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Structural and Morphological Alterations of *Candida albicans* Cells after Treatment with Atratoxin B_1 from *Holothuria atra* (Jaeger)

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Abstract – Atratoxin B_1 which was found to inhibit the growth of *Candida albicans* caused structural and morphological alteration of the cells. Increased accumulation of vesicles and membranous bodies in the cytoplasm, and alterations of the cell membrane and cell wall were most obvious. Sequential lytic events of the cells eventually resulted in complete disintegration of the cytoplasmic structures. These results suggested that atratoxin B_1 functioned by either blocking the biosynthetic step during cell wall synthesis, altering cell wall metabolism or dissolution of the cell organelles. These changes caused a progressive destruction of the cell wall leading to cell lysis.

Key words – Structural alterations, morphological alteration, *Candida albicans*, atratoxin B_1, *Holothuria atra* (Jaeger).

Introduction

Natural products from marine organisms has long been an important group of substances used as pharmaceutical compounds. Like most tropical countries, Malaysian waters are highly diversified with marine organisms which differ from those present in the temperate waters. The association between various organisms as defence mechanisms and predation, can be seen in the forms of morphological and chemical manifestations. One of the obvious chemical methods is the secretion of toxic compounds with the role to reduce the competition among the dwellers in a specific ecosystems. These compounds affected the physiological conditions of the targeted preys and can also be fatal. It is well known that marine invertebrates such as holothuroids, jelly fish, anemones, sponges and nudibranches, have been reported to exhibit the ability to secrete compounds which will act as defence mechanism or to maintain a stable association in their respective niches. These extracellular compounds which were found to be toxic to even higher animals can be exploited as potential drugs against pathogenic microorganisms, cancerous cells or as physiologically active compounds in animal systems. We have reported findings on the studies of extracts from a local sea cucumber, *Holothuria atra* (Jaeger) as antifungal agents (Ibrahim and Khaw, 1990; Darah *et al.*, 1991; Darah and Ibrahim, 1992; Darah *et al.*, 1994). *H. atra* (Jaeger) is an abundant species in Malaysian waters and it is of little interest to the local communities compared to the other species, *Stichopus variegatus*, which is traditionally known to have medicinal value. *In vitro* investigations demonstrated an intracellular extract of *Holothuria atra*, known as atratoxin B_1, which was extracetd from the tissues of the

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cell wall of the organism, exhibited significant activities against various species of dermatophytes (Darah *et al.*, 1995). However, these toxins did not show any significant effect against bacteria (Ibrahim and Khaw, 1990). It has been shown by microscopic observation that the mode of action of atratoxin B_1 on fungi was by disfunctioning the cell wall. The binding of the toxin to the sterol component of the cell wall also resulted in the deformation of the cell wall. The investigation on the effects of atratoxin B_1 on yeast cells, has not been fully carried out. We initiated the study by performing experiments to see the effect of atratoxins on yeasts. Special emphasis was given to the studies on the morphological and ultrastructural alterations of *Candida albicans* with the objectives to provide evidence for any specific alterations in the cell structure. Based on these observations, the mode of action of the extract on to the yeast cells was elucidated.

Experimental

Preparation of the atratoxin from *Holothuria atra* (Jaeger) – *H. atra* of various sizes (10~30 cm long) were obtained from several locations, consisting of Kendi Island and Muka Head, Penang and Song Song Island, Kedah, West Malaysia. The atratoxins from *H. atra* were extracted and purified using the method which was described previously (Ibrahim and Khaw, 1990).

Yeast isolates and the cultivation in liquid medium – A clinical isolate of *Candida albicans* HUSM1, obtained from the School of Medical Sciences, Universiti Sains Malaysia, were used throughout the study. The yeast culture was grown and maintained on Sabouraud glucose agar slant at 30°C. Cultivation was carried out in SDB for a known period of time to observe the growth profile. Growth profile in the presence of atratoxin was also determined to see the effect of atratoxin B_1 on the growth. Yeast cells (1 ml, 1×10^9 cells/ml) obtained from the cell culture after 2, 12 and 36 hr were transferred into fresh culture broth containing 10 mg/ml of atratoxin B_1. The atratoxin was also added to the growing culture of *C. albicans* after 12 hr (exponential growth phase) and after 36 hr (stationary growth phase) and the cultivation was continued for a maximum duration of 48 hr.

