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THE IN VITRO ACTIVITY OF CHITOSAN NANOPARTICLES VERSUS METRONIDAZOLE AGAINST BLASTOCYSTIS HOMINIS

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Abstract

Blastocystis spp. is a protozoan parasite with controversial clinical significance and consensus for treatment. Metronidazole was considered the main line of treatment, but currently wide range of efficacy rate, drug resistance and many undesirable side effects motivated the search for a novel effective safe therapy. Chitosan nanoparticles have offered a new pharmacological tool for the treatment of many parasites especially protozoan parasites.

In vitro antiprotozoal activity of Chitosan Nanoparticles (Cs NPs) on *Blastocystis spp.* was assessed through challenging with graded concentrations (20, 40, & 50µg/ml) compared to MTZ (100µg/ml), parasite count and viability was assessed after 24, 48 & 72hr. of incubation.

Chitosan nanoparticles offered a remarkable inhibition activity on *blastocystis* growth and viability, which were proportional to its concentration and exposure duration, Cs NPs 20µg/mL gave a significant growth inhibition percentage after 24hr & 48hr (56.4% & 50.5%) respectively, regarding Cs NPs 40µg/ml, it was (73.1% & 86.9%) respectively. While highly significant decrease in mean parasitic count 99.2% was occurred when 50µg/ml Cs NPs was used for 72hr compared to 89.8% MTZ for the same duration. Meanwhile, metronidazole (100µg/ml) was effective in treatment of blastocystis more than Cs NP 20µg/ml, but it was less effective than Cs NP 40µg/mL and Cs NP 50µg/ml for all duration time.

Key words: *Blastocystis* spp., *in vitro*, Cs NPs, Metronidazole.

Introduction

Blastocystis spp., an obligate common intestinal protozoan infects domestic, pet, and wild animals as well as humans of worldwide distribution (Zanetti *et al,* 2020). *Blastocystis* spp were reported in Kuwaiti infected children (Zaki *et al,* 1991), in Jordanian gastroenteritis preschool children (Nimri, 1993), in Saudi Arabian healthy food handlers (Amin, 1997), in Egyptian polluted canal water (El Shazly *et al,* 2007), in Lebanese symptomatic and asymptomatic patients (El Safadi *et al,* 2013), in Qatar among immigrant workers (Abu Madi *et al,* 2015), in Lebanon in infected Syrian Refugee (Khalid *et al,* 2021)

Blastocystis spp. is the most common eukaryotic organism found in human fecal samples (Salehi *et al,* 2017). Humans' transmission is via fecal-oral route, with high zoonotic among animal handlers (Abdo *et al,* 2021). Most people with *Blastocystis* spp neither showed signs nor symptoms (Tan, 2008), but it was found in patients particularly in immunosuppressed individuals with diarrhea and other digestive problems (Bednarska *et al,* 2018). Thus, it is an opportunistic gastrointestinal parasite (Ahmed *et al,* 2022).

The main symptoms associated with blastocystosis include bloating, anorexia, abdominal pain, fatigue, constipation, flatulence, urticarial skin rash, acute and chronic diarrhea or even irritable bowel syndrome, which tender growth and development particularly among children <5 years old (Yakoob *et al,* 2004). Pathogenicity of the *B. hominis* can persist from some weeks to many years in untreated ones

(Yakoob *et al,* 2011).

Although Metronidazole® (MTZ) has been the first line of *B. hominis* treatment with efficacy up to 100% (Stensvold *et al*, 2010), yet drug resistance developed (Dunn *et al,* 2012). Besides, its side effects as carcinogenic and teratogenic effects (Adil *et al,* 2020).

The nanoparticles proved to be a new hopeful therapeutic agent with a crucial role in treatment (Sun *et al*, 2019). Chitosan is a natural nontoxic polysaccharide derived from deacetylation of chitin and approved by US/ FDA as effective antiprotozoal, antimicrobial and antifungal agent (Garg *et al,* 2019). Also, chemical modifications of chitosan gave its derivatives better water solubility and antimicrobial property (Yan *et al,* 2021).

Chitosan nanoparticles were well used as drug delivery system for malignant malaria (Teimouri, 2016), cryptosporidiosis (Mammeri *et al,* 2018), and leishmaniasis (Riezk *et al,* 2020). Also, Said *et al.* (2012) reported Cs NPs efficiency in treating giardiasis.

