Exposure to sublethal concentration of chlorine enhances the cytotoxicity of *Acanthamoeba castellanii*

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Abstract

Background: Chlorine is a common disinfection agent commonly added to public water facilities and supplies. Acanthamoeba species are free-living amoebae commonly found in water and can become opportunistic pathogens. In this study, we examine the cytopathic and phagocytic properties of *Acanthamoeba castellanii* trophozoites following exposure to the sublethal concentrations of chlorine.

Methods: Two hours exposure to five ppm hypochlorite calcium was considered the sublethal concentration for *A. castellanii* trophozoites. To compare the pathogenic potential of treated and untreated *Acanthamoeba* trophozoites, cytotoxicity, adhesion assays in RAW 264.7 macrophages, osmo, and thermotolerance tests were carried out. Bacterial uptake was assessed in treated cells to evaluate their phagocytic characteristics. Oxidative stress biomarkers and antioxidant activities were compared in treated and untreated trophozoites. Finally, the mRNA

expression of the mannose-binding protein (MBP), cysteine protease 3 (CP3), and serine endopeptidase (SEP) genes was determined in cells.

Results: In chlorine-treated trophozoites, cytopathic effects were more extensive and resulted in the detachment of macrophage monolayers. Treated trophozoites could not grow at high temperatures (43 °C). Besides, they showed osmotolerance to 0.5 M D-mannitol but not to 1 M. Results demonstrated a higher bacterial uptake rate by chlorine-treated trophozoites than untreated cells. The treated and untreated cells had significantly different glutathione and glutathione/glutathione disulfide ratios. Antioxidant enzyme activities, total antioxidant capacity, and malondialdehyde level were increased significantly in chlorine-treated cells. Quantifying mRNA expression in chlorine-treated trophozoites revealed that virulence genes were upregulated.

Conclusion: Chlorine can form resistance and virulent amoebae if it is not used at a proper concentration and exposure time. Identification of stress responses, their mechanisms in *Acanthamoeba,* and their relation to amoeba virulence would give us a better perception of their pathophysiology.

Keywords: *Acanthamoeba castellanii,* Chlorine, Oxidative stress, Cytotoxicity

Introduction

The genus *Acanthamoeba* is considered an important pathogen of our time due to its ability to cause opportunistic and non-opportunistic infections in humans (Marciano-Cabral & Cabral 2003). These eukaryotic organisms belong to a group of amoebae known as free-living amoebae (FLA) and reside in soil, water, and engineered water systems such as swimming pools and

healthcare plumbing systems (Scheid 2018; Montalbano Di Filippo et al., 2015). *Acanthamoeba* is known to cause keratitis and meningoencephalitis, sinusitis, and cutaneous lesions in immunocompromised individuals, such as AIDS patients (Torno et al., 2000). It has two stages in its life cycle: an active phagotrophic trophozoite and a quiescent double-walled cyst stage. The cyst stage enables the amoeba to remain viable in harsh conditions, including chlorine-treated water (Bowers and Olszewski, 1983). Chlorine is a highly efficient disinfectant added to public water supplies to kill disease-causing pathogens, such as bacteria, viruses, and to a lesser degree parasitic protozoa that present in water supply reservoirs, on the walls of water mains, and in storage tanks (Galal-Gorchev 1996). For disinfection purposes, chlorine dosage should be at the breakpoint concentration (i.e., consuming chlorine demand) or slightly higher. The concentration should be sufficient to destroy waste products and pathogens and oxidize organic contaminants. Numerous reports have been in the literature on isolating pathogenic FLA in chlorinated domestic and recreational waters. Although many studies are being conducted to find effective compounds against amoebae, it seems that the changes in surviving amoebae after exposure to these compounds have been neglected. Given the above issues, this study examined *A. castellanii* trophozoites for their virulence, cytopathic, and phagocytic properties following exposure to the sublethal chlorine concentrations.

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 % dimethyl sulphoxide. Peptone-yeast extract-glucose [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 medium was routinely used for maintaining amoeba cultures in 25-cm^2 cell culture flasks (Corning) at 25 °C. For subculture, trophozoites were suspended by tapping the flask vigorously and using a cell scraper (Corning), centrifuged at 300 × *g*, and washed in Page's Amoeba Saline [PAS; 1.6 mM $MgSO_4$. $7H_2O$, 0.4 mM $CaCl_2$, 0.1% sodium citrate. $2H_2O$, 2.5 mM NaH_2PO_4, 2.5 mM K_2HPO_4, 0.05 mM $Fe(NH_4)2(SO_4)2$. $12H_2O$], centrifuged at 300 × *g*, and suspended in 5 ml of fresh PYG medium. Using a hemocytometer, cell viability and concentration were assessed using trypan blue staining. 2.5×10^5 amoebae were then inoculated in a new flask containing a fresh PYG medium. Every seven days, this subculture procedure was repeated.

