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Effect of chlorine on survival and ultrastructural changes of *Acanthamoeba castellanii*

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Introduction

Free-living amoebae that belong to the genus *Acanthamoeba* are widespread in the environment, including water. They are responsible for human infections and can host pathogenic microorganisms. Under unfavorable conditions, they form cysts with high levels of resistance to disinfection methods, thus potentially representing a threat to public health. Many public water systems add chlorine (a process known as "chlorination") to their water supply for the purpose of disinfection. Disinfection kills or inactivates harmful microorganisms, which can cause illnesses such as typhoid, cholera, hepatitis, and giardiasis. Acanthamoebae are pathogenic amoebae and may provide a reservoir for pathogenic bacteria; it is consequently important to understand the response of these amoeba to chlorination, and our study was indeed aimed at examining cellular changes in Acanthamoebae following chlorination.

Materials and methods

Growth of *A. castellanii*

The stock culture of *A. castellanii* (ATCC 30010) was stored at -80 °C in a solution containing 7.5 % dimethylsulphoxide. Amoeba culture was routinely maintained in 25-cm^2 cell culture flasks (Corning) filled with 5 ml of peptone-yeast extract-glucose medium [PYG; 2% proteose peptone, 0.1% yeast extract, 1.6 mM $MgSO_4 . 7H_2O$, 0.4

mM $CaCl_2$, 0.1 M sodium citrate. $2H_2O$, 2.5 mM Na_2PO_4. $7H_2O$, 2.5 mM KH_2PO_4, 0.5 mM $Fe(NH4)_2(SO4)_2$. $12H_2O$, 0.1 M glucose] pH 7.0 at 25 °C unless indicated otherwise. In order to encyst the amoebas, active trophozoites were inoculated on agar plates (without bacteria). After seven days, the cysts were rinsed out from the agar surface with a sterile AS solution, which is a modified Neff's amoeba saline (Garajová et al., 2019). The cells were concentrated by centrifugation at 800 × g for 20 min and subsequently resuspended in a PAS medium for the subsequent analyses.

Treatment condition and viability assay

The 6-well plate (Nunc™, Wiesbaden, Denmark) was seeded with 10^6 trophozoites or cysts of *A. castellanii* in two ml of PAS per well and incubated at 25 ˚C for one h to allow amoebal settlement and adhesion before exposure. The medium was gently removed for treatments. The trophozoites were exposed to different concentrations (1-10 ppm) of calcium hypochlorite. The selected concentrations are the concentrations that are commonly used for chlorine. Exposure was done in three repetitions. Also, for each repetition and each time, a control sample containing 10^6 unexposed trophozoites diluted with 500 microliters of sterile distilled water was considered. The viability of trophozoites was evaluated at 30 min and one h, and every one hour up to five h. The percentage of encystation of treated trophozoites was assessed as well. The viability of trophozoites/cysts was estimated using a hemocytometer and trypan blue stain. Based on the results, the best time and concentration for LC50 (50% lethality) were determined and trophozoites were exposed to that concentration for ultrastructural examination.

Field emission scanning (FESEM) and transmission electron microscopy (TEM) study

The effect of two h treatment with 5 ppm chlorine on the cell surface and cell ultrastructure was examined by FESEM and TEM, respectively. For FESEM, the cells were fixed with 2.5% glutaraldehyde overnight. The samples were further dehydrated with a series of graded alcohol (20%, 40%, 60%, 80%, 90%, and 100% ethanol), mounted on aluminum stubs, and dried using a critical point dryer. Samples were then coated with gold particles. The morphology of *A. castellanii* trophozoites after treatment with PAW was subsequently examined under FESEM (Mira4, Tescan, Czech) at the Central laboratory of Shahid Chamran University of Ahvaz, Ahvaz, Iran.

