

**THERAPEUTIC AND IMMUNO-MODULATORY EFFECT OF SILVER
NANOPARTICLES-LOADED WITH METRONIDAZOLE
ON EXPERIMENTAL BLASTOCYSTOSIS**

By

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Abstract

Blastocystis is one of the commonest enteric protozoa in many hosts. This study evaluated the effect of silver nanoparticles (AgNps) loaded with metronidazole (MTZ) on mice infected with *Blastocystis*. They were subjected to parasitological, histopathological, immunological, and immunohistochemical studies. The parasitological results revealed a powerful effect of Ag NPs loaded with MTZ over either MTZ or Ag Nps; where the reduction of cysts count in stool were (90.7%, 87.33% & 59.33%) respectively as compared to infected non-treated group. Histopathological results showed that AgNPs loaded with MTZ were the best in reducing inflammatory changes followed by MTZ then AgNPs. Regarding IgA, IgM & IgG, Ag NPs loaded with MTZ treated mice showed the best result followed by MTZ treated mice then Ag NPs treated mice; where the reduction of mean concentration of IgA were (30.5%, 26.24% & 2.13%) respectively, while that of IgM were (15.2%, 12.8% & 2.8%) respectively and IgG were (23.1%, 12.3% & 5.2%) respectively as compared to infected non-treated group. The synergistic effect of both Ag NPS & MTZ provoked the best reduction of CD3 & CD20 expression and augmented counter modulation of the immune response to restore normal immune homeostasis.

Key words: *Blastocystis*, Metronidazole, Silver nanoparticles, Experimental mice

Introduction

Blastocystis spp. proved to be one of the commonest enteric protozoa in man and other hosts (Batista *et al*, 2017). *Blastocystis* infection is globally prevalent parasite, affecting more than 1 billion peoples worldwide (Andersen and Stensvold, 2016). In Egypt, the prevalence was 16.7% by Elnazer *et al*. (2017) and 1.8% by El Drawany *et al*. (2019). Subtype 3 was the prevalent zoonotic one worldwide, with a controversy in its pathogenic potential (Lepczynska and Dzi-ka, 2019). Blastocystosis may be asymptomatic or presented with enteric symptoms, as nausea, vomiting, diarrhea, or constipation, abdominal pain, bloating and irritable bowel syndrome or urticaria and rashes (Rendragraha *et al*, 2019). This depended on the parasite virulence and host immune status. The gut is rich with many immune cells, predominately intraepithelial, B & T lymphocytes as the main effector cells that interact with epithelial cells to allow normal homeostasis (Valsecchi *et al*, 2004). B cells are the

main one for humoral immunity producing antibodies in response to antigenic stimulation, develop to memory B cells, act as antigen-presenting cells & secrete signaling cytokines as well (Mauri and Bosma, 2012). T lymphocytes play a central role in cell-mediated immunity (Terabe and Berzofsky, 2008). As *Blastocystis* efficiently adheres to the intestinal epithelial cells, excreting cysteine protease enzymes (Puthia *et al*, 2005; Wawrzyniak *et al*, 2013); it affects the gut immune homeostasis, initiating immunological reactions that include cellular and humoral immune responses with activation of T & B lymphocyte, secretion of cytokines, immunoglobulins & antimicrobial peptides (Yason *et al*, 2016). CD3 & CD20 used by immunohistochemical staining as markers of tissue T & B lymphocytes respectively (Abu El-Fetouh *et al*, 2015).

Despite the controversy of pathogenicity, the symptoms have presupposed treatment if became persistent without other detected pathogens (Mokhtar *et al*, 2016). Many anti-

protozoal drugs either single or in combination with others gave high failure rates (Le Busque *et al*, 2018). Although metronidazole (MTZ) is a golden blastocystosis treatment, yet with wide range of side effects as carcinogenicity, embryogenicity and teratogenicity, as well as variable inappropriate responses ranged from 0 to 100% (Batista *et al*, 2017).