Treatment condition

Our previous study found that two hours of exposure to five ppm of hypochlorite calcium can be considered the 50% lethal concentration (LC_{50}) for *A. castellanii* trophozoites (REFERENCE). In this study, to evaluate the impact of sublethal concentration of chlorine on cytopathic, phagocytic, and antioxidant responses of *A. castellanii* trophozoites, cells were treated with LC_{50} of hypochlorite calcium.

A 6-well plate (Nunc™, Wiesbaden, Denmark) was seeded with 10^5 trophozoites of *A. castellanii* in 2 ml of PAS per well and incubated at 25 ˚C for one h to allow amoebal settlement and adhesion before exposure. After gently removing the medium, 2 ml of 5 ppm hypochlorite calcium was added. Amoebal culture in PAS was set up as the control. A hemocytometer and trypan blue stain were used to determine the viability of trophozoites after two hours.

Cytotoxicity and adhesion assays

Cytotoxicity assays were performed to compare the pathogenicity of treated and untreated *Acanthamoeba* trophozoites on macrophage cells (Martín-Navarro et al., 2010). Briefly, the Raw 264.7 macrophage cell were grown to monolayers in 6-well plates. RAW 264.7 (macrophage cell line) was purchased from the Iranian biological resource center. Cells were cultured in RPMI-1640 supplemented 10% heat-inactivated (56 °C, 30 min) FBS and 1% Penicillin (50 u/mL) Streptomycin solution (50 µg/mL) (Sigma, St. Louis, Mo., USA). Three times a week, cultural mediums were changed. As monolayers completely covered flask bottoms, subcultures were performed. Raw 264.7 cell monolayers with 10% fetal calf serum were incubated with untreated or treated *Acanthamoeba* trophozoites (10^5 amoebae/well). Cytopathic effects were monitored periodically for up to 24 hours. Hematoxylin staining was used to assess cytopathic effects following this incubation period. Moreover, supernatants were collected for cytotoxicity testing by measuring LDH release following manufacturer instructions (Paadci, Iran). Positive control wells were incubated for 30 minutes with 1% Triton X-100 after the incubation period. Untreated RAW 264.7 macrophage cells were considered as the negative control. By using the plate reader, samples were measured for absorbance at 490 nm. The percentage of cytotoxicity of each sample was calculated using the formula: (sample absorbance – negative control absorbance) / (positive control absorbance – negative control absorbance) × 100% (Anwar et al. 2018).

An adhesion assay was conducted to compare the ability of treated and untreated trophozoites binding to macrophage cells (Sissons et al. 2004). Briefly, the cell line was cultured in a confluent monolayer in a 6-well plate 24 h before the assay. Treated or untreated amoebae (10^5 cells/well) were added to the plate and incubated in 5% CO_2 at 37 °C. Incubation for one hour was followed by washing the unbound amoebas with PBS and counting them using the

hemocytometer. The percentage of bound amoebae was calculated as 100%–% unbound amoebae.

Thermo- and osmotolerance tests

The osmo- and thermotolerance of treated and untreated trophozoites were compared using bacterial cocultures in non-nutrient agar and axenic growth condition. According to Khan et al. (2001), cocultures were performed using an initial inoculum of 10^4 trophozoites placed in a 10 mm diameter central area in the NNA plates (90 x 15 mm). An osmo-tolerance test was conducted at 28 °C using plates containing 0.5 and 1.0 M mannitol. Assays for thermotolerance were performed at 26°C, 37°C, and 42°C without mannitol. During ten days, the area occupied by trophozoites was measured every 24 hours, starting from the inoculum area. It was measured that the radius of the area occupied by trophozoites was moderate (+: r <10 mm), high (++: r >10 mm), or very high (+++: r >20 mm). Triplicate tests for temperature and osmotolerance were conducted (Corsaro et al., 2017).

