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COMMERCIAL EYE DROPS TRIGGERS NECROTIC EFFECT IN *Acanthamoeba* sp.

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ABSTRACT

Eye infection due to microbial infection is hard to treat and painful. Two types of eye drops that are commonly used to relieve eye pain offered by pharmacists have been tested in this study namely solution A (containing tetrahydrozoline hydrochloride) and solution B (containing gentamicin and dexamethasone). The efficacy and the activities of these eye drops were tested on *Acanthamoeba* sp. (a clinical isolate from an *Acanthamoeba* keratitis patient) to study the cytotoxicity effects of the solutions on the *Acanthamoeba.* The *Acanthamoeba* were exposed to solution A and B for 24 hr and cell viability was assessed using MTT assays, morphological changes using the light microscope and through acridine orange and propidium iodide (AO/PI) staining for cytoplasmic biochemical activities. The IC_{50} value for *Acanthamoeba* cell viability was 45.1% and 20.3% for solutions A and B respectively. Morphological observation shows the inhibition of acanthapodia formation on the surface of the cells. Solution A and B-treated *Acanthamoeba* appeared in the red color of the cytoplasm upon staining with AO/PI indicating a necrotic mode of cell death. This is due to loss of membrane integrity of *Acanthamoeba* cell membrane after exposed to solution A and B at their IC_{50} value. It is shown that solutions A and B can cause cell death in trophozoite of *Acanthamoeba* cells at moderate IC_{50} value. Unfortunately, the necrosis mode of cell death is not a preferable type of cell death for treating *Acanthamoeba* infection. Therefore, it can be concluded that solutions A and B are not suitable to treat eye infected with *Acanthamoeba* sp. as it does not promise absolute healing as the solution concentration needed is quite high and the cell death mechanism is necrosis.

Key words: Ophthalmology, gentamicin, necrosis, eye infection, *Acanthamoeba* keratitis

INTRODUCTION

Nowadays several drugs are available in the market to treat microbial infections in the eye. However, their efficacy is not promising due to the constituents present in it. They might be efficient against targeting the microorganism of the eye, however, it could affect the corneal layer of the host at the same time. The death of the microorganisms and debris released by the dead particles could cause negative consequences against the safety of the eyes. Generally, the microorganisms that cause infections to the eyes are viruses, bacteria, fungi, and even protozoa. In this study, we aimed to determine the effectiveness of two types of commercial eye drops that are readily available in the market against the protozoa phylum, *Acanthamoeba* species. *Acanthamoeba* sp. rarely infects the eye but it can cause blindness if it enters the eye. This might occur due to the use of contaminated water for face wash and contact lenses. The largest case of *Acanthamoeba* keratitis is mainly from the infection caused by the use of contact lenses. Currently, Polyhexamethylene biguanide (PHMB) and chlorhexidine have been used to treat *Acanthamoeba* keratitis (Lorenzo-Morales *et al*., 2015). Though these drugs are effective, results revealed that most of the commercial drugs end up

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with serious microbial keratitis. Microbial keratitis is the infection caused by microorganisms such as bacteria, fungi in the corneal layer due to infections. This type of adverse effect indicated that the current treatments are not completely successful (Seal *et al.*, 2003; Lim *et al.*, 2008). To avoid these limitations, the mode of action of these available drugs against the microorganisms should be inquired and clarified. Therefore in this study, we tested two commercial eye drops namely Solution A containing (tetrahydrozoline hydrochloride) and Solution B (containing gentamicin and dexamethasone acetate) to assess theirs *in vitro* effects against *Acanthamoeba* sp. based on the dose-response and the mode of cell death determination on the infective stage of *Acanthamoeba* sp.

MATERIALS AND METHODS

Preparation of culture media

Protease yeast glucose (PYG) media was prepared to culture the *Acanthamoeba*. Protease and Yeast (3.75 g) and the D+ glucose (7.5 g) were weighed and added into Page amoeba saline solution (PAS) solution. Finally, distilled water was added to the solution to make up 1liter.

Cultivation of *Acanthamoeba* sp.

Acanthamoeba cells (isolated from Hospital Kuala pur) were cultured in a T-25 tissue culture flask that contains 10 mL of Protease-Yeast Glucose media. Then, the cells were kept in the incubator at 30°C to reach confluence and subcultured further for every 4 days.

