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EFFICACY OF ZINC OXIDE NANOPARTICLES AS WATER DISINFECTANT AGAINST *CRYPTOSPORIDIUM PARVUM*

By
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Abstract

Cryptosporidiosis is a considerable cause of global diarrhea-related morbidity and mortality, especially among children and immunocompromised individuals. In addition, various studies suggested a link between cryptosporidiosis and cancer. There is no fully effective treatment or vaccination for cryptosporidiosis. Moreover, these highly infectious oocysts are usually resistant to the routinely used disinfection measures, and subsequently repeated waterborne outbreaks occur. Zinc oxide nanoparticles (ZnONPs) are widely used safe material that show good antimicrobial properties. The study investigated the efficacy of ZnONPs in disinfecting water *Cryptosporidium parvum* oocysts.

Cryptosporidium parvum oocysts were incubated with different concentrations of ZnONPs or chlorine for one hour. Then, we further investigated the ability of the ZnONPs-pretreated oocysts to induce pathology in experimental animals. Evaluation was performed regarding onset of oocyst shedding, oocyst score, degree of intestinal pathology and apoptosis. Our results showed that the oocyst pretreated with 90 and 120 µg/ml ZnONPs concentrations had the latest onset of oocyst shedding and the lowest shedding scores. Moreover, they presented the mildest degrees of pathology and apoptosis.

Keywords: *Cryptosporidium parvum,* Zinc oxide nanoparticles, water disinfection.

Introduction

Cryptosporidium is a waterborne intestinal zoonotic parasite that causes many serious health problems, which may be fatal, especially in children and immunocompromised individuals. Moreover, it is an important defining-illness for acquired immunodeficiency syndromes (Kotloff *et al,* 2013). In Egypt, there was an incidence <25% in children with cancer (Mohammad *et al,* 2021). Added to its potentially fatal diarrhea, a link between *Cryptosporidium* and malignancy, especially colonic carcinoma, was suggested by some epidemiological studies (Kalantari *et al,* 2020). It is also suspected that even asymptomatic infections negatively affect the health of the host (Huang *et al,* 2004).

The global prevalence of cryptosporidiosis varies greatly and is higher in developing countries and areas of limited access to safe water sources. Unfortunately, this global prevalence is expected to rise in the coming years due to worldwide climatic changes and increased water demands (Ashigbie *et al,* 2021; Rahman *et al,* 2022). Ocysts of *Cryptosporidium* are characterized by their high resistance to environmental factors, they can survive for several days even in presence of the commonly used disinfectants. Besides, their infective dose is very low. As few as 10 oocysts can cause infection even in immunocompetent host (Gharpure *et al,* 2019). This was the reason for the occurrence of many outbreaks of cryptosporidiosis especially with the usage of recreational water (Pinto and Vinayak, 2021).

Cryptosporidium oocysts were found in all water types. The highest prevalence was in wastewater and the lowest was swimming pool water and marine water (Daraei *et al,* 2021). Due to its resistance to commonly used disinfectants, *Cryptosporidium* is a real problem for the wastewater reuse industry (King *et al,* 2017). The absence of an effective vaccination and the incomplete efficacy of the only approved anti-*Cryptosporidium*

therapy, nitazoxanide makes water disinfection a main step in controlling such parasite (Pinto and Vinayak, 2021).