The present study aimed to evaluate the Cs NPs *in-vitro* activities with different concentrations in treating *Blastocystis hominis.*

Materials and Methods

Morning stool samples were collected from patients attended the Gastroenterology Outpatient Clinic, Suez Canal University Hospitals from April, 2022 to June 2022.

The labeled stool samples were immediately examined macroscopically for pinworms, gravid segments and the likes. Samples were examined microscopically as direct wet smears with or without Lugol's iodine stain, and by formol ether concentration technique stained with modified Ziehl-Neelsen and/or Trichrome stains (Garcia, 2007). *Blastocystis*-positive samples without any other parasites were selected for in-vitro cultivated.

Blastocystis-positive samples were cultivated in duplicate tubes in 5ml of modified Jones' medium rice starch-free, supplemented with 10% horse serum, 100 UI/ml penicillin®, and 100µg/ml streptomycin®. Culture tubes were incubated at 37°C with daily microscopic inspection for positivity up to three days. Those with live parasites exceeded 1×10^3 vacuolar forms were subcultures every 3-4 days in fresh medium (Souppart *et al,* 2009), otherwise was discarded.

Drugs: 1- Cs NPs were prepared based on ionotropic gelation of chitosan with tripolyphosphate (TPP) anions (Calvo *et al,* 1997). Chitosan was liquefied in acetic aqueous solution 1% with sonication. Tripolyphosphate (TPP) as 1mg/ml was prepared as suspension in distilled water. An equal volume of acidic chitosan solution (0.3%) & TPP was added with continuous magnetic stirring at room temperature, to have Cs NPs, centrifugation (10.000rpm) for 30min. at 4°C. The supernatant was removed, and the purification CS NPs was obtained by distilled water removal by sodium hydroxide remnant. Finally Cs NPs were stored at 4-8°C till used (Elzatahry *et al,* 2008). Characterization of Cs NPs in size (20±5nm) of nanoparticles and zeta (ranged +42.8 mV) were done using photon correlation spectroscopy and laser Doppler. 2- Metronidazole MTZ (Flagyl) was used as a reference drug (Sawangjaroen and Sawangjaroen, 2005). Stock solution of 1mg/ml was prepared (100µg/ml) and stored in a dark bottle at 4°C (El-Deeb *et al,* 2012).

In vitro exposure to Cs NPs & MTZ: A *Blastocystis* inoculum size $1x10^5$parasites/ml from cultures was prepared, and counted by the Neubauer cell counting chamber. Inoculum was put into each set of Jones medium culture tubes with different concentrations of Cs NPs as 20, 40, & 50µg/ml (Elmi *et al,* 2021) or MTZ 100µg/ml for parasite growth and viability. Non-treated control (NTC) parasitic culture was submitted to the same procedure. Culture tubes were used in triplicate for every concentration of each nanoparticle, MTZ and the NTC cultures.

In vitro anti-*Blastocystis* activity of Cs NPs & MTZ: a- Growth was assessed by parasite number and morphologic changes after 24hr,

48hr & 72hr of incubation. Changes were evaluated by a Neubauer cell counting chamber as mean parasitic counts, percentage inhibition of *Blastocystis* multiplication by Cs NPs (20, 40, & 50µg/ml) and MTZ 100µg/ml compared to NTC was calculated as: Growth inhibition % (X)

$$X = \frac{a - b}{a} \times 100$$

a = Parasite mean number in control cultures (NTC), and b = mean number in treated ones.

B- Viability was assessed after staining with 0.4% Trypan blue solution as a microscopy indicator for dead parasites as increased permeability to the dye, which penetrated the cell then it became pink, and vies was versa with active living ones.

Statistical analysis: Data were tabulated; computerized and analyzed using statistical software program IBM SPSS 19.0, as mean ± SD of triplicate determinations and growth inhibition percent. Independent sample t test was used to assess significance between different means. Values of $P < 0.05$ were considered significant and $P < 0.001$ highly significant.

Ethical considerations: All procedures were conducted according to the ethical approved by the Institutional Human Ethics Committee, Reference number 4809# Faculty of Medicine, Suez Canal University. An informed written consent was taken from each patient included in the study after explanation of the purpose of the study; infected patients were given an appropriate treatment.

Growth and viability profiles of the non-treated control culture showed marked increase in the mean *Blastocystis* count and viability at 48hrs compared to 24hr, followed by a slight decrease at 72hr.