Nanomedicine is the nanotechnology implementation for diagnosis, treatment, prevention and control of diseases (Abaza, 2016). Silver nanoparticles (Ag NPs) proved effective against both extracellular and intracellular parasites (Adeyemi *et al*, 2018). It significantly decreased the *Cryptosporidium parvum* oocysts count and viability in a safe, effective and cheap manner (Hassan *et al*, 2019).

In the present study, Ag NPs loaded with MTZ was evaluated for the augmented therapeutic and immune modulating effects on Albino mice infected with *Blastocystis*.

Material and Methods

Blastocystis spp. culture was kindly obtained from Parasitology Department, Ain Shams Faculty of Medicine, and was sub-passaged on Jones' medium, isolated and infective inoculum was prepared as 10.000/ml.

Drug: Metronidazole (MTZ, Sanofi Aventis Co., Cairo) stock solution of 1mg/ml was prepared as 120µg/ml for oral syrup (Mokhtar *et al*, 2016), and Ag NPs were synthesized (Mulfinger *et al*, 2007). Ag NPs loaded with MTZ: were prepared by adding 2mg/ml Ag NPs solution to 100 mg/ml. MTZ.

Experimental animals: Fifty male clean laboratory bred 7 weeks old & 20gm weight Swiss Albino mice were purchased from Tudor Bilharz Research Institute, Giza. They were handled under conventional conditions according to National Institutes of Health guidelines for animal experimentation, and approved by the Ethical Committee at Faculty of Medicine for Girls, Al-Azhar University.

Experimental design: Mice were divided into five groups of 10 mice each. G1: Heal-

thy controls, G2: Infected untreated controls, G3: Infected MTZ-treated, G4: Infected Ag NPs treated and G5: Infected and treated by Ag NPs loaded with MTZ. Mice were infected with 10,000 cysts of *Blastocystis*/mouse by oral gavage (Ismail *et al*, 2016). To prove infection, fresh stool samples were collected from each one on 4th day post infection (PI) for microscopic examination. Two weeks PI, G3, G4 & G5 were treated with MTZ as 120µg/kg/day (Fahmy *et al*, 2015), Ag NPs as 25µg /kg/day (Mulfinger *et al*, 2007), and Ag NPs loaded with MTZ (120µg/kg/day) respectively for 10 consecutive days.

Drugs' efficacy: All groups were sacrificed by neck rapid decapitation 4 weeks PI for examinations.

Parasitological examination: Intestinal contents were evacuated by gentle scraping and stools were collected to count *Blastocystis* by direct wet mount (unstained and iodine stained), and formal ether concentration technique (Garcia and Bruckner, 2007).

Histopathological examination: 1cm colon segment was excised, opened, fixed in 10% formalin, embedded in paraffin blocks, sectioned at 4 to 5µm thickness, mounted on slides, stained by H & E and examined microscopically (Drury *et al*, 1967).

Immunological examination: Anti-*Blastocystis* antibodies; IgA, IgM & IgG levels were evaluated by indirect ELISA using crude *Blastocystis* antigen (Engvall and Perlmann, 1971).

Immunohistochemical examination (IHC): Done on 2 separate sections from each group by CD3 mouse monoclonal antibody (1/50, code M7254, clone F7. 2.38) or CD20 mouse monoclonal antibody (1/200, code M0-755, clone L26), then incubation with labeled polymer by using 2 sequential 30 min. incubation (En-Vision & System-HRP, code K4006; Dako Denmark A/S).

Final reaction was obtained by 10min incubation with 3, 3' diaminobenzidine & substrate chromogen as brown colored precipitate at antigen site after the manufacturer's instructions. Slides were digitized by an Ol-

ympus microscope with 1/2×photo adaptor, using 100x. Images were analyzed on an Intel Core I3- based computer by using video test morphology software (Russian Federation, Saint-Petersburg), for immuno-histologic analysis for stain density.

Statistical analysis: Data were analyzed by SPSS program (statistical package of social science, SPSS Inc., Chicago, IL, USA) version 16 for Microsoft Windows. Mean and standard deviation measured central tendency

Table 1: *Blastocystis* cysts/ml of intestinal fluid and % of reduction in all studied groups.