Water disinfection is usually performed by many conventional methods e.g., chlorination, ultraviolet (UV) treatment and ozonation. These methods have disadvantages that make them unideal for water disinfection. Chlorination is the most widely used method for water disinfection because of its relatively low cost. Despite that it forms a potentially carcinogenic disinfection byproduct (DBP) that results from its interaction with waterborne pathogens especially when used in high doses to overcome the chlorine-resistant pathogens. Similarly, ozonation can generate toxic bromate after reacting with bromide ions in water. Moreover, its cost is higher than chlorine. Neither ozone nor UV treatments leave a residual in treated water and subsequently there will be no re-infection safeguard in the distributing networks. In contrast to traditional disinfection methods, nanotechnology offers new possibilities for DBP-free water treatment (Collivignarelli et al. 2018; Dimapilis et al. 2018). Silver nanoparticles (AgNPs) showed satisfying antiprotozoal activity against *C. parvum* waterborne infections (Cameron *et al,* 2016; Abou Elez *et al,* 2023). Despite that, relatively high cost and narrow window between efficacy and toxicity limits their practical application. These limitations were found to be relatively lower with another metal oxide that also showed lower organ damage, zinc oxide (ZnO) (McGuffie *et al,* 2016; Wang *et al,* 2017; Das *et al,* 2019).

The ZnONPs specific physical and chemical properties, e.g., large surface to volume ratio, small size, antimicrobial activity, photocatalytic and semiconducting properties, introduced zinc oxide nanoparticles (ZnONPs) to be deeply investigated as one of new generation physical therapies for cancer, nano-antibiotics and osteo-inductive mediators for regenerating bone tissue sunscreens, and thermal control paints (Osmond and Mccall, 2010; Fereshteh *et al,* 2013; Carofiglio *et al,* 2020). Moreover, ZnONPs are used in many food industries, being safe to use (Espitia *et al,* 2012). Usage of ZnONPs in water and wastewater disinfection was encouraging, as It overcame the limitations of conventional water treatment methods, mainly DBP formation. But, there was lack of information regarding the performance of ZnONPs as antimicrobial agent of water pathogens (Dimapilis *et al,* 2018).

The present study aimed aimed at evaluating the efficacy of different ZnONPs concentrations against the resistant *Cryptosporidium parvum* to conventional water disinfectants, using ZnONPs-pretreated parasite to induce pathology in experimental mice.

Material and Methods

Ethics statement: Mice were kept under standard housing environment of food and temperature in the animal house of Theodor Bilharz Research Institute, TBRI (Giza). All experimental procedures were done in accordance with international ethical guidelines after approval by the institutional ethics committee (number 10/2022PARA27).

Study design: A total of 50 inbred dexamethasone-immunosuppressed pathogen-free male BALB/c mice (6-8 weeks, & 18-20g) were randomly divided into 3 main groups. GI: 10 mice served as crude *C. parvum* infected control. GII: 10 mice served as chlorine-pretreated *C. parvum* infected one. GIII: 30 mice infected pre-treated with ZnONPs, which was classified into subgroups A-C. Each subgroup included 10 mice.

Immune suppression of animals: Immune-suppression started 14 days before infection by oral dexamethasone (Kahira Pharmaceuticals & Chemical Industries Co., Egypt) at a dose of 0.25µg/g/day (Moawad *et al,* 2021).

Preparation of disinfectants: Chlorine disinfectant was prepared by dissolving High test hypochlorite (HTH) 65% in sterile distilled water to a concentration of 5 mg/liter (Adeyemo *et al,* 2019). A stock of ZnONPs suspension with average particle size of 11-21 nanometers (nm) (Sigma Aldrich., USA) was serially diluted in sterile distilled water

to get concentrations of 60, 90, 120µg/mL for subgroups A, B & C respectively (Delavari *et al,* 2014).

Oocyst preparation and disinfection: Intestinal contents of naturally infected calves were stained by modified Ziehl-Neelsen (MZN) and microscopically examined for the oocysts. *C. parvum* DNA was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen, USA) then genotyped as *C. parvum* by PCR of *Cryptosporidium* oocyst wall protein (COWP) gene (Hassan *et al,* 2019). After genotype confirmation, oocysts were concentrated and purified using Sheather's sugar flotation solution (JORVET, USA), treated with 0.6% sodium hypochlorite, and washed with phosphate buffered saline (PBS). The stock of *C. parvum* oocysts was effectively mixed and equally distributed in five antiseptic tubes marked as I, II, IIIA, IIIB, & IIIC, counted by hemocytometer to get a concentration of $1x10^5$ oocysts/ml of the disinfecting solution according to group. Tube (I) served as a crude control suspended in distilled water without disinfectant. Tube (II) served as chlorine treated mice. Serial dilutions of ZnONPs, 60, 90 &120µg/ml were added to IIIA, IIIB, & IIIC tubes respectively. All tubes were kept at room temperature in daylight for one hour with frequent mixing of the contents.

