Zinc Oxide Nanoparticles Kill Giardia and Protect Against Intestinal Damage

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Background: Giardiasis is the most common diarrheal disease among children and travelers and a life-threatening agent in some immunocompromised patients. Zinc is an important element in resistance to Giardia-induced intestinal damage. Objectives: evaluation of the efficacy of zinc oxide nanoparticles (ZnO-NPs) as a therapy for giardiasis and if its action as a source of zinc can give a chance to benefit from its immune stimulating and tissue protecting properties. Methodology: Therapeutic effects of ZnO-NPs were studied alone or in combination with metronidazole (MTZ) in mice experimentally infected with Giardia intestinalis. Antiparasitic efficacy, serum zinc levels, intestinal cell function, intestinal pathology, apoptosis, and local intestinal immunity were assessed. Results: The present study showed that, despite the more reduction of Giardia-cysts induced by MTZ, ZnO-NPs achieved better functional, histopathological, and immunological improvement of the intestinal mucosa compared to MTZ. The best results were reached with MTZ/ZnO-NPs combination. Conclusion: ZnO-NPs killed Giardia, protected intestinal cells and helped their regeneration. These effects can be related to improved zinc levels that was also reflected on potentiation of local intestinal immunity. Besides, ZnO-NPs could decrease the incidence of apoptosis preserving properly functioning intestinal cells.

INTRODUCTION

Giardiasis is a worldwide diarrheal illness caused by the protozoan parasite Giardia intestinalis (syn. Giardia lamblia or Giardia duodenalis). It is the most common - and sometimes the earliest - parasitic infection in children. It is also the most common infection in travelers. More than 500000 new cases are reported every year with a prevalence ranging from 2% - in developed- to 20:30% in developing countries. It can be a life-threatening infection in some immunocompromised patients if they are refractory to treatment.

Despite its noninvasive behavior, G. intestinalis can induce many pathological changes in the small intestine which lead to insufficiency of intestinal disaccharidases with malabsorption of nutrients and electrolytes that present clinically as diarrhea. Zinc is one of the most affected electrolytes that markedly decrease in serum during giardiasis. This trace element plays an important role in diarrheal pathology. That’s why both World Health Organization (WHO) and United Nations Children’s Fund (UNICEF) recommended to double the daily requirements of zinc in diarrheic children and introduced zinc as an important ingredient of oral rehydration solution. Both giardiasis and zinc have an inverse relationship. Giardia infection usually leads to zinc deficiency by preventing its absorption. On the other side, zinc is important in resistance to giardiasis induced pathology. Its administration during giardiasis can prevent the associating weight loss and even enhances parasite clearance by up-regulating the host’s immune response with production of specific antibodies and immune cells. It also works beyond giardiasis by helping regeneration of intestinal epithelium and even improving its absorptive ability after diarrhea.

Despite the presence of multi-choices of anti-giardiasis drugs, all of them are associated with many side effects that range from gastric upset to leukopenia and hemolytic anemia. The problem of the drug-resistant Giardia and current drugs’ adverse effects encouraged trials to discover new chemical or natural alternatives that mainly target the parasite and protects host tissues from post-infection sequelae. In targeted drug delivery, nanoparticles (NPs)-based drugs are important candidates. Zinc oxide (ZnO) is one of the five zinc compounds that are currently listed as a “safe compound” by Food and Drug Administration (FDA) organization to be used by humans. Its NPs (i.e. ZnO-NPs) could improve the activity of several serum enzymes and were used as a new additive to meet the nutritional zinc requirements. In addition, ZnO-NPs have many antimicrobial properties that are mediated by...
altering the permeability of membranes and inducing oxidative stress that leads to bacterial cell death.  
Based on these data, the current study was designed to evaluate the efficacy of ZnO-NPs as a therapy for giardiasis and if its action as a source of zinc can give a chance to benefit from the immune stimulating and tissue protecting properties of zinc.

METHODOLOGY

Ethics Statement
All animal experiments were performed at Theodor Bilharz Research Institute, TBRI (Giza, Egypt). Mice were kept under standard housing conditions in the animal house of TBRI and were maintained on a zinc-free diet purchased from National Research Centre. Room temperature was kept at 20-22°C. Experimental procedures were performed in accordance with the international ethical guidelines after the approval of the institutional ethical committee of TBRI.

