in the inner wall structure. The cytoplasm is dense, and a lot of contractile vacuoles are observed. The treated cysts are broken into small pieces and the cyst wall loses its continuity. The PVP-I may produce pores in the cyst wall and rupture the plasma membrane of the cell inside and rupture of the cyst wall in the end.

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