Treated groups' was significantly compared with non-treated control. The growth of *B. hominis* was inhibited after adding MTZ and Cs nanoparticles to cultured tubes. The growth inhibition occurred was in a dose dependent manner proportional to the exposure duration. The highest *Blastocystis* inhibition (99.2%) was with Cs NPs 50µg/ml at 72hr. This was highly significant as compared to other doses at 72hr exposure time. Although MTZ 100µg/ml was more effective in treating *Blastocystis* than Cs NPs 20µg/ml, but it was less effective than Cs NPs 40µg/ml & Cs NPs 50µg/ml for all times. The effective dose and duration was 50µg/ml for 72hr.

Cs NPs caused an inhibition effect on *Blastocystis* morphology mainly (40 & 50µg/ml), with size range of treated cultures was significantly smaller than NTC (P <0.001). Cultures treated with MTZ (100µg/ml) also showed reduced size. All concentrations of Cs NPs and MTZ treated cultures showed increased granular form count at 48hr & 72hr, replaced the typical vacuolar forms compared to NTC.

Results

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

Table 1: *Blastocystis* mean counts ($X10^4$) and inhibition % incubated with graded concentrations of Cs NPs &MTZ

Group	Duration of incubation for growth inhibition (G.I.)					
	24hr.		48hr.		72 hr.	
	M±SD	G.I. %	M±SD	G.I. %	M±SD	G.I. %
Control	45±3.6	0	108.3±7.5	0	97.3±6.1	0
MTZ(100)	14.6±0.5*	67.5%	31.9±1.1**	70.5%	9.9±0.2**	89.8%
Cs NPs 20*	19.6±2.4	56.4%	53.6±2.1	50.5%	23.7±1.3	75.6%
Cs NPs 40	12.1±1.4*	73.1%	14.1±0.5**	86.9%	6.0±0**	93.8%
Cs NPs 50**	5.3±2.3	88.2%	8.6±0.5	92.1%	0.8±0.1	99.2%

*P <0.05 =significant difference & **P <0.001= highly significant difference as compared to NTC

Discussion

Treatment of *B. hominis* is a matter of debate as a complete knowledge for its pathogenicity is not proven, but there is an overall agreement for the necessity of its medical treatment. Nanoparticles are a novel pattern of treatment which is currently studied as lot, it was investigated as an immune system stimulant, antibacterial, antifungal and antiparasitic drug with the successful promising outcomes

(Sakar *et al,* 2022).

To the best of the authors' knowledge, investgating the in vitro activity of Cs NPs, as a new alternative modality of treatment against *Blastocystis hominis* is attempted in this research for the first time.

In the present study, the non-treated control group showed significant number increase (108.3±7.5) after 24hr with slight decrease (97.3±6.1) after 48hr. This growth manner of untreated cultures was also reported by other authors (Roberts *et al,* 2015).

MNZ was used as a standard anti-Blastocyst drug; its efficacy for growth inhibition was shown to be the only significant after 24hr (67.5%) with highly significant inhibition after 48hr (70.5%) and 72hr (89.8%) this result agreed with Yakoob *et al.* (2011).

MTZ is assumed to override *Blastocystis hominis* through nucleic acid synthesis disruption (Nasirudeen *et al*, 2004; Raman *et al,* 2016). Meanwhile, variable degrees of MTZ sensitivity were reported in different studies (El Deeb *et al*, 2012; Roberts *et al*, 2015; Mokhtar *et al,* 2016). This variability may be attributed to the different geographical locations and/or intra-subtype differences (El-Sayed *et al,* 2017).

In the present study, *B. hominis* challenged with different concentrations of Cs NPs caused different grades of growth inhibition proportional to NPs concentrations and exposure duration. The highly significant decrease in mean parasitic count was 99.2% when 50µg/ml Cs NPs was used for 72hr compared to 89.8% MTZ for the same duration was less effective in 24hr & 48hr. But, Cs NPs 20µg/ml gave a significant result after 24hr & 48hr, of 56.4 & 50.5 respectively, and Cs NPs 40µg/ml gave 73.1 & 86.9 respectively. This agreed with Elmi *et al.* (2020), they used Cs NPs to treat *Plasmodium falciparum, Trichomonas vaginalis* and *Giardia lamblia,* reported inhibition rates of 59.5%, 99.4%, & 31.3%, respectively. Also, Gomes *et al.* (2022) studied experimental intranasal immunization with chitosan microparticles and leishmanial vaccine (LACK-DNA), they found that chitosan enhanced the vaccine protection inducing of specific life immunity against visceral leishmaniasis. Moreover, *P. berghei*-infected mice proved to be treated either by chitosan based chloroquine (Tripathy *et al,* 2013) or through the nanoform, improved the efficacy and bioavailability of curcumin in its treatment (Akhtar *et al*, 2012).