Evaluation the phagocytic ability

To compare the phagocytic ability of treated and untreated cells, trophozoites of *A. castellanii* were co-cultured with *Escherichia coli*, and their bacterial uptake rate was determined. Coculture experiments were performed in Page's amoeba saline at 25 ˚C. Culture flasks (25 cm^2) were seeded with trophozoites (10 ml/flask at a ca. 2×10^4 cells/ml PAS) and incubated at 25 ˚C for one h to allow amoebal settlement and adhesion before infection. After gently removing the medium, 10 ml of a bacterial suspension at a concentration of ca. 2×10^5 CFU/ml PAS with a multiplicity of infection (MOI) of 10 bacteria per amoeba was added to each flask. Bacterial and

amoebal monocultures were set up as controls (Zarei et al. 2019). Two hours after co-cultivation at 25 °C, 100 g/ml gentamicin sulfate solution (Sigma-Aldrich, St. Louis, MO) was added to the co-culture flasks to kill extracellular bacteria. To recover intra-amoebal bacteria, the amoebae were washed with PAS to remove the gentamicin and lysed for 5 minutes with 0.5% sodium deoxycholate. In this way, amoebal trophozoites were lysed (100%) without affecting bacterial viability. TSA plates were used to count viable intra-amoebal bacteria in cocultures and monocultures (Zarei et al., 2019). The bacterial uptake rates were calculated by dividing the recovered bacteria by the number of viable trophozoites × 100 (Jeong et al., 2007).

Biochemical analysis

For biochemical analysis, lysates of chlorine-treated trophozoites and untreated trophozoites were prepared by RIPA lysis buffer. Briefly, media was aspirated, and 1×10^4 cells were washed gently with 10 ml ice-cold PBS. 500 µl of RIPA Lysis Buffer (sodium chloride (5 M), Tris-HCl (1 M, pH 8.0), nonidet P-40, sodium deoxycholate (10 %), SDS (10%) with PMSF as the protease inhibitor was added to the plate and swirled to distribute buffer. Plates were kept on ice for all steps. A spatula scraped the cells, and the lysates were collected. After incubating on ice for 15 minutes, lysates were sonicated three times for two seconds, with one minute of rest on ice between each pulse. Lysates were incubated for an additional 15 minutes, centrifuged at 12,000 x g for 5 minutes at 4 °C, and the supernatants were collected, aliquoted, and stored at -40 °C.

Markers of oxidative stress glutathione (GSH), glutathione disulfide (GSSG), superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (TAC) and malondialdehyde (MDA) levels in cell lysates were determined. Glutathione colorimetric assay kits

(ArsamFarazist, Iran) were used to measure GSH and GSSG concentration. GSH and GSSG concentrations are expressed as µmol/ mg of protein.

CAT activity was measured by the method described by Korolyuk et al. (1988). Briefly, the reaction mixture, including 50 µl Tris HCl buffer (0.05 mM), 100 µl H_2O_2 (10 mM) in Tris HCl, and 5 µl cell extract, was added to a 96 well plate. Ammonium molybdate 4% was added to each well after ten minutes at room temperature, and absorbance was measured at 410 nm against a blank (the reaction mixture without cell extract). CAT activity was expressed as IU/mg of protein, and it was calculated using this formula: CAT activity (µmol/min)= (Control absorbance - Sample absorbance)/Control absorbance × Control concentration/10.

The activity of SOD was measured based on the manufacturer's instructions (Ransel Kit, Randox Laboratories Ltd. GB). The enzyme activity in the lysates was expressed as mU/ mg of protein.

Malondialdehyde level was measured using Placer et al.'s thiobarbituric acid reaction method (1966). The thiobarbituric acid reactive substances were determined at 532 nm. The values of MDA were expressed as nmol/mg protein.

The samples' total antioxidant capacity (FRAP value) was measured according to Benzie and Strain (1998). In brief, buffer acetate and TPTZ solution in HCl were mixed to prepare FRAP (ferric reducing antioxidant power). It was then mixed with $FeCl_3$. A mixture of eight µL of serum and 240 µL of the mentioned working solution was incubated for ten minutes at room temperature. At 532 nm, samples were measured for optical density. Total antioxidant capacity was expressed as nmol/mg protein.

Bradford's method measured total protein concentrations in cells using bovine serum albumin as a standard.

RNA extraction and analysis of some virulence genes expression by real-time PCR (RT-qPCR)

Chlorine-treated *Acanthamoeba* was analyzed for mannose-binding protein (MBP), cysteine protease 3 (CP3), and serine endopeptidase (SEP) messenger RNA (mRNA). Trizol Reagent (Invitrogen, USA) was used to extract total RNA from treated and untreated trophozoites. Following the manufacturer's instructions, the mRNA enrichment was performed using the RNeasy mini kit (Qiagen, USA), and the contaminating genomic DNA was removed using DNase I (Invitrogen, Carlsbad, CA, USA). The reverse transcription of RNA to cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, USA).