The structural and morphological studies of the yeast cells after exposure to atratoxin B_1 – One milliliter of the yeast cell suspension of concentration of 10^6 cells/ml was added into 15 ml of molten Sabouraud glucose agar and poured into a Petri dish. The inoculated dish was incubated at 30°C for 48 hours. Similarly, two milliliters of the atratoxin B_1 at the concentration of 10 mg/ml was also added in the molten agar and was incubated for 0, 12, 24, 48 and 72 hours at 30°C. Culture which was treated with sterile distilled water was used as control. A small block of yeast containing agar was withdrawn from the inoculated dish at various time intervals (0, 12, 24, 48 and 72 hours) and was fixed and observed under the electron microscope (Cambridge) by either the transmission (Mares, 1989) and scanning (Borgers *et al.*, 1989) electron microscopy.

Results and Discussion

As mentioned earlier on, the studies on the use of atratoxins from *Holothuria atra* (Jaeger) as antifungal agent have been carried out extensively in our laboratory. We also carried out similar studies on the use of the extract for the treatment of candidiasis caused by *Candida albicans*. Strains of *C. albicans* were isolated from the patients who contracted candidiasis from University Hospital, School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian,

Kelantan, Malaysia. Using the isolates, we have shown that atratoxin B_1 was able to inhibit the growth of all the isolates tested based on the agar diffusion method. One of the isolates, *C. albicans* HUSM1 was used to further study the growth inhibition in liquid medium (Fig. 1). As shown in the figure, a normal growth profile was obtained in SDB in the absence of atratoxin, however in the presence of atratoxin, the cells obtained from either the lag, exponential or stationary phase completely failed to undergo multiplication. The atratoxin B_1 was also added to the growing cells after 12 and 36 hr and the cultivation was continued up to 48 hr. It was observed that, the growth of the cells ceased immediately, suggesting that atratoxin B_1 is able to cause cell disfunctioned when in contact although lysis does not occur. The results indicate that the toxin is a potential compound for the treatment of candidiasis based on the fact that it significantly exhibited yeastocidal effect on *C. albicans* cells.

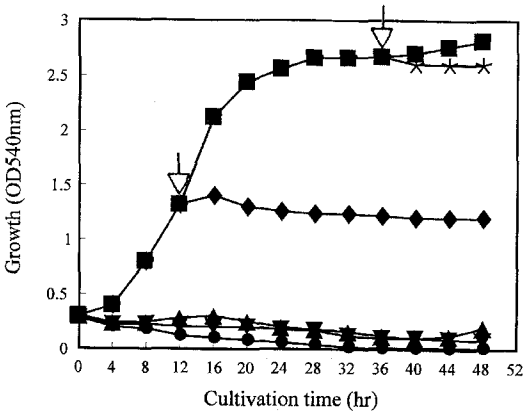

Fig. 1. Growth profiles of *Candida albicans* HUSM 1 in the absence and presence of atratoxin B_1 from *Holothuria atra* (Jaeger). Without the addition of atratoxin B_1 (■), with the addition of atratoxin at 10 mg/ml to the cell culture using the inoculum obtained from a 2 hr (●), 12 hr (▲) and 36 hr (▼). The atratoxin was also added to the growing cells after 12 hr (◆) and 36 hr (*). Arrows indicate time of atratoxin addition to growing cells and the growth profiles thereafter was determined. The cells are grown in 35 ml of SDB in a 100 ml conical flask.

The yeastocidal effect by the atratoxin on *C. albicans* was studied using the electron microscope, both the transmission and scanning electron microscopy. The atratoxin-induced changes in *Candida albicans* based on the comparative studies from the electron micrographs of treated and untreated cells are shown. Untreated cells of *C. albicans* (Fig. 2a) showed that the yeast cells were oval in appearance and distinct cells are observed to be present in the budding stage, showing the daughter cells emerge from the surface of the mother cells These buds are seen as spherical or slightly elongated cells.

After 12 hours of treatment, the antimicrobial effect of the atratoxin was evident. By comparison with the control cells (Fig. 2a), the cell walls of the candidal cells appeared to render the surface of the cells sticky, causing the cells to bind together leading to a state of gross alteration and distortions (Fig. 2b). The atratoxin B_1 treated samples after 24 hours showed the initial ruffling of the normal smooth cell wall surface, depressions, cavitation and wrinkles (Fig. 3a). After 48 hours treatment, it was found that the cells of *C. albicans* exhibited severe alterations of the cell wall with formation of invaginations and the cells collapsed (Fig.