Study Design
Mice were divided into five groups, 10 mice each. Group I (GI) served as negative controls (non-infected non-treated mice). The remaining 4 groups were infected with G. intestinalis. Group II (GII) was the positive control group. Group III (GIII) was treated by oral metronidazole (MTZ). Group IV (GIV) was treated with oral ZnO-NPs. Group V (GV) was treated with both oral MTZ and ZnO-NPs.

PROCEDURES:
Parasite and mice infection:
Trophozoites of Giardia assemblage B were obtained from mice previously infected by cysts from human stool of infected patients. Patients’ stool was genotyped as a part of a genotyping study for Giardia (unpublished work). For experimental inoculation, actively motile trophozoites were suspended in phosphate buffered saline (PBS) and were adjusted to obtain a concentration of $1 \times 10^5$ trophozoites in 200 μL which is the infecting dose for each mouse. Before inoculation, a 6–9 h fasting period with no water restraint was required to facilitate infection procedure. The trophozoites were inoculated directly into the duodenum of male BALB/c mice (6–8-week-old, 18-22 gm weight) using a syringe fitted with a cannula needle to prevent tissue damage. Stool samples were regularly examined for Giardia cysts to confirm the establishment of infection. In the present study, infection was established in all mice 7 days post infection (d.p.i).

Metronidazole treatment:
Mice of GII and GV were treated with MTZ suspension (Rhone Poulenc Rorer, Sanofi Aventis, Cairo, Egypt) in a dose of 500 mg/kg/day orally for 7 successive days after the establishment of infection.

Zinc oxide nanoparticles (ZnO-NPs) treatment:
ZnO-NPs powder with a mean particle size of 20 nanometers (Nanostructured & Amorphous Materials, Inc., USA) was used. Mice of GIV and GV were treated with an oral dose of 10 mg/kg of ZnO-NPs suspended in 100 μL sterile distilled water once daily for 7 consecutive days after the establishment of infection.

Euthanizing animals and sample collection:
On day 15 p.22 mice were euthanized by decapitation. Blood was collected to measure serum zinc level. Blood samples were centrifuged at 800g for 10 min. The separated serum was stored at -20°C until used. The intestinal lumens of euthanized mice were flushed with ice-cold saline to wash out food particles. The collected stool was used for counting Giardia cysts. The proximal part of jejunum was fixed in formalin 10% for histopathological and immunohistochemical studies and detection of trophozoites. The remaining small intestinal lumens were cut opened and the mucosa was scraped - using a glass slide-, homogenized in 0.9 % saline then centrifuged at 4000 x g force for 10 min at 4°C. The collected supernatants were used for disaccharidases and secretory IgA assays.

Assessment of serum zinc levels:
Serum zinc levels were assessed by spectrometry as described by D’Haese et al. Measurements were carried out at 213.9 nm using a hollow cathode zinc lamp with a coefficient of variation of 2.6% and a recovery of 97%.  

Counting of Giardia cysts:
The collected stool was centrifuged at 400g for 15 minutes. The sediments were resuspended in a known volume of saline. Giardia cysts were counted using a hemocytometer. N.B. Giardia cysts were counted in the whole amount of washed out stool of each mouse then divided by the weight of stool. Cyst count was expressed as number/g of stool.

Assessment of intestinal mucosal cells function by sucrase and maltase enzyme assays:
Biochemical assays of sucrase and maltase activities were used as functional markers of Giardia-induced mucosal injury. Enzyme activities were determined according to Belosevic et al. and expressed as units per gram of protein. Total protein content was determined by Bradford protein assay using bovine serum albumin as a protein standard.

Assessment of intestinal secretory IgA:
Supernatants of intestinal homogenates were used to estimate total secretory IgA levels using Quick Detect TM Secretory IgA (Mouse) ELISA Kit (Biovision, USA). Steps were performed according to the manufacturer’s instructions.