Cryptosporidium parvum challenge infection: Before challenging infection, fecal samples from all mice were examined to con firm no parasitic infection by direct wet smear and MZN technique for three consecutive days. Each mouse was orally inoculated with one mL of oocyst suspension according to group. MZN-stained stool samples were examined day-to-day for oocysts to confirm infection onset day (Leitch and He 1994).

Oocyst shedding: Fresh fecal pellets were regularly examined for oocysts every 3 days until the end of the experiment at 4, 7, 10, 13, & 16 days post infection (dpi). Samples were suspended in 10% formalin, homogenized, smeared on microscopic slides, and stained with MZN. Oocysts were counted using a hemocytometer and an oocyst shedding score was calculated per smear for each mouse (0= no oocysts; 1= one to 10 oocysts; 2= 11 to 50 oocysts; 3 = 51 to 100 oocysts; 4 >100 oocysts) (Leitch and He 1994)

Histopathological study: On 16^{th} day pi, mice were euthanized by decapitation. Distal jejunum and ileum were dissected out, put into 10% formalin. Paraffin blocks were prepared, and sequential tissue sections were stained with hematoxylin and eosin (H&E), examined microscopically, and classified into mild, moderate, and severe pathology according to the detected structural alterations (Laurent *et al,* 1999).

Evaluation of tissue apoptosis: Expression of caspase-3 in intestinal tissue was assessed by using anti-caspase-3 antibody (Abcam-USA). Positive staining was identified when cell membrane and/or cytoplasm were colored brown. Immunohistochemical grading of caspase-3 staining was determined by histoscore (H-score). Membrane intensity staining was given a number from (0, 1+, 2+ & 3+) and multiplied by brown stained cells percentage in each tissue. A score of 0-300 was given to each specimen [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)] after Fraser *et al.* (2003).

Statistical analysis: Data were represented in mean ±SD, median, number (No.) and percentage. After Homogeneity testing, one-way ANOVA (F) (with LSD as post Hoc) and Kruskal Wallis (K) (with Tamhane as Post Hoc) compared variables among groups. Repeated measures ANOVA (F) (with Mauchly's W test for sphericity testing and Bonferroni correction) compared among different consecutive measures in each group. Chi-square test ($\chi 2$) with Z test to compare column proportions to associatebetween categorical variables and whenever any of the expected cells were less than five, Fischer's exact test was used. Two-sided P value <0.05 was considered significant.

Results

The ZnONPs-pretreated mice in subgroups B & C started to shed oocysts after <6 days

of infection (6.50±0.70 & 6.70±0.48 respectively; $p = 0.567$; F=30.17 - $p < 0.001$) scoring the slowest onset of oocyst shedding with significant differences compared with other groups ($p < 0.01$). Low ZnONPs concentration, used for subgroup A, also elicited a marked delay in oocyst shedding onset (5.50±0.84) and ranked, with chlorine treated mice (4.80±1.13; $p= 0.05$), second to subgroups B & C. Differences between first and second ranked groups were significant ($p < 0.001$). Infected control mice (3.40±0.51) started oocyst shedding earlier than all the studied groups with statistically significant differences compared with them ($p < 0.01$).

All ZnONPs-pretreated oocysts showed statistically significant lower oocyst scores compared with the crude controls ($p < 0.05$) throughout the duration of study (4^{th} day K=39.08 - $p<0.001$; 7^{th} day F=5.96 – $p <0.001$; 10^{th} day F=32.07 - $p <0.001$; 13^{th} day K=41.87 - $p<0.001$; 16^{th} day K=43.50 - $p <0.001$). Lowest oocyst scores in the study were detected in subgroups C & B, followed by subgroup A & chlorine-pretreated mice. Differences between first and second ranked groups were statistically significant in all days of oocyst score assessment ($p<0.05$).