The reaction mixtures were placed in a thermal cycler (T100 Thermal Cycler, Bio-Rad) and cycled for 10 minutes at 25°C, 120 minutes at 37°C, and 5 minutes at 85°C. After that, the samples were stored at -80 °C until they were used. qRT-PCR amplification was performed using specific primers MBP, CP3, and SEP genes (Table 1).

Each target gene's PCR efficiency was determined, and a melt curve analysis was performed to ensure that only one gene product was amplified. The Real-time PCR assay reaction mixture (10 µl) containing five µl of Power SYBR Green PCR Master Mix (Applied Biosystems), 0.25 µl of each primer (Table 1) (10 mM), two µl of cDNA template, and two µl of RNase-free water were amplified using AB Applied Biosystems Real-Time PCR system (AB Applied Biosystems, USA). The thermal conditions were as follows: initial denaturation at 95 ◦C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 61 °C for 30 s, and a final extension at 72 ◦C for 1 min. Each run was verified with a cDNA template-free negative control, and the

gene for actin-related protein 2 (ARP2) was used as an internal control since it is constitutively expressed.

Calculation of the relative gene expression

Based on the expression of the specific mRNAs in the untreated trophozoites, the threshold for positive real-time PCR was calculated using equation $2^{-\Delta\Delta Ct}$. A relative fold change in gene expression was measured in three replications, and values > 2 or - 2 were considered indications of gene expression increases or decreases.

Statistical Analysis

Results were analyzed using the Independent Samples *t*-test (SPSS 20, SPSS Inc., Chicago, IL). The significance levels are expressed at a 95% confidence ($p \leq 0.05$).

Results

Cytotoxicity and adhesion increased in chlorine-treated trophozoites

In a cytotoxic assay, the treated trophozoites with the sublethal chlorine concentration caused more severe damage to Raw 264.7 macrophage cells within 12 h of incubation, as determined by hematoxylin staining. In 24 h, cytotoxic effects increased, and monolayers detached (Fig 1). In order to determine whether the cytopathic effects were a result of lysis or disrupted monolayers, LDH release was measured. LDH was not released by *Acanthamoeba* incubated alone, and macrophage cells showed 6% cytotoxicity. There were significant differences in cytotoxicity at 12 and 24 h in treated trophozoites, as shown in Table 2. In addition, treated trophozoites adhered to macrophage cells in a high and considerable percentage (92.5%), but compared to the untreated group (86.4%), the difference was not significant (Fig 2). According to cytotoxicity

and adherence results, treated trophozoites adhered to and degraded cell monolayers, and cytotoxicity rates were significantly different (Fig 1).

Thermo- and osmotolerance decreased in chlorine-treated trophozoites

Results of thermo and osmotolerance tests are presented in Table 2. At 43°C, untreated trophozoites developed, but chlorine-treated cells developed at 37°C and did not grow at 43°C. Despite the lower growth of treated trophozoites at 0.5 M mannitol and the absence of growth at 1 M, untreated cells survived and developed at both concentrations.

Chlorine-treated trophozoites engulfed more bacteria than untreated trophozoites

An uptake assay was performed after two hours of cocultivation at 25°C in order to compare the efficiency of bacterial uptake by treated or untreated *A. castellanii* trophozoites. Results demonstrated that there was a higher bacterial uptake rate by chlorine-treated trophozoites than in untreated cells ($p < 0.05$) (Fig 1). Bacteria are more likely to be engulfed by chlorine-treated *A. castellanii* trophozoites.

Antioxidant responses triggered in chlorine-treated trophozoites

Under oxidative stress conditions caused by chlorine treatment, *A. castellanii* antioxidant enzymes showed significant differences in their activities. The treated and untreated cells had significantly different glutathione and GSH levels and GSH/GSSG ratios ($P = 0.04$, $P = 0.006$ and $P = 0.001$, respectively). CAT and SOD activities were significantly increased in chlorine-treated cells ($P = 0.03$, and $P = 0.03$, respectively). Chlorine treatment of *A. castellanii* trophozoites significantly altered TAC and MDA levels (Table 3).