Histopathological examination of small intestine:
Paraffinized blocks of proximal jejunum were cut into thin sections, mounted on clean glass microscopic slides and stained with haematoxylin and eosin (H&E) stain. The slides were examined using a multi-head
microscope, Olympus SC100, and analySIS getIT software. Villous height and crypt depth were digitally measured. Intraepithelial lymphocytes (IELs) were counted along villus units. IELs were expressed as number per 100 epithelial cells 21.

Assessment of intestinal cell apoptosis:
Apoptosis of intestinal cells was detected by immunohistochemical staining of caspase-3 using anti-caspase-3 antibodies (Neomarkers, USA). Positive cells for caspase-3 immune stain appeared as intracellular brown punctuations 30. Immunohistochemical grading of caspase-3 stain was determined by histo score (H-score) where the intensity of membrane staining was given a number from (0, 1+, 2+ or 3+). Percent of stained cells in each tissue was multiplied by the intensity of staining. \[1 \times (\% \text{ cells} 1+) + 2 \times (\% \text{ cells} 2+) + 3 \times (\% \text{ cells} 3+)\]. Then, a score of 0-300 was given for each field followed by a mean score for all fields 31.

Statistical analysis
Data entry, coding, and analysis were conducted using SPSS (20), IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp. Data of this study were of the quantitative type, so they were expressed in Mean and Standard Deviation (SD). Analytic statistics were conducted using ANOVA to estimate the difference between means of more than two parametrically distributed quantitative variables. Post Hoc test was used to assess the difference in two means of two individual groups after a significant ANOVA. The level of significance of the present data was 95%, so, p-value >0.05 was considered a non-statistically significant difference, while p-value < 0.05 was considered a statistically significant difference.

RESULTS

Cyst reduction with MTZ is better than ZnO-NPs
Treatment with MTZ (GIII) achieved a statistically significant higher reduction of Giardia cysts than sole ZnO-NPs (GIV) treated group (p<0.001). Despite that, the highest percent of cyst reduction was recorded with combined MTZ/ZnO-NPs treatment (GV). (p<0.001) [Figure 1. a].

Improved serum zinc levels with ZnO-NPs treatment
Treatment of Giardia-infected mice with ZnO-NPs (GIV) was associated with a statistically significant increase in serum zinc levels compared to either the positive control (GII) or MTZ treated groups (GIII) (p<0.001). The best improvement of serum zinc levels was detected with combined MTZ/ZnO-NPs therapy in GV (p<0.001) [Figure 1.b].

Intestinal functions are preserved with ZnO-NPs
Unlike cyst count, improved intestinal functions - as reflected by sucrase and maltase levels – were higher in ZnO-NPs treated group (GIV) than sole MTZ treated group (GIII) with a statistically significant difference between both groups (p<0.001). The best results were recorded in GV that received combined MTZ/ZnO-NPs therapy [Figure 1.c].

Local intestinal immunity is better with ZnO-NPs treatment
ZnO-NPs treated group (GIV) presented a statistically significant higher level of secretory IgA and IELs compared to sole MTZ treated group (GIII) (p<0.001). Regarding IgA, combined MTZ/ZnO-NPs therapy (GV) was better than all other groups with a statistically significant difference compared to them (p<0.001). IELs count was lower in combined MTZ/ZnO-NPs than sole ZnO-NPs treated group (p<0.001) [Figures 1. d & e].
Figure (1) Comparison between the assessed parameters among the studied groups regarding. a) *Giardia* cyst counts/g of stool. b) Serum zinc levels (μmol/L). c) Intestinal disaccharidases (sucrase and maltase) levels (units/g). d) Secretory IgA levels (pg/ml). e) IELs numbers among studied groups. f) Histopathological measures of the small intestine (villous height and crypt depth) (μm). g) H scores of caspase-3.

N.B.1- Columns with different symbols have a statistically significant difference.

2- Percent numbers refer to percent of change (either increase or decrease) compared to positive control group (GII).
**Reduction of intestinal pathology with ZnO-NPs**

Reduction of intestinal pathology – that was presented as decreased villous height and increased crypt depth – was higher in ZnO-NPs treated group (GIV) than the sole MTZ treated one (GIII) (p<0.001). The best improvement of intestinal pathology was detected in the group that received combined MTZ/ZnO-NPs therapy (GV) with a statistically significant difference compared to the other groups (p<0.001) [Figures 1.f and Figure 2].