Determination of IC_{50} value by using MTT assay

Acanthamoeba sp. (1×10^4 cell/well) was seeded into a 96-well microplate and incubated at 30°C. After 24 hr of incubation time, the culture media was removed and replaced with culture media containing Solution A and solution B at various concentration ranges from 0.7% to 60% with five replicates for each solution. After 24 hr, 20 µL of MTT solution (0.2 mg/mL) was added into each well and incubated for another 4 hr at 30°C. Then, DMSO was added to dissolve the purple formazan crystal and the absorbances of the samples were measured using a microplate ELISA reader at 570 nm. The IC_{50} value was derived from the plotted graph, cell viability versus concentration of solution A and B.

Morphological observation after treatment using a light microscope

Morphological changes in *Acanthamoeba* sp. were observed directly under an inverted light microscope *via* cell treatment with 3 mL of Solution A and B at their IC_{50} value concentration in 6-well plates. After 24 hr of treatment, the changes were recorded.

Determination of mode of cell death

Determinations on the mode of cell death on *Acanthamoeba* were based on Nakisah *et al*. (2012) and was carried out in 6-well plates *via* treating the *Acanthamoeba* cells (1×10^4/mL) with Solution A and B at their IC_{50} value concentration at 30°C for 24 hr. Then, the untreated and treated *Acanthamoeba* sp. were centrifuged at 3000 rpm for 15 min. Later, the supernatants were discarded and the pellets were rinsed with PBS solution. Next, the pellets were resuspended with acridine orange/ propidium iodide (AO/PI) solution. The cell suspensions were incubated in the dark for 10 min and placed onto a slide and covered with a coverslip. Finally, the slides were visualized using a fluorescence microscope (Olympus IX50, USA) at 400 magnification time.

RESULTS

IC_{50} determination by using MTT assay

Initially, the toxic effect of the solution A and B were tested against *Acanthamoeba* sp. through MTT assay and their 50% inhibition concentration (IC_{50}) values were obtained. Figure 1 shows the

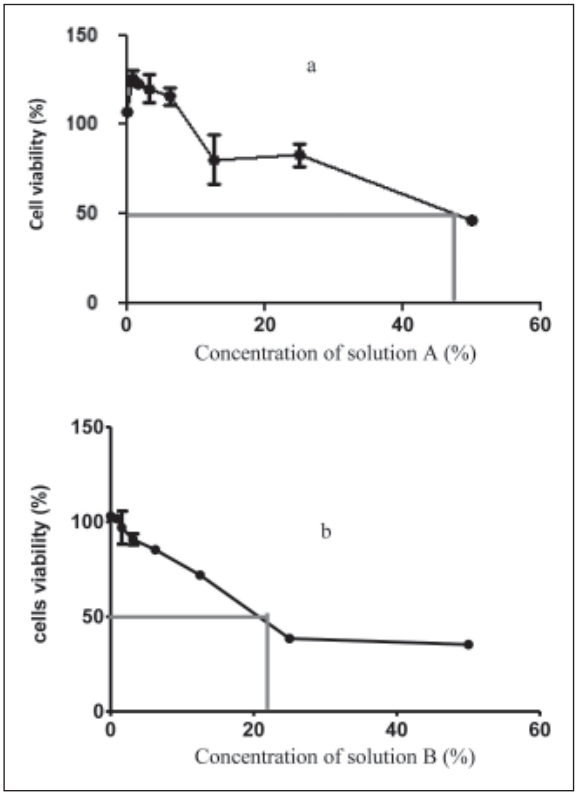

Fig. 1. Percentage of cell viability using MTT assay after 24 hr after the treatment of Solution A (a) and B (b). The IC_{50} value obtained is 45.1(%) and 20.5 (%) Mean ± SEM, *n*=5.

percentage of *Acanthamoeba* sp. cell viability against the various concentration of Solution A and B with the IC_{50} values. The IC_{50} values of Solution A and B were 45.1% and 20.3%, respectively. The combination of gentamicin and dexamethasone exhibits more toxicity (*in vitro*) towards *Acanthamoeba* cells in comparison with solution A that contains tetrahydrozoline hydrochloride.