At first, an aluminum plate with dimensions of 1×1cm, designed and sterilized, was placed in each well of the 6-well cell culture plate. 10^6 trophozoites was counted, slowly transferred to the aluminum plate in the wells, and finally exposed to the LD50 concentration of chlorine (5 ppm) for two hours. After the two-hour incubation period, under sterile conditions, the liquid inside the wells was slowly removed, and the aluminum plates inside the wells were allowed to dry. Then, the cells on the aluminum

plates were fixed using cold 2.5% glutaraldehyde for 2 hours. Finally, 2.5% glutaraldehyde was removed and after drying the aluminum plates, the cells on the plates were dehydrated with 20-100% alcohol series for 5 minutes for each series. The plates were placed in a pre-designed container and stored in a place with a suitable temperature and free from dust. Finally, it was transferred to the Electron Microscope Center located in the Central Laboratory of Shahid Chamran University of Ahvaz for morphological studies and other studies. With this method, three-dimensional images of the structure of the sample were obtained.

For TEM, the trophozoites were washed in 0.1 M Milloning's buffer and fixed in 2% glutaraldehyde solution buffered with 0.1 Milloning's buffer at pH 7.2 for two h (trophozoites). To facilitate thin section preparation, fixed protozoa were embedded in 2% agar. Agarized pellets were then fixed in 1% osmium tetroxide, dehydrated in a graded acetone series, and embedded in Spurr's resin. Ultramicrotome sections were stained with 1% uranyl acetate followed by 2.5% lead citrate and examined on a TEM (Hitachi S4160, Korea) at the Central Laboratory of Pasteur Institute, Tehran, Iran.

Results & Discussion

As the graphs in Figures 1 and 2 show, common concentrations of chlorine that are used as disinfectants were not able to completely destroy *A. castellani* trophozoites and cysts. The percentage of viability of trophozoites treated with 10 ppm chlorine for 0.5, one and two hours were obtained 67.2%, 49.5% and 39.8%, respectively. While, for *A. castellani* cysts were 72.1%, 60.8% and 51.4%, respectively. Therefore, the results of the treatment showed that the cysts are more resistant. LC50 for trophozoites were 5ppm for two hours. Ultrastructural changes of amoeba treated with LC50 of calcium hypochlorite showed cell membrane changes. The creation of pores and bubbles were observed in FESEM. It shows an increase in the number of vacuoles, changes in mitochondria, and a decrease and change in the number of pseudopods of the trophozoite treated with chlorine using the TEM. It shows the changes in the endoplasmic reticulum structure and the presence of dense granules. Changes in the nucleus and the presence of lamellar bodies caused by the destruction of mitochondria were other findings using the TEM. Chlorine as a disinfectant is widely used to disinfect surfaces and remove biofilms, in water distribution systems and mainly in drinking water treatment. The mechanism of chlorine effect is the production of free radicals, including hydroxyl radicals, through the Fenton process. Excessive production of free radicals during a process called oxidative stress causes damage to different parts of the cell such as proteins, DNA and cell membrane (Maillard. 2004). These irreversible damages lead to changes in cell function or cell death.

Conclusion

This study showed that the commonly used concentrations of chlorine as a disinfectant could not completely destroy trophozoite and amoeba cysts. However, ultrastructural changes occur on the surface of amoeba trophozoite cells after exposure to chlorine. These changes mainly included changes in cell surface morphology and changes in the structure of the nucleus, endoplasmic reticulum, vacuolation, and changes in mitochondria. The production of free radicals by chlorine and the creation of oxidative stress led to changes in the cell or cell death. This study suggests the need to review the instructions for disinfectants such as chlorine. Because in addition to being pathogenic, the amoeba is crucial for being a reservoir and transmitting microbial and even viral agents.

***Keywords**: Acanthamoeba castellanii, Chlorine, Survival, Ultrastructural changes*

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Declaration of conflict of interest

The authors declare there is no conflict of interest.

Statement on ethics

The paper reflects the authors' own research and analysis in a truthful and complete manner. The paper is not currently being considered for publication elsewhere. It properly credits the meaningful contributions of co-authors and co-researchers.