The CS NPs improved the ivermectin antifilarial activity (Ali *et al,* 2013). Also, the CS NPs showed promising anti-*Toxoplasma* potentials (Gaafar *et al,* 2014; Teimouri *et al,* 2018). Besides, El-Gendy *et al.* (2021) reported a successful therapeutic action of Cs NPs & MTZ when investigated either separately or augmentation of MTZ therapeutic effect when loaded it on Cs NPs for treating giardiasis experimentally infected hamsters.

CNPs have many special properties that help in its efficacy for overriding microbial agents, e.g., it has a muco-adhesion property that drives for opening tight junction (Khan *et al*, 2020). Also, Cs NPs have antimetabolite activity as it can serve for membrane and nucleic acid synthesis inhibition that occur due to its permeation into the cell nucleus, combining with DNA next inhibiting the synthesis of mRNA and DNA transcription, subsequently rupture and leakage of intracellular component (Yan *et al,* 2021). This action is helped by its remarkable property of low molecular weight (Másson *et al,* 2021).

Other chitosan nanoparticles derivative that have relatively larger molecular weight act through other mechanism as it has condensed positive charge, that binds to the negatively charged elements on the microbial cell wall forming non-pourus layer around the cell, accordingly alteration of cell permeability and blockage of the cell transport (Pardeshi and Belgamwar 2016). Also, Ahmed *et al.* (2019) with *Cryptosporidum*, proposed that Cs NPs overcame the micro-organism by forming the dimples or pits, which permit leakage of oo-

cyst component occurring by its adsorption inside the cell due to its large surface area property. They added that Cs NPs action was through inhibiting DNA synthesis or through making pits that help the leakage of cell wall component to disruption

In the present study, MTZ treated *Blastocystis* showed cell shrinkage and compressed organelles together. This agreed with Huppertz *et al.* (1999) who reported that apoptotic cells underwent cytoplasmic fluids loss and proteins denaturation with marked morpholo gical changes. Also, Nasirudeen *et al.* (2004) reported that *Blastocystis* apoptosis was represented by the cell shrinkage and compaction of organelles in cytoplasm.

Blastocystis parasites in the culture tubes treated with Cs NPs showed some morphological characters in the form of size reduction, granular form was more verifiable and abundant in the microscopic field than vacuolar form, and this striking observation was reported (El-Deeb *et al,* 2012). In their study on the in-vitro treatment of the *Blastocystis* spp. ST3 with *Ferula asafetida*, pointed out those viable vacuolar forms were replaced by granular forms, which lost viability overtime and finally disintegrated this observation was reported (Vdovenko, 2000), who proposed that granular forms may represent the sub-sequent step of the degenerative changes in the parasitic cell. In this context the results could be elucidated.

Conclusion

Chitosan nanoparticles showed potent inhibitory effect on the *Blastocystis* spp. There was highly significant effect on the cultured parasites with the increase in dose and exposure time.

However, the metronidazole alone was effective in the treatment of blastocystosis than Cs NP, but the drug was less effective than Cs NP 40µg/ml and Cs NP 50µg/ml for all the exposure times.

Future studies on the inhibitory effect on the *Blastocystis* different genotypes are ongoing and will be published in due time elsewhere.

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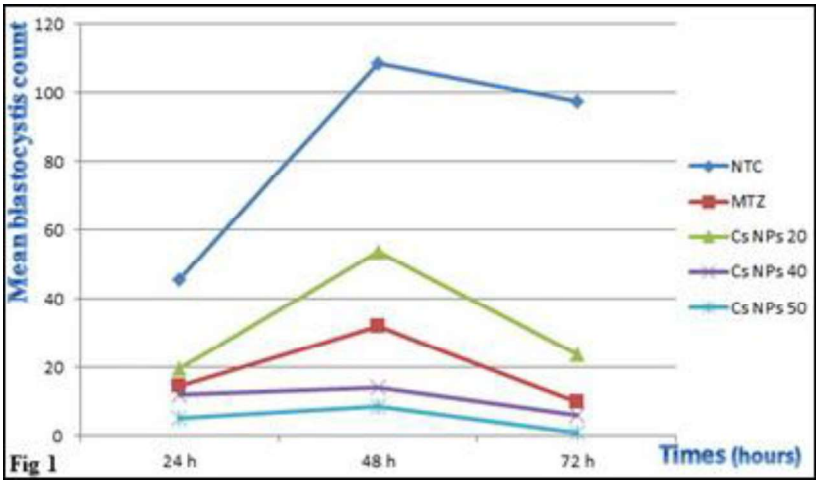
Explanation of figures