Morphological changes observation on *Acanthamoeba* sp. treated with solution A and solution B

After 24 hr of treatment with Solution A and B, significant morphological changes were observed on the *Acanthamoeba* sp. To distinguish the differences between the morphological changes that occurred in *Acanthamoeba* sp., the treated *Acanthamoeba* sp. were compared with the untreated cells which serve as a negative control. Figure 2 shows the comparison of morphological changes between untreated (Figure 2a) and treated *Acanthamoeba* sp. (Figures 2b & 2c) observed under the inverted microscope. Based on the observation, the untreated cells were in trophozoite shape with acanthapodia, nucleus, and vacuoles, however, the shape of the cells treated with Solution A and B were round and cystic shape.

Membrane integrity and mode of cell death determination on *Acanthamoeba* sp. by AO/PI staining

The observation of the stained cells under the fluorescent microscope was used to determine the cell viability based on the membrane integrity and lysosome activities that occurred on the *Acanthamoeba* cells. AO/PI stained *Acanthamoeba* cells after exposure with the Solution A and B treatment were viewed under the fluorescent microscope (Figure 3). Compared to untreated cells, the treated *Acanthamoeba* cells underwent membrane disintegration and leakage occurs that leads the PI to enter into the cytoplasmic region. The green color represents the healthy cells whereas the red one represents the *Acanthamoeba* cells that undergo necrosis.