Virulence genes upregulated in chlorine-treated trophozoites

In order to ascertain the impact of a sublethal chlorine concentration on the expression of some virulence genes, MBP, CP3, and SEP expression were examined. Quantifying mRNA expression in chlorine-treated trophozoites revealed that all the mentioned genes were upregulated. Fig 2. shows the gene mRNA expression in chlorine-treated trophozoites.

Discussion

In recent years, many studies have been conducted to obtain effective antimicrobial compounds, and good progress has been achieved in this issue. But few studies have focused on the changes in microbes after exposure to inappropriate concentrations of antimicrobial compounds. While it is essential to consider microbes cellular changes after inappropriate intervention in the environment. Therefore, in this study, we investigated the effects of sub-lethal concentrations of chlorine on *Acanthamoeba*. There were two reasons for choosing and evaluating chlorine in this study. The first reason was that chlorine is the most common water disinfectant. Another reason was the chlorine mode of action. Chlorine is an oxidative biocide that can remove electrons from susceptible chemical groups and oxidize them. At a cellular level, low levels of oxidation can be highly reversible, and organisms have evolved many defenses against these effects. At higher biocide concentrations, these defense mechanisms can be overwhelmed (Finnegan et al., 2010). Chlorine and other chlorine-based disinfectants are oxidative agents that generate hydroxyl radicals via a Fenton-type reaction, and these reactive oxidative species can disrupt cellular structures, metabolic processes, and membrane structures (Wang et al., 2010). Besides, in *Acanthamoeba*, oxidative stress has a crucial role in its pathogenesis processes like cytolysis

and tissue invasion. Therefore, when *Acanthamoeba* encounters chlorine, its oxidative stress responses are triggered. Now, it is necessary to investigate the effect of inappropriate chlorine concentration on these responses and, ultimately, on the pathogenicity of amoeba. In this study, in macrophage culture, trophozoites that survived after two h treatments with five ppm chlorine showed higher cytopathic effects. A significant difference in adhesion and cytotoxicity rates was observed for the treated trophozoites. It is proved that when environmental forces induce damage in macromolecules, a cellular stress response is set in motion. This includes the changes in gene expression, DNA repair mechanisms, protein turnover modifications, and redox balance restoration (Jimenez 2014). It seems that stress-triggered regulatory systems may involve in the amebic survival process and virulence potential. In bacteria, oxidative stress triggers the efflux pump system, contributing to antibacterial resistance and virulence enhancement. Stress responses in trophozoites treated with the sub-lethal chlorine concentration were associated with the stimulation of amebic virulence factors. However, more studies are needed to understand the virulence-related stress response mechanisms in *Acanthamoeba* trophozoites.

In this study, treated trophozoites with sublethal chlorine exposure were capable of surviving at temperatures of 37 °C, but they could not grow at high temperatures (43 °C). Furthermore, treated trophozoites showed osmotolerance to 0.5 M D-mannitol but not 1 M. Generally, the in vitro growth of *Acanthamoeba* isolates under relatively high osmotic stress or at a relatively high temperature can be related to virulence since virulence is at least partially associated with an isolate's capacity to adapt and remain viable in the tissues of a mammalian host. But according to Landell et al. (2013), thermotolerance and osmotolerance tests are not necessarily indicative of pathogenicity or virulence, and in vivo testing and cell culture are necessary to characterize *Acanthamoeba* isolates for pathogenicity.

To assess the phagocytic ability of chlorine-treated trophozoites, the uptake assay was performed after two h of cocultivation of *A. castellanii* trophozoites and *E. coli* at 25°C. Amoebae consume bacteria as a food source to fulfill their nutritional needs by taking up bacteria through phagocytosis. Phagocytosis in *Acanthamoeba* is a receptor-dependent process involving intracellular signalling and cytoskeletal rearrangement (Gonzalez robles et al., 2014). Tyrosine kinase-mediated signaling, phosphatidylinositol 3-kinase, and MBP play important roles in *Acanthameoba* uptake of *E. coli* (Alsam et al., 2005). Different studies imply that stress can regulate phagocytosis in different ways, depending on the kind of phagocytic receptor involved, the level of stress, and the physiological state of the cells. From the host point of view, macrophages and amoebae display similarities in their interactions. Both cells ingest particles into phagosomes and have lysosomal enzymes responsible for digestion and nutrient acquisition (German et al., 2013). Some studies indicated that high oxidative stress attenuates macrophage function, primarily resulting in reduced phagocytic capacity. Besides, they showed that lower oxidative stress could improve phagocytosis and maturation of macrophages (Brown et al., 2009; 2012). This study showed that the induction of oxidative stress via chlorine exposure and the amoeba's adaptive responses increased phagocytosis in trophozoites.