![Figure (2): H & E stained tissue sections of small intestine (scale bar = 50 µm). a) A Normal intestinal tissue of negative control group with normal villi and crypts. b) *Giardia*-infected intestinal tissue with marked villous shortening and damaged villi, deep crypts and a mild increase in IELs (referred by the green arrow). c) ZnO-NPs-treated group with improved villous height, crypt depth and a marked increase in IELs (referred by the green arrows).](image1)

**Reduction of intestinal cell apoptosis with ZnO-NPs**

Like the intestinal pathology, apoptotic changes - assessed by immune histochemical staining of the apoptotic marker, caspase-3 – were significantly decreased in ZnO-NPs (GIV) treated group than the MTZ treated one (GIII) (p<0.001). Combined MTZ/ZnO-NPs therapy achieved the best protection from apoptosis. GV had the lowest H-score of caspase-3 [Figure 1.g and Figure 3].

![Figure (3): Immunohistochemical staining of tissue sections of small intestine with the apoptotic marker caspase-3 (scale bar = 50 µm). a) A Negative immune staining of caspase-3. b) *Giardia*-infected intestinal tissue with a strong expression of caspase-3 (referred by green arrows). c) ZnO-NPs treated group with a decreased expression of caspase-3 (referred by green arrows).](image2)

**DISCUSSION**

The current study was designed to evaluate the efficacy of ZnO-NPs as a therapy for giardiasis and if its action as a source of zinc can give a chance to benefit from the immune stimulating and tissue protecting properties of zinc. Assemblage B of *G. intestinalis* was used because of its frequent incidence in our community and wide range of its animal reservoirs. Regarding cyst count reduction - that was higher with MTZ treatment (unlike serum zinc levels)–, some sort of additive effect was detected in combined therapy that achieved 100% cure rate. The action of ZnO-NPs can be related to increased zinc levels with its immune stimulating properties that could suppress *Giardia*. Our results can be explained by findings of Baek et al. 32, Ahmadi et al. 19, Mao & Lien 33, and Wang et al. 34 who reported that ZnO-NPs can act as a source of zinc and compensate its deficiency. As reported by the mentioned authors, the ZnO-NPs-induced increase in serum zinc levels was...
achieved within hours of its administration. The antimicrobial properties of ZnO-NPs reported by Aderibigbe\textsuperscript{20} can explain the ZnO-NPs-induced killing of 
\textit{Giardia}. They related their antimicrobial action to the resulting microbe-targeted oxidative stress. Despite that, the more potent killing of \textit{Giardia} - and subsequently less damaged mucosal cells with better absorptive power - that occurred with combined MTZ/ZnO-NPs therapy in GV achieved the highest percentage of increase in serum zinc levels and \textit{Giardia} cyst reduction.

Despite being the first published study as anti-
\textit{giardiasis} treatment, ZnO-NPs similarly scored potent antiparasitic effects with other organisms e.g. \textit{Leishmania} \textsuperscript{35} and \textit{Eimeria} \textsuperscript{23}. The authors related their results to the ZnO-NPs-induced increase in cellular permeation, oxidative stress, and binding of NPs to microbial DNA, proteins, and lipids that disrupt many metabolic pathways of pathogens leading to their destruction.

Disaccharidases are considered reliable markers of enterocyte maturity and function \textsuperscript{36} that decrease by \textit{giardiasis}-induced intestinal damage. That’s why these enzymes were used to assess intestinal function in the current study. Improved intestinal functions - as reflected by sucrase and maltase levels – that was higher in ZnO-NPs treated group can be related to the improved level of the intestinal protecting trace element, zinc. Best results were recorded in GV that received combined MTZ/ZnO-NPs therapy. The additive effect between both drugs - that induced better improvement of serum zinc levels and 100\% killing of the cause of pathology i.e. \textit{Giardia} was reflected on intestinal function. The positive correlation between zinc and intestinal function is supported by many other studies which similarly reported that zinc deficiency is associated with disturbed intestinal function and subsequent reduction of disaccharidases.