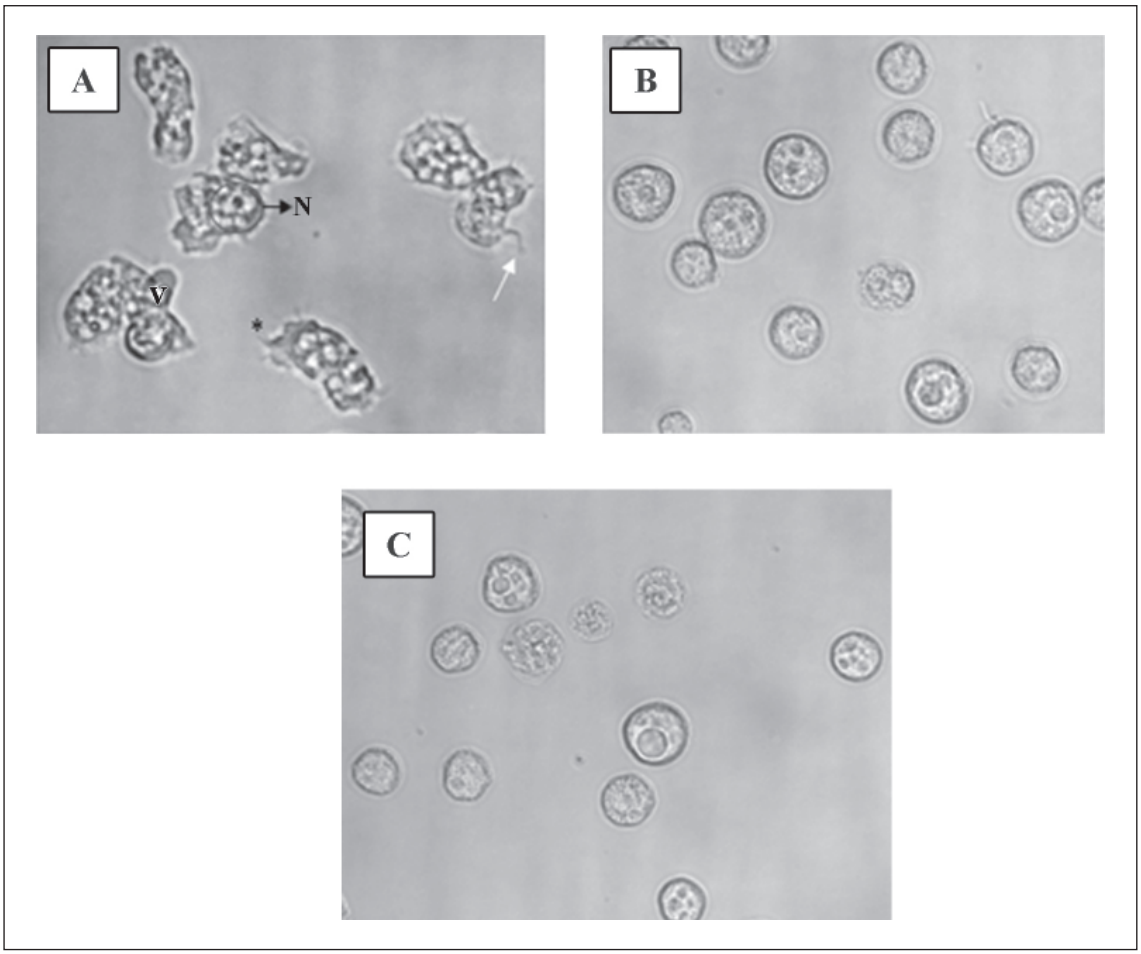

Fig. 2. Image of morphological structures of *Acanthamoeba* sp. under the inverted light microscope observation where (A) untreated cells show nucleus structure (N), distinctive acanthopodia (white arrow), pseudopodia (asterisk), and numerous vacuole (V) can be seen and (B and C) treated cells with the Solution A and B can be seen with the rounded shape of a cell indicates the encystment process from its trophozoite stage.

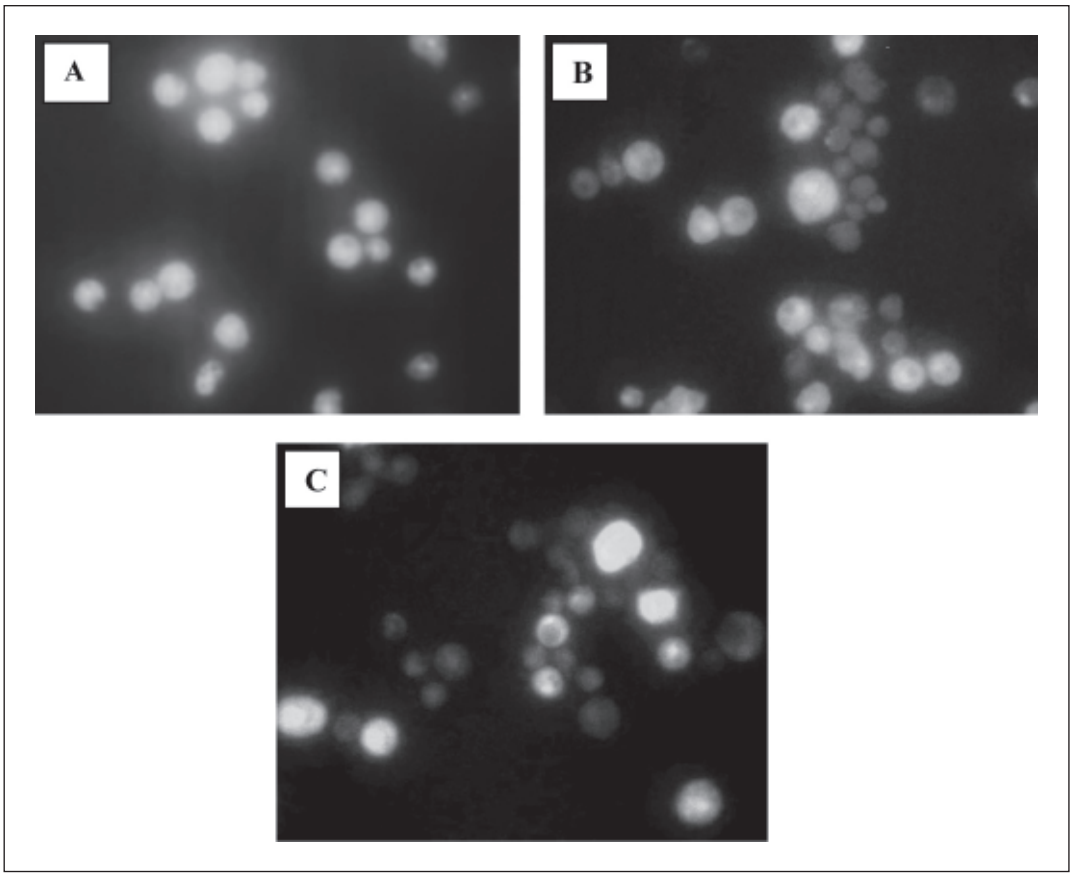

Fig. 3. The image of stained *Acanthamoeba* sp. after been observed under the fluorescent microscope where (A) is the untreated cells display the green cytoplasm (B and C) the treated cells appeared with the green-yellow nuclei after 24 hr incubation time. Magnification was 400X.