Fig. 1: Mean count of *Blastocystis* in cultures challenged with graded concentrations of Cs NPs & MTZ compared to non-treated culture (NTC).

Fig. 2: *Blastocystis* showed large size in non-treated cultures (black arrow) with A- Vacuolar form: wet mount (x400), B- Multivacuolar form and cyst form: Trichrome stain (x400), C- Granular form and cyst form: wet mount (x1000), & D- Granular form iodine stain (x1000).

Fig. 3: a. Predominance of granular form with small size and distorted morphology (red arrow) in Cs NPs treated cultures after 24h (x400), & b. MTZ treated culture after 24h.with relatively larger size (blue arrow) than Cs NPs treated cultures (x400).


Mean blastocystis count
120
100
80
60
40
20
0
24 h
48 h
72 h
Times (hours)
NTC
MTZ
Cs NPs 20
Cs NPs 40
Cs NPs 50
Fig 1

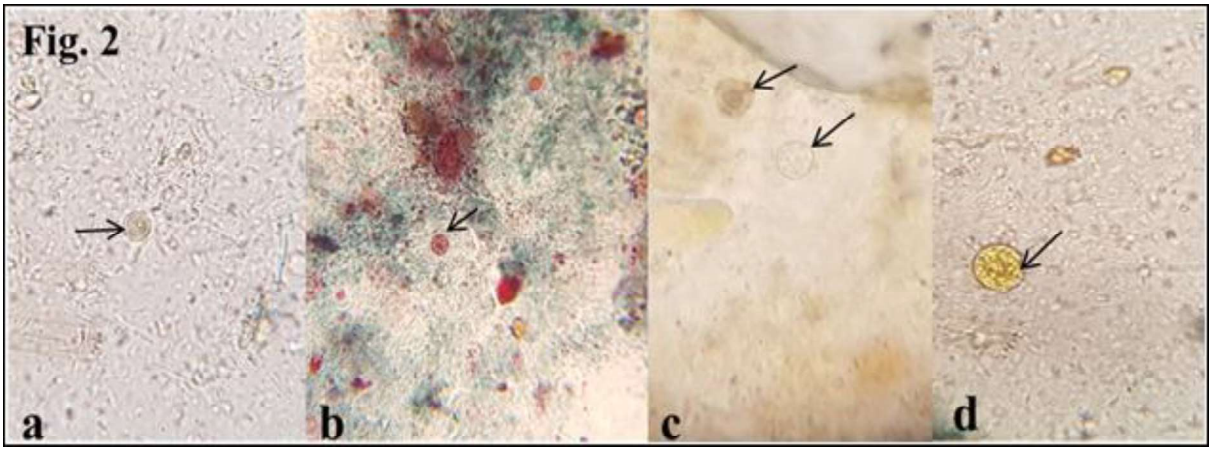


Fig. 2
a
b
c
d

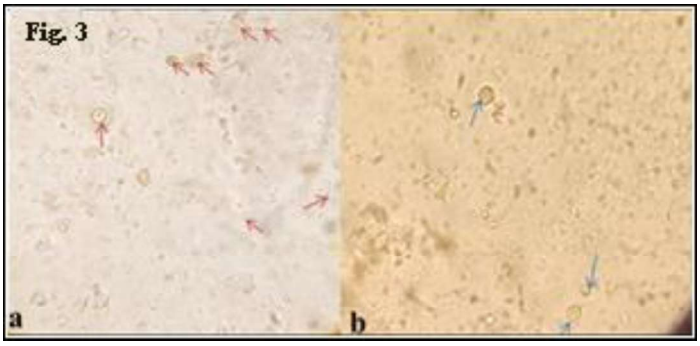


Fig. 3
a
b