All crude control mice showed severe pathological degree with significant differences compared with ZnONPs-pretreated subgroups that were pathology free (FE=41.35 - $p<0.001$). Moreover, pathological degree decreased by increasing ZnONPs concentrationons. Subgroup C showed lowest degree (mi-ce 80% showed mild and 20% showed moderate pathology). Subgroup B ranked 2^{rd} (mice 60% showed mild but, 40% showed moderate pathology). Subgroup A ranked 3^{rd} among ZnONPs-pretreated sub-groups (mice 40% showed mild, but 60% showed moderate pathology). Chlorine pretreated oocysts caused severe pathology in mice (30%, moderate, 60% mild & 10% none.

The intestinal apoptosis was in accordance with the degree of pathology and showed marked reduction of apoptosis in ZnONPs pretreated groups (F=143.08 - $p<0.001$). The lowest H-score of apoptotic marker caspase-3 was detected in subgroup C (47.0±8.23), which showed significant differences compared to others ($p < 0.001$). Second rank for subgroup B was (69.0±8.75). Although H-score of subgroup A (102.0± 13.98) ranked third, it remained statistically lower than both chlorine (GII) and crude control (GI) groups (130.0±17.63 & 182.0±18.13 respectively; $p < 0.001$ groups).

Details were given in figures (1, 2, 3 & 4).

Discussion

The lack of an effective cryptosporidiosis therapy, especially in immunocompromised patients, makes the preventive measure a main strategy for its control especially with the growing increase in health problems that leads to suppression of the immunity (Dhal *et al,* 2022). Because *C. parvum* is resistant to the commonly used water disinfectants (Betancourt and Rose, 2004), introduction of new disinfectants is an important step to control this potentially fatal disease. In the current study, ZnONPs proved to be less toxic. This agreed with Das *et al.* (2020), who reported that treating *Xiphophorus hellerii* fish infection with AgNPs was associated with moderate damage of gill, intestine, and liver with 83.3% survival rate.

However, ZnONPs-treated fish presented minimal tissue damage with a 100% survival rate. Safety of Zinc oxide was also approved by the Food and Drug Administration (FDA) as one of five zinc compounds that are "safe compounds" for human use. Its nanoparticles showed promising antimicrobial properties through changing membrane permeability and inducing oxidative stress (Sirelkhatim *et al,* 2015). Moreover, ZnONPs have many specific properties that make it a practical material to be used compared with other AgNPs. In addition to its low cost, stability, and easy preparation, it has a wide therapeutic window between efficacy and toxicity. It also showed a good selectivity for bacteria over mammalian cells (McGuffie *et al,* 2016). These criteria encouraged to select ZnONPs as a candidate water disinfectant.

In the present study, over vital dye staining for ZnONPs-pretreated *C. parvum,* was not avoided. This agreed with Tarazona *et al.* (1998), who reported that immunosuppressed mice model gave more or less cryptosporidiosis picture in humans without being affected by the hosts' immunity. Also, Mahittikorn *et al.* (2014), who found that as vital dye staining, overestimated non-viable oocysts compared to animal model.

In the current study, infection establishment and onset of oocyst shedding significantly varied among groups, latest in ZnONPs-pretreated oocysts with higher concentrateons. This agreed with Zambriski *et al.* (2013) who reported that the less infective *C. parvum* dose*,* the more delay in shedding onset and progression. Thus, NPs killed or at least attenuated *C. parvum* oocysts with the dose-dependent manner (Nasser 2016). The present non-significant difference between B & C subgroups gave a good result without high concentrations. Also, the present concentration killed $1x10^5$ oocysts/ml was extremely higher than the concentrated naturally occurred in wastewater that didn't exceed 200oocyst/ml (Nguyen *et al,* 2016).