Groups	Mean ±SD	T-test	P value	Reduction%
Infected non treated	2500±500			
MTZ treated	316.7±175.6	7.1	0.002*	87.33
Ag NPs treated	1016.7±500.8	3.6	0.022*	59.33
Ag NPs loaded with MTZ treated	233.33±144.34	7.5	0.002*	90.7

*Significant difference (P ≤0.05).

Histopathological: Infected non-treated intestine showed large number of *Blastocystis* in lumen, glands and sub-mucosa. Mucosa showed ulceration, villous atrophy and disturbed crypt-villous ratio with chronic inflammatory infiltrates. The group treated with Ag NPs loaded with MTZ gave the best result as it showed very few *Blastocystis* in glands, with normal structure of mucosa, lamina propria, and normal crypt-villous ratio, followed by group treated with MTZ and the least was Ag NPs treated ones. The IgA, IgM & IgG: Ag NPs loaded with MTZ treated group showed the best cure rate followed

and quantitative data dispersion. Student t-test compared between groups. Significance was at P <0.05 with confidence level 95%.

Results

Parasitological: Ag NPs loaded with MTZ gave the best result (Tab.1) followed MTZ then Ag NPs; where the reduction of *Blastocystis* cyst mean count /ml intestinal fluid were 90.7%, 87.33% and 59.33% respectively, compared to infected non treated group with significance (P <0.05).

by MTZ treated group then Ag NPs treated group; where the percentages of reduction of mean concentration of Ig A (pg) /ml serum were (30.5%, 26.24% & 2.13%) respectively and that of IgM were (15.2%, 12.8% & 2.8%) respectively, but IgG were (23.1%, 12.3% & 5.2%) respectively compared to positive control, with significant difference in G3 & G5 (P<0.05).

IHC sections showed different expression of intraepithelial lymphocytes (IELs) infiltrated within crypts and Struma using CD3 & CD20 (Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 & 16).

Table 2: IgA, IgM & IgG evaluation in studied groups by indirect ELISA

immunoglobulins groups		IgA	IgM	IgG
normal	mean± SD	9.03±1.1	437.67±30	672±24.27
infected non treated	mean± SD	14.1 ± 2.3	579.3 ± 21.03	987.7 ± 22.
MTZ treated	mean± SD	10.4 ± 0.7	505.3 ± 6.51	866 ± 45.83
	t-test	2.7	5.8	4.15
	p. value	0.05*	0.004*	0.014*
	% of reduction	26.24%	12.8%	12.3%
Ag NPs treated	mean± SD	13.8 ± 0.9	563 ± 46.1	936 ± 55.75
	t-test	0.2	0.56	1.5
	p. value	0.8	0.61	0.21
	% of reduction	2.13%	2.8%	5.2%
Ag NPs loaded with MTZ treated	mean± SD	9.8 ±1.3	491.3±10	760 ± 51.6
	t-test	2.8	6.5	7.02
	p. value	0.05*	0.003*	0.002*
	% of reduction	30.5%	15.2%	23.1%

*Significant difference (p ≤0.05).

Discussion

Effective treatment of symptomatic blastocystosis only without other pathogenic age-

nts became a great challenge since the failure of metronidazole and other remedies (Roberts *et al*, 2014, Batista *et al*, 2017).

Metal NPs were loaded with several drugs, e.g. praziquantel, rifampicin, amphotericin B, chloroquine and albendazole in the treatment of different parasitic infection e.g. schistosomiasis, malaria, leishmaniasis and visceral larva migrans (Abaza, 2016). The present study explored the effectiveness of AgNPs loaded with MTZ on experimental blastocystosis by parasitological, histopathological, immunological and immunohistochemical examinations. To the best of the available knowledge, this is the first study in which blastocystosis was treated with Ag NPs loaded with MTZ