DISCUSSION

Cell viability, cytotoxicity, and fifty percent inhibition concentration (IC_{50}) on *Acanthamoeba* sp. determination by using MTT assay

From this study, it is clear that the viability of the *Acanthamoeba* cells treated with Solution A and B decreased gradually as the concentration increases (Figure 1). The IC_{50} values for solution A and B were 45.1% and 20.3% respectively. Though these solutions reduced the cell viability, high IC_{50} values revealed that the efficacies of these samples are less against *Acanthamoeba* cells. Toxicity is the degree of toxic substance that may harm or affects the cells. The IC_{50} value is an important parameter to understand the cellular toxicity of the samples. In this study, the higher IC_{50} value might be due to the encystment of the *Acanthamoeba* cells and the inactivity of the drug (Martín-Navarro *et al.*, 2013). The drug sensitivity testing and the development of effective therapeutic strategies might be limited due to the complexity in isolation and maintenance of the cells (Ahmad *et al.*, 2013). The drug or compounds with lower IC_{50} values could be a potential complement for *Acanthamoeba* keratitis therapy or even first-line treatment (Martín-Navarro *et al.*, 2013). High IC_{50} percentage of the solutions against *Acanthamoeba* sp. indicated their low efficacy in affecting the cell's viability which might not help in reducing eye infection incorporated with *Acanthamoeba* sp. *Acanthamoeba* infection produces exquisite pain and ulceration on the ocular surface resulting in cytolysis and apoptosis of the cornea tissue and thus the dissolution of corneal stroma also can occur (Clark & Niederkorn *et al.*, 2006).

Morphological observation on *Acanthamoeba* sp. under the light microscopy

The morphological changes in the *Acanthamoeba* cells due to the treatment of Solution A and B at IC_{50} value concentration were viewed under light microscopy. After 24 hr of treatment with solutions A and B, the morphology of the cells revealed acanthapodia and cyst characteristics. The same type of modification has not been observed in the untreated cells. It can be seen under a light microscope.

Likewise, the untreated *Acanthamoeba* cells were in an inactive state, the trophozoite stage was witnessed whereas, in the treated *Acanthamoeba* cells, the inactive state was observed with dormant cyst and double-membrane formation. These results specified that the treated *Acanthamoeba* cells tended to protect them from the stress environment conditions *via* altering their structure (Booton *et al.*, 2009).

The cysts are often found in the established *Acanthamoeba* keratitis in the corneal stroma. According to Ibrahim *et al.* (2014), the encystment of the *Acanthamoeba* cells is due to the chemical compounds. Additionally, Byers *et al.* (1991) reported that the occurrence of encystment is also an alternative way to develop a repetitive cycle through cell division under environmental stress or other conditions. The encystment process of *Acanthamoeba* cells starts with shortened acanthapodia and the formation of a double membrane. Acanthapodia is the spiny surface projections made up of hyaline cytoplasm that could penetrate the host cells. The pathogenicity of the organisms is preserved by the maintenance of the encystment rates (McClellan *et al.*, 2001). The encystment rate is important to continue their life in a harsh environment. The irregular shape of the *Acanthamoeba* cells is due to the existence of the acanthapodia and the contractile vacuole. High pathogenicity will occur in the trophozoite stage which is the active stage. High pathogenicity is due to the ability of acanthapodia which adhere on the surface and invade tissues that could infect the host. The pathway of the host signalling cell will get interferes due to the binding of *Acanthamoeba* cells to the host (Lorenzo-Morales *et al.*, 2015). The host cell damage is due to the role of the *Acanthamoeba* phospholipases as it facilitates the *Acanthamoeba* virulence. Also, during the adhesion process, the production of toxin secretions such as oxygen free radicals and proteases will increase and that could damage the human connective tissues. According to the findings of Marciano-Cabral and Cabral (2003), there are distributions of trophozoite and cyst in the alveoli of Granulomatous Amoebic Encephalitis (GAE) patients.