Free radicals and oxidant species may behave as deleterious and toxic products involved in cellular dysfunction. DNA, lipids, and proteins are damaged when these species are overproduced. Living organisms are equipped with a variety of antioxidants that serve to counterbalance the effects of oxidants. In this study, the antioxidant response of exposed *Acanthamoeba* to chlorine as the oxidative agent was investigated. Like other aerobic organisms, *Acanthamoeba* protects itself against oxidative stress assaults through antioxidant networks consisting of enzymatic and non-enzymatic components. Glutathione (GSH) is the smallest

intracellular protein thiol molecule in the cells, preventing cell damage caused by reactive oxygen species (ROS). Reduced glutathione (GSH) is considered to be one of the most important scavengers of ROS, and its ratio with oxidized glutathione (GSSG) may be used as a marker of oxidative stress (Jones and Go 2010). The GSH and GSH/GSSG were significantly lower in the chlorine-treated cells. The GSH/GSSG ratio tends to decrease in severe oxidative stress. Therefore, our results showed that treated trophozoites struggled with oxidative stress due to chlorine. Prominent among antioxidants are the SODs, a group of metalloenzymes that detoxify the high reactive O^{-}_{2} to H_2O_2 and ground state oxygen (O_2) by a dismutative reaction. Catalases are enzymes that prevent cell oxidative damage by degrading hydrogen peroxide to water and oxygen (Yamakura and Kawasaki 2010). This study showed that the activities of SOD and CAT were significantly greater in the treated trophozoites. Oxidative stress due to chlorine triggered antioxidant responses of trophozoites, and elevation of antioxidant enzymes declare the fight of amoeba against oxidative stress. The induced oxidative stress due to chlorine increased the MDA level as a marker of lipid peroxidation. MDA and protein carbonyl content were also significantly increased by H_2O_2 exposure, according to Motavallihaghi et al. (2018).

Total antioxidant capacity measures the number of free radicals scavenged by a test solution used to evaluate cells' antioxidant capacity. The measure of TAC considers the cumulative action of all the antioxidants present in the cell, thus providing an integrated parameter rather than the simple sum of measurable antioxidants (Suresh et al., 2009). TAC differed significantly between this study's treated and untreated cells, confirming that the amoeba fosters its total antioxidant capacity to overcome oxidative conditions. In *Acanthamoeba*, Motavallihaghi et al. (2022) found increased SOD, CAT, GR, and GPX activities under oxidative stress induced by H_2O_2. Pathogenic *Acanthamoeba* initiates the invasive process by attaching amoebic mannose-binding

receptors to mannosylated glycoproteins of host cells. This early interaction will release proteases, determinants for extracellular matrix disruption and cell death by necrosis and apoptosis (Neelam and Niederkorn 2017). Pathogenic *Acanthamoeba* has exhibited increased extracellular protease activities, and there is a link between pathogenicity and the increased levels of extracellular proteases. Pathogens use the secretory system to express virulence factors and cope with stress conditions, which might favor their adaptation to specific biological niches (Krishnan and Askew, 2014). The genus *Acanthamoeba* elaborates serine and cysteine proteases. Although several studies have implicated proteases with *Acanthamoeba* pathogenicity, they may also be salient to *Acanthamoeba* survival, multiplication, and cellular transformation (Dudley et al. 2008). Exposure to oxidative stress due to chlorination and combatting for survival and transforming to cysts led to significantly higher expression of virulent genes like MBP, CP3, and SEP.

Conclusion

This study showed that chlorine, as the antimicrobial and oxidative agent, is a double-edged sword against *A. castellanii*. It can form resistance and virulent amoebas if it is not used at a proper concentration and exposure time.

The proper concentration of a disinfectant is important to achieve the best results. Considering the length of the disinfection time, which depends on the germicide potency, is also essential.

The importance of the existence of resistance and invasive free-living amoeba in the environment cannot be neglected due to their health importance and bacterial protection. Identification of stress responses, their mechanisms in *Acanthamoeba,* and their relation to amoeba virulence would give us a better perception of their pathophysiology. This information

would be helpful for designing effective antiamoebic agents. The existence of amoebae should be considered in water and environment disinfection programs.

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Conflict of Interest

The authors declare there is no conflict of interest.

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