The higher improvement of local immunity markers - either humoral (IgA) or cellular (IELs)- that associated ZnO-NPs treatment can be explained by the action of ZnO-NPs as a source of zinc which by itself is important for normal functioning innate and acquired immune responses. It is essential for normal function and development of innate immune cells e.g. neutrophils, NK cells, and macrophages. It is also important for the growth and function of both T and B lymphocytes and subsequently immunoglobulin production - including IgA - \textsuperscript{12,41,42}. IgA antibodies play an important role in controlling \textit{Giardia} infection. Their increase induces a potent local immune response and rapid eradication of this parasite \textsuperscript{41}. Improved IgA levels can explain the decreased \textit{Giardia}-cyst count that occurred in ZnO-NPs treated groups in the present work. Increased IELs with ZnO-NPs treatment may be another cause of the associating cyst reduction. It is documented that IELs’ cytotoxicity against \textit{Giardia} is higher than splenic cytotoxic cells \textsuperscript{44}. Unlike Scott et al. \textsuperscript{45} - who reported that the main pathology of \textit{Giardia} is mediated by T lymphocytes other than IELs and that IELs number didn’t differ from the uninfected group-, we recorded a statistically significant higher IELs number in the \textit{Giardia}-infected groups - compared to the uninfected control- although the highest increase was detected with ZnO-NPs treatment. The more decrease in IELs that occurred with combined MTZ/ZnO-NPs therapy than sole ZnO-NPs can be related to more killing of \textit{Giardia} that occurred in GV and subsequently less stimulation of immune cells.

Improved immunity was reflected on pathology. Reduction of intestinal pathology – that was higher in ZnO-NPs treated group than MTZ treated one – can be related to improved serum zinc levels which help in the process of regeneration of intestinal epithelium \textsuperscript{14,15} even after a short period of zinc supplementation \textsuperscript{46}. The best improvement of intestinal pathology was detected in the group that received combined MTZ/ZnO-NPs therapy (GV). This can be explained by the more killing of the cause of pathology i.e. \textit{Giardia} that was added to zinc-induced improvement.

Similarly, Dkhil et al.\textsuperscript{23} reported that, besides the good antiparasitic activity of ZnO-NPs against the coccidian parasite, \textit{Eimeria papillate}, they could also improve the infection-induced intestinal pathology. Wang et al. \textsuperscript{34} also reported that ZnO-NPs could improve intestinal pathology (improved duodenal and ileal villus length, crypt depth, and villus surface) when used as a substitute of ZnO and colistin sulphate combination in weaned piglets.

Like the intestinal pathology, the higher reduction of apoptotic changes that occurred with ZnO-NPs can be related to increased serum zinc levels (in ZnO-NPs treated group) which increases intestinal cell resistance to apoptosis as reported by Duff and Ettarh\textsuperscript{15}. Another explanation of the anti-apoptotic action of ZnO-NPs was reported by Shoae-Hagh et al. \textsuperscript{46}. They related the viability protective and anti-apoptotic actions of ZnO-NPs on cultured pancreatic islets to its antioxidant action that decreases stress on living cells. The more potent removal of the cause of apoptosis –i.e. \textit{Giardia}- was associated with more reduction of apoptosis. This was noticed in GV that received combined MTZ/ZnO-NPs treatment and scored the lowest H-score of caspase-3. The \textit{Giardia}-induced apoptosis and the subsequent loss of intestinal epithelium may be a cause of the associating decrease of IELs in the positive control group\textsuperscript{48}.

**CONCLUSION**

All these results can give a conclusion that, ZnO-NPs killed \textit{Giardia}, protected intestinal cells and helped in their regeneration. These effects can be explained by improved zinc levels that was also reflected on
potentiation of local intestinal immunity – increased IgA and IELs. Besides, ZnO-NPs could decrease the incidence of apoptosis preserving properly functioning intestinal cells. Results were the best in combined MTZ/ZnO-NPs therapy that induced more killing of Giardia.

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- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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