Membrane permeability determination on *Acanthamoeba* sp. by AO/PI staining

This paper report the first evidence on observation on the cytoplasmic activities with the effect of two eye drops on *Acanthamoeba* sp. Based on the results, we had confirmed the efficacies of the two eye drops on the *Acanthamoeba* sp. and their potential effects of using these two eye drops towards *Acanthamoeba*-infected eyes. Observation under fluorescence microscopy by using AO/PI revealed two important events on the *Acanthamoeba* cytoplasmic activities. The plasma membrane integrity and the mode of cell death either necrosis or apoptosis.

The role of the plasma membrane is to protect the cells from the surroundings, provide cell shape, and act as the selective barrier that regulates the substances which can enter and exit out from the cells. Adverse environments will disrupt the membrane integrity of the cells that could induce apoptosis, necrosis, or autophagic cells. In this study, the *Acanthamoeba* cells that were exposed under the stress conditions *via* treatment with Solution A and B for 24 hr were stained with the combination of acridine orange and propidium iodide (AO/PI). The stained treated *Acanthamoeba* cells were observed under the fluorescent microscope to differentiate the viable and non-viable *Acanthamoeba* cells. It also helps to visualize the mode of death either apoptosis or necrosis. Acridine orange (AO) and Propidium iodide (PI) are the dyes used to detect the mode of cell death. AO is a nucleic-acid selective dye that binds to the native or denatured nucleic acid of a cell *via* crossing the intact membrane. Propidium iodide is a dye that cannot cross the membrane of live cells; however, it binds to the RNA.

The results obtained from the fluorescent microscope after staining is shown in Figures 3b and 3c. It revealed that the *Acanthamoeba* cells undergo the necrosis mode of cell death. Necrosis occurs when the cells are exposed to the extreme variance from the physiological conditions that may affect the plasma membrane to damage. The plasma membrane is evoked by the agents like complement and lytic viruses due to the physiological conditions. Necrosis begins with the impairment of the cell's ability to maintain homeostasis which leads to an influx of water and extracellular ions. As a result, the intracellular cells especially the mitochondria and the entire cells will get swollen and rupture. The lysosomal enzymes will be released into the extracellular fluid due to the ultimate breakdown of the plasma membrane. The physiological significance of the necrosis process will significantly affect the group of contiguous cells. Mainly this process is evoked due to the non-physiological disturbances and phagocytosis by the macrophages. Moreover, the effect of necrosis is the stimuli of the host inflammatory response (Choudhury *et al.*, 2011). The inflammatory observed with extraintestinal amoebiasis is the part of the fatal contact of host cells with amoebae.

The AO/PI treated cells indicated the presence of dead cells, a necrotic type of cells. This strongly suggested that the complete damage of cells is more serious than apoptosis. Necrosis is considered to be

the unnatural death process of cells. Necrotic cell death is often associated with extensive tissue damage and thus results in an intense inflammation response (*in vivo*). In this study, the orange stained organelles in the cytoplasm were appeared due to the permeable-cationic dyes that enter acidic molecules through an intact membrane. The mode of cell death that occurred on *Acanthamoeba* cells is also the unwanted mode of cell death, the necrosis which can induce the inflammatory effect to the host and thus results in adverse effects to the human. Thus, the efficacy of Solution A and B as the treatment to cure AK is weak as it induces the necrosis mode of cell death.

CONCLUSION

Overall, after 24 hr of treatment, the two eye drops exhibited a high percentage of IC_{50} concentration against the *Acanthamoeba* sp. The morphological changes and alteration on the *Acanthamoeba* cell structure were observed under a fluorescence microscope through AO/PI staining that supported to determine the mode of cell death. Most of the *Acanthamoeba* cells after the treatment with solutions A and B underwent necrosis mode of cell death which is the undesirable type of cell death in eyes that could result in the inflammatory response to the host owing to the leakage of the chemical substances or necrotic tissues from *Acanthamoeba.*

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