In the present work, the dose-dependent oocyst inhibition detected was by ZnONPs pretreatment. This agreed with Joe *et al.* (2017), who found that by disturbing oocyst wall glycoproteins allowed entry and thus, dissolution of ZnONPs increased toxic Zn2+ ions in the infective agents. Also, Siddiqi *et al.* (2018) and Prasad *et al.* (2021) reported that ZnONPs photosensitive properties were activated on exposure to light forming reactive oxygen species that impair parasite's virulence related surface glycoprotein molecules with oocyst infectivity attenuation. No doubt, NPs small size allowed easy diffusion via cell membrane pores to exert a direct toxic action (Sabourian *et al,* 2020). Besides, it bound with DNA molecules and disrupt vital biochemical processes of *C. parvum* (Czyżowska and Barbasz, 2022).

In the present study, in spite of the oocyst shedding similarity between chlorine-pretreated and ZnONPs-pretreated subgroup A, a severe inflammation was detected in chlorine pretreated mice. This agreed with Saleh *et al.* (2019), they reported that the ZnONPs reduced the pathogenesis of *Pseudomonas aeruginosa* by inhibiting Quorum sensing-controlled virulence factors rhamnolipids, pyocyanin, pyoverdin, hemolysins, elastase and proteases and genes responsible for control. Besides, Abdelghafar *et al.* (2022) reported that ZnONPs decreased *Pseudomonas aeruginosa* and *Staphylococcus aureus* cell surface hydrophobicity attenuated their bacterial biofilm-forming capacity.

In the current study, ZnONPs prevented *C. parvum* oocysts attachment to the intestinal tissue of mice. Ojcius (1999) reported that attachment was an important for *Cryptosporidium* to reside on the epithelial cells apical surface of intestine and didn't invade deeper layers of humans' gastrointestinal mucosa. Dhal *et al.* (2022) reported that attachment prevention could reduce cryptospodiosis pathological effect.

In the present study, *C. parvum*-induced pathology was associated with apoptosis of some intestinal cells as a trial of the parasite to maintain infection. This agreed with Mele *et al,* (2004), who reported that main morphological changes of apoptosis were mediated by a family of intracellular cysteine proteases, caspases. So, the present decreased apoptosis scores in ZnONPs-pretreated mice were due to the less pathological changes with attenuated oocysts virulence.

Conclusions

The 90 &120µg/ml ZnONPs concentrations gave the best disinfection of water *C. parvum* oocysts' infectivity, and thus, intestinal pathology and apoptosis. This was a pilot study that identified ZnONPs as a potential water disinfection candidate.

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Explanation of figures

Fig. 1: Column chart presentation of onset days of oocyst shedding in groups. Latest onsets detected in subgroup C followed by subgroup B, with significant differences compared with others.

Fig.2: Curve presentation of progression of oocyst score in groups. Slowest progressions detected in subgroups B & C.

Fig. 3: Pathological changes. A: Column chart presentation of pathologic degree in groups. Highest % of mild pathology in subgroup C followed by subgroup B, B: H&E-stained jejunum of subgroup IIIC showed mild inflammatory infiltrate in lamina propria of intestinal villi

(yellow arrow), No shortening or blunting of villi, C: H&E-stained jejunum of subgroup IIIA showed moderate inflammation (green arrow) chronic inflammatory cells in lamina propria and shortening of villi, & D: H&E-stained jejunum of chlorine-treated group showed severe inflammatory changes in the form of severe chronic inflammation, shortening, and blunting of villi (green arrow) (x200).

Fig. 4: Apoptotic changes of groups. A: Column chart presentation of H-score in groups. Lowest H-score was detected in subgroup C followed by subgroup B, B: Jejunum of subgroup IIIC showed low caspase-3 expression, C: Jejunum of subgroup IIIA showed moderate caspase-3 expression, & D: Jejunum of infected control GI showed strong caspase-3 expression (x200).