Blastocystis expresses cysteine proteases, which play a crucial role in host cells invasion (Amaya *et al.*, 2015), facilitated by connective tissue and extracellular matrix proteins degradation. These proteases affect the epithelial integrity, causing intestinal inflammations, damage and increased permeability in experimental model (Puthia *et al.*, 2005; Basyoni *et al.*, 2018). In the present work, histopathological examination of the infected non treated group revealed detrimental effects on the intestinal mucosa; including villous shortening, atrophy and infiltration of lamina propria with inflammatory cells. *Blastocystis* were detected within the lumen and in the villi. This is in agreement with Ismail *et al.* (2016) and Basyoni *et al.*, (2018). Also, there was increased production of the three tested anti- *Blastocystis* antibody isotypes, IgA, IgM, and IgG in serum of the infected non treated group in comparison of the non-infected control group. This agreed with Zierdt *et al.* (1995) who reported that IgG was elevated in 25 of 28 *Blastocystis* infected humans in a study conducted by ELISA, Hussain *et al.* (1997) who reported significant increase in IgG2 antibody in irritable bowel syndrome patients infected with *Blastocystis*, and Kaneda *et al.* (2000) found that serum Ig A, IgM & IgG in 70% of infected asymptomatic individuals. But, Chen *et al.* (1987) found lack of specific antibody response in patients with *Blastocystis* caused intermittent diarrhea. It is supposed that the

antibodies production is a response to surface coat fragments of *Blastocystis* that was transported from the gut lumen to tissue lymphocytes (Hussain *et al.*, 1997). Tan *et al.* (2002) reported that this surface coat might act as immunological barrier to protect the parasite by promoting production of ineffective B cells that produce non-functioning immunoglobulins. *Blastocystis*-secreted cysteine proteases were immunogenic that controlled and modulated the host immune system by degradation of secretory IgA (Puthia *et al.*, 2005; Rendragraha *et al.*, 2019).

CD3 is a protein structure on the surface of T lymphocytes, associated with T-cell receptor (TCR) complex (Yang *et al.*, 2005), while CD20 is a protein expressed on B cells surface (Nadler *et al.*, 1981). The CD3 & CD20 expressed in the infected untreated group was increased as compared to non-infected control. This agreed with Abu El-Fetouh *et al.* (2015) who found higher CD3 and CD20 mean cell counts in the infected compared to control mice. The surface coat fragments of *Blastocystis* secreted proteases and cytokines transported from the gut lumen to the tissue stimulate multiple immune cells including T & B lymphocytes (Long *et al.*, 2001). Iguchi *et al.* (2009) reported that the increased transcription of IFN- γ , IL-12, & TNF- α in mucosa of *Blastocystis* infected rats, and that the parasites enhanced the activation (or influx) of T cells, B cells, and other immune cells in local tissues.

In the present study, Ag NPs loaded with MTZ showed a powerful effect over either MTZ or Ag NPs. The counts of *Blastocystis* cysts were greatly reduced, histopathological inflammatory changes were markedly improved, with marked reduction in Igs levels and the best distribution of CD3 and CD20 found in group treated by Ag NPs loaded with MTZ. These results may be attributed to the intelligent design at the Nano scale that yields a compound including drug Nano carrier for efficient drug delivery. Nano carriers have many advantages including its small size, which overcomes physiological

barriers and facilitates cells entry. Increased its solubility improves bioavailability and ability to be delivered to the target sites with controlled release (Karunaratne, 2007). MTZ gave an acceptable effect with moderate *Blastocystis* cysts count reduction, moderate improvement of histopathological inflammatory changes and the immunological response. This was in accordance with the results of Basyoni et al. (2018) who reported (79.3%) percentage of reduction of *Blastocystis* cysts count and moderate improvement of inflammatory reactions and Fahmy et al. (2019) who reported 83 % percentage of reduction and repairing of the histopathological changes; with MTZ treatment of experimental blastocystosis. On the other hand, Roberts et al. (2014) reported complete failure of MTZ and reported that it should no longer be considered the recommended treatment for *Blastocystis* infection. Also, Batista et al. (2017) found inadequate response to MTZ and their systematic review showed greatly variable responses to MTZ treatment ranging from 0% to 100. This variability may be due to differences in drug resistance, *Blastocystis* subtypes, technical methods or geographical conditions. Ag NPs was the least effective in the present study. Ahmed et al. (2015) were the first to study the effect of Ag NPs on *Blastocystis* where it gave in vitro percentage of growth inhibition slightly higher than or comparable to MTZ. Adeyemi et al. (2018) reported moderate in vitro anti *T gondii* effect of Ag NPs and great selective in vitro anti trypanosomal effect, but only trypanostatic effect in vivo. Gopinath et al. (2017) stated that Ag NPs interact with bacterial membranes by either adhesion and/or penetration into the membrane bilayers, affecting its integrity and cause intracellular protein and electrolytes leakage. This represents an evidence of apoptosis in *Blastocystis* upon treatment with Ag NPs (Huppertz et al, 1999). Cameron et al. (2016) reported that its mechanism of action is a dual effect where the nanoparticles of silver interact with the cell wall,