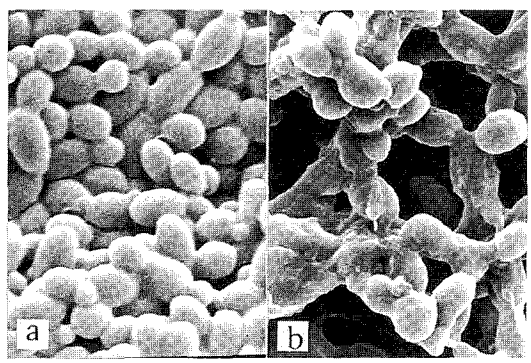

Fig. 2. Scanning electron photomicrographs of normal and atratoxin treated cells. a: normal cells, b: treated with atratoxins for 12 hr. Magnification (×3900).

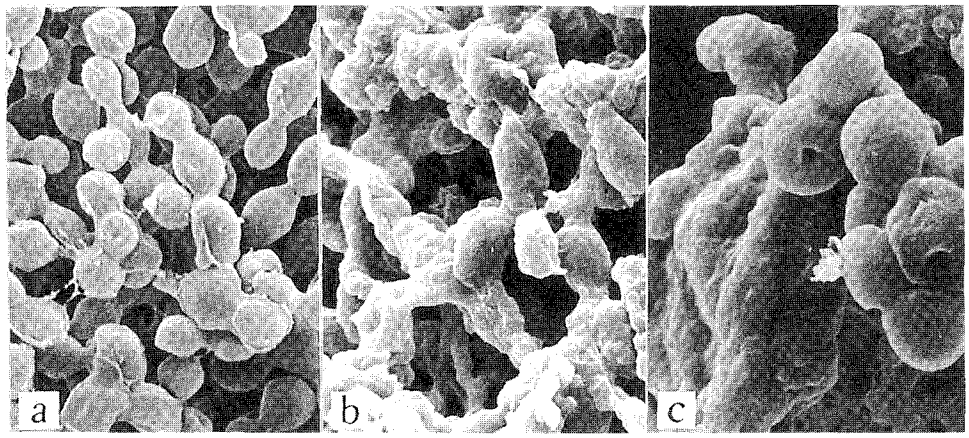

Fig. 3. Scanning electron photomicrographs after exposure with atratoxins. a: 24 hr, b: 48 hr and c: 72 hr exposure. Magnification (×3900).

3b). After 72 hours of treatment, complete necrotic (collapsed cells) and cavitated cells were seen. It is thought that the cells lost its metabolic functions completely during this stage (Fig. 3c).

Scanning and transmission electron microscopy studies, suggested that the cells had undergone distinct morphological and cytological alterations. Fig. 4 shows the structural arrangement of actively growing yeast cells under the transmission electron microscopy (Fig. 4a). It was observed that the dense cytoplasmic structure and uniform rounded typical of most yeast cells are clear. The formation of clear and uniform cell wall (as seen by the cell wall thickness) was also observed both in the cell wall of the daughter cells and the mother cells. After 12 hours of exposure to atratoxin B_1 (Fig. 4b), the cells exhibited notable alterations in the cell membrane (plasma membrane) and the cell wall. The cytoplasmic volume decreased, and the cell membrane started to invaginate leaving a state of structural disorganisation within the cell cytoplasm.

The sequence of morphological alterations after 24 hours of treatment with atratoxin B_1 is shown in Fig. 5(a~c). As shown in the figure, it was observed that at least 3

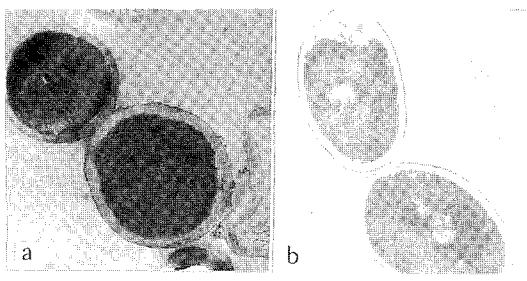

Fig. 4. Transmission electron photomicrographs of *C. albicans* cells of normal and treated cells. a: normal cells (magnification × 17,875), b: treated for 24 hr (magnification × 13,750).

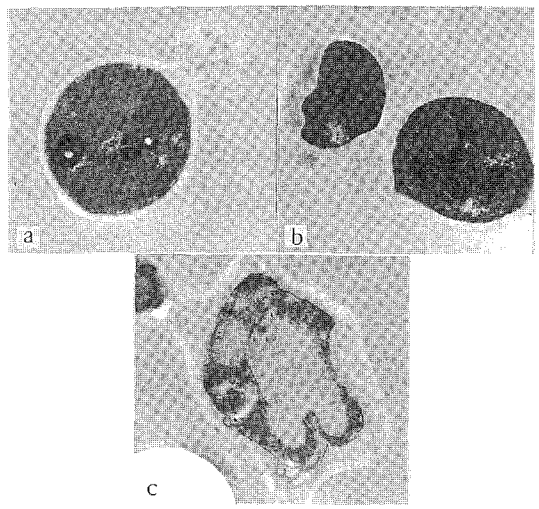

Fig. 5. *C. albicans* cells exposed to atratoxin between 24-48 hr. Magnification (×17,875).