damaging it and the Ag⁺ ions get entrance into the cyst destroying it. It is proved that Ag NPs have different targets in the parasite and that generation of reactive oxygen species contributes greatly to its anti-parasitic effect (Adeyemi et al, 2018). Although Ag NPs are less effective than MTZ, yet it has the advantage of being naturally safe compounds, with no cytotoxicity in suitable doses on human cells (Ahmed et al, 2015). The synergistic effect of both Ag NPS and MTZ provoked the best results in reduction of CD3 and CD20 levels and augmented counter modulation of the immune response to restore normal immune homeostasis

Conclusion

AgNPs loaded with MTZ efficiently treated blastocystosis and improved immunological pictures as promising therapeutic agent for human infection. Not all lymphocytic infiltrates of CD3 & CD20 in the colon epithelium were lymphocytic colitis especially in man. So, physician must evaluate treatment.

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Explanation of figures

- Fig. 1 A&B: Normal control intestinal section showed normal mucosa, lamina propria and normal intestinal crypt-villous ratio (H&E, x200).
- Fig. 2: Intestinal section in infected non treated mice showed large number of *Blastocystis* in lumen of intestinal glands (black arrows). Boxed area showed magnified vacuolar forms of *Blastocystis* with four peripheral nuclei and central vacuole (H&E x400).
- Fig. 3: Intestinal section in infected non treated mice showed large number of *Blastocystis* in lumen (red arrow) and glands crossing lamina propria to submucosa. Intestinal mucosa showed ulceration of lining epithelial cells (black arrow), broadening and disturbed crypt-villous ratio; Chronic inflammatory infiltrate in lamina and submucosa (yellow arrow) (H&E, x200).
- Fig. 4: Intestinal section in infected mice treated with MTZ showed mild number of *Blastocystis* in lumen (black arrow), mucosa with superficial erosions (red arrow) slight healing of mucosa and normal intestinal crypt-villous ratio (H&E, x200).
- Fig. 5: Intestinal section in infected mice treated with Ag NPs showed *Blastocystis* in lumen (black arrows), showed with dense inflammatory cell infiltrate (H&E, x200).
- Fig. 6: Intestinal section in infected mice treated with Ag NPs loaded with MTZ, showed few *Blastocystis* in glands only (black arrows), normal structure of mucosa, lamina propria and normal intestinal crypt-villous ratio (red arrow) (H&E, x400).
- Fig. 7: Colonic section showed few scattered CD3 cells in negative control (IHC 200x).
- Fig. 8: Colonic section showed few scattered CD20 cells in in negative control (IHC 200x).
- Fig. 9: Dense CD3 in crypts and stromal tissue of infected untreated mice (IHC 400x).
- Fig. 10: Dense CD20 in crypts and stromal tissue of infected untreated mice (IHC400x).
- Fig. 11: Dense CD3 stromal and crypt infiltrates of infected and Ag NPs treated mice (IHC100x).
- Fig. 12: Dense CD20 stromal and crypt infiltrates of infected and Ag NPs treated mice (IHC200x).

Fig. 13: Moderate inflammatory cell infiltrate of CD3 of MTZ treated mice (IHC 400x).

Fig. 14: Moderate inflammatory cell infiltrate of CD20 of MTZ treated mice (IHC 200x).

Fig. 15: Improved mice showed few inflammatory cells of CD 3 in intestinal mucosa in Ag NPs loaded with MTZ treated mice (IHC 200x).

Fig. 16: Improved mice showed few inflammatory cells of CD20 in intestinal mucosa in Ag NPs loaded with MTZ treated mice (IHC 200x).