different forms of morphological alterations can be observed. In the first form as shown in Fig. 5a, the mother cell was very dense with its vesicles and membranous bodies dispositioned within the cell, which is seen as dense cytoplasmic aggregations. The mother cell is joined to the cell wall of the growing bud, however the cytoplasmic materials failed to be transferred to the daughter cell. Therefore, the small cell protrusion is actually an extension of the profused cell wall of the daughter cell. Another form of alteration is shown in Fig. 5b. In this case, the mother cell was able to transfer the cytoplsmic materials to the daughter cell, although the daughter cell exhibited destructive effect by the toxins. This can be clearly shown by the irregular shape of the daughter cells showing invaginations in the cell walls. When the cells were allowed for a longer period of time of 48~72 hours (Fig. 5c), both the mother cell and daughter cells undergo structural degeneration. This can be clearly shown in the deformation of the shape and the formation of large vacuoles within the cytoplsm. Cytoplasmic shrinkage, irregular cell membarne, invaginations and thick cell wall are obvious. The mother cell is still linked to the daughter cells and separation does not occur. Ultimately, these cells lysed and disintegrated. The collapsed of the cells is indicated by the secretion of the cytoplasmic materials leading to cell shrinkage. After 72 hours of incubation, more than 90% of the cell population was completely lysed as the osmotic balance in the cell was distrupted (Fig. 5c). Cytoplasmic shrinkage and cytopathological changes were observed followed by the formation of large vesicles within the cells. It is also speculated that the cytoplasmic organelles may have become abnormal and the cytoplasmic matrix was disintegrated.

Based on the electron micrographs shown, it is clear that the most interesting feature of the antimicrobial activities of atratoxin B_1 is its ability to cause lysis on the growing cells of *C. albicans*. Lysis was prevented by osmotic stabilization in bacteria and therefore this is the reason for the ability of the penicillin-treated bacteria to retain the protoplast or sphaeroplast (Baguley, 1979). Lysis occurs during the growth of the yeast and does not occur when protein synthesis was blocked (Cassone *et al.*, 1979). In view of these data, it appears that atratoxin B_1 affect a biosynthetic step(s) in cell wall synthesis in *C. albicans*. We suggest that atratoxin B_1 primarily impairs the structure of the cell membrane and its function. Cell membrane properties such as fluidity and permeability and the activity of membrane bound enzymes (Cabib, 1981) might have been altered as a consequence of the impairment. Since there is compelling evidence that normal cell membrane activity is a prerequisite for regular synthesis and deposition of cell wall constituents, cell wall abnormalities also may be attributable to cell membrane alterations. Changes in the permeability of the cell membrane could cause a decreased in cell volume, evidenced by the disjunction of the cell membrane from the cell wall.

In principle, cell lysis may be caused either by an interference with cell membrane function or by a disturbance of the delicate balance between synthesis and degradation of compounds involved in the cell membrane construction, which only exist during the stage of cell multiplication (Meingassener and Sleytr, 1982). Our results have confirmed that the balance of cell wall growth during bud formation is rapidly and critically affected by atratoxin B_1. Under normal conditions, the cell wall of yeast is synthesized and remodelled during budding so that no significant changes in wall thickness and layering of the wall of the budding cell are seen (Cassone *et al.*, 1981; Itokawa *et al.*, 1988). In contrast,

atratoxin B_1-treated cells showed extreme alteration of the wall of the budding cells.

It is difficult to explain the nature and the significance of the numerous vesicles and membranous bodies, which represent a typical cytological change in spatially related to cell membrane. The formation of vesicles, as well as the involvement of the cell membrane and cell wall may be the consequences of the interaction of atratoxin with fungal metabolism on the assumption that treated cells tried to eliminate unphysiologically synthesized materials by exocytosis. These changes are identical with those observed in *Trichophyton mentogrophytes* (Cassone *et al.*, 1981) and *Microsporum canis* (Darah *et al.*, 1996), but they differ in that their vesicles penetrated the cell wall of dermatophyte but not in the case of *C. albicans*. This differences reflects different cell wall structures of both species.

Our study suggested that atratoxin B_1 acted on the cell wall of the yeast which subsequently resulted in the formation of cytopathological and membrane structural degeneration leading to cell lysis and death. At present, we have successfully formulated the extract to be used for the treatment of induced candidiasis in animal models. The results of the work will be reported subsequently.

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