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Investigating the Effect of Silver, Chitosan, and Curcumin Nanoparticles on *Blastocystis* spp. and Comparing it With Metronidazole In Vitro

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Abstract

Background: *Blastocystis* is an anaerobic gastrointestinal protozoan that causes infections in humans and a wide range of animals. It was found that the host specificity and the pathogenic potential of different isolates are correlated with sequence variations in the SSU-rRNA gene. The identification of the organism at the species level is still an unclear challenge. The use of natural nature substances against infectious organisms has been promising, and the optimization of these substances in the direction of better delivery such as the form of nanoparticles (NPs) of natural substances has recently been considered.

Objectives: The present study aimed to investigate the effect of silver, chitosan, and curcumin NPs on *Blastocystis* spp. and compare it with metronidazole *in vitro* conditions.

Materials and Methods: The parasite was cultivated in Robinson's medium and was then identified by polymerase chain reaction (PCR), and the subtype of the parasite was determined, which was subtype 3. Then, the methyl thiazolyl tetrazolium (MTT) test was performed to determine the toxicity level of the prepared drugs/substances using Caco2 cells. This study investigated the concentrations of silver NPs (10, 25, and 50 µg/mL), chitosan (75, 50, 25, and 12.5 µg/mL), and curcumin (250, 500, and 1000 µg/mL), and their effect on 24- and 48-hour time points after exposure to the parasite. Then, the final number of parasites was counted after staining with trypan blue by a Neubauer slide, and the values of IC50 and selectivity index (SI) were calculated for each substance.

Results: Chitosan and curcumin NPs had SI of 2.04 and 13.15, respectively, which were more effective than metronidazole, and silver NP was 0.143. However, chitosan NP had the best anti-parasitic effect. Based on the obtained results, chitosan and curcumin NPs were more effective against blastocystis than against metronidazole.

Conclusion: Chitosan and curcumin NPs (liposomal curcumin) have a good inhibitory effect on blastocystis compared to metronidazole, but silver NP did not perform better than metronidazole.

Keywords: *Blastocystis*, Nanoparticles, Silver, Chitosan, Curcumin

Background

Blastocystis is one of the most common human intestinal parasites.[1] It was first described 100 years ago, but little is known about its pathogenicity, genetic diversity, host range, and treatment. It has a global distribution in developing countries, probably due to poor sanitation.[2] It is common in many animals, including mammals, birds, and amphibians. Up to 22 subtypes (STs) have been described so far, and subtypes ST1-9 and ST12 have been found and reported in human cases with varying prevalence.[3] ST3 has been found in most human epidemiological studies.[1] *Blastocystis* is transmitted by fecal-oral route between humans and animals.[4] Several studies have shown possible transmission through contaminated water, and it has been stated that inadequate provision of basic facilities plays an important role in transmission.[2] After more than a hundred years, there are still many ambiguities regarding the parasite and its clinical importance.[5] This parasite is colonized in the intestine and is usually found in the feces of people with gastrointestinal clinical symptoms without identifiable symptoms.[6] Although some studies regarded this parasite as the only cause of gastrointestinal and skin diseases, they explained the clinical symptoms such as abdominal pain, diarrhea, vomiting, nausea, swelling, anorexia, and skin symptoms that are usually less noticeable such as itching.[7] Nowadays, metronidazole is considered the choice drug for *blastocystis* treatment.[8] However, this drug has side effects such as headache, tongue swelling, hives, itching, dizziness, nausea, dry tongue, as well as vomiting.[9] In addition, it has potential side effects (carcinogenic and mutagenic) and adverse effects on the fetus, and drug resistance to this drug is increasing.[10] Recently, the use of natural substances against infectious agents, especially parasites, has been increasing because they have fewer clinical complications and are easily accessible. The nanoparticles (NPs) are used for better drug delivery to optimize these natural substances.[11] The main aim of

the present study was to investigate the effect of silver NPs, chitosan, and curcumin (liposomal curcumin) on *blastocystis* and compare it with metronidazole *in vitro* conditions.

Materials and Methods

The parasite was prepared from the archive of Tarbiat Modares University Parasitology and Entomology department, cultivated in Robinson's medium, and then identified by polymerase chain reaction technique. Afterward, the subtype of the parasite was determined, which was ST3. To determine the toxicity level and anti-parasite effects of the prepared drugs/substances, a methyl thiazolyl tetrazolium (MTT) test was performed using Caco2 cell lines. The concentrations of silver NPs (10, 25, and 50 µg/mL), chitosan (75, 50, 25, and 12.5 µg/mL), and curcumin (250, 500, and 1000 µg/mL) and their effect on 24 and 48 hour-time after exposure to the parasite were investigated. The final number of parasites was counted after staining with trypan blue by a hemocytometer slide. The value of IC50 and selectivity index (SI) was calculated for each drug/substance. Twenty thousand Caco2 cells were counted and placed in 96-well plates in triplicates. Then, 200 µL of DMEM/F12 medium and 10% of fetal bovine serum (FBS) were added to each well and incubated at 37°C for 24 hours. After 24 hours, the supernatant was discarded, and 100 µL of culture medium containing new FBS and 100 µL of metronidazole drug with concentrations of 0.5, 1, 2, 4, 8, and 10 µg/mL, silver NPs with concentrations of 1, 5, 10, 25, and 50 µg/mL, curcumin NPs with concentrations of 250, 500, and 1000 µg/mL, and chitosan NPs with 25, 50, 75, and 100 µg/mL concentrations were added and incubated for 24 hours. The rest of the empty wells were filled with sterile phosphate-buffered saline to prevent the evaporation of the existing solutions (the same was done in the case of 48 hours). After 24 hours, the supernatant was drained and 10 µL of MTT solution and 90 µL of culture medium containing FBS were added and incubated for 3-5 hours. The supernatant was drained again, and 100 µL of DMSO solution was added to each well and left for 15 minutes.

The effectiveness of any drug/substance compound depends on its SI, which means that the drug must kill the parasite, and on the other hand, it must be non-toxic or have a low toxicity effect on the host, so the index is calculated as follows:

$$SI = IC50\ Cell/IC50\ Blastocystis$$

Results

Finally, after several times of pegging, the light absorption was read by an enzyme-linked immunosorbent assay reader at a wavelength of 570 nm. The percentage of cell viability rate was then calculated.

The Proximity of Parasites and Drugs

Under sterile conditions, 10 000 parasites were counted by Robinson's medium, and 200 µL were poured into 2 mL volume microtubes. Then, 200 µL of metronidazole and mentioned drugs were poured three times, and a negative control with phosphate-buffered saline and a positive control with metronidazole were considered for all tests. Afterward, all the microtubes were incubated in a 37°C incubator for 24 and 48 hours. To count, 20 µL of parasites were mixed with 20 µL of trypan blue solution, and after pipetage, 10 µL were injected into the hemocytometer slide and counted in 16 chambers. Live parasites were colorless, and dead parasites were observed as stained. The growth inhibition percentage was calculated according to the following formula:

$$GI\% = \frac{\text{1-GR extract}}{GR\ control} \times 100$$

After comparing the percentage of growth inhibition of all three NPs, chitosan NP had the highest percentage of growth inhibition. The percentage of growth inhibition of the silver NP for 24 hours was the same at concentrations of 10 (55%), 25 (75%), and 50 (64%), while the percentage of growth inhibition of curcumin NP for 48 hours were found at concentrations of 250 (53.33%), 500 (69%), and 1000 (73%). Moreover, the growth inhibition percentage of 50 µg/mL of silver NP in 48 hours (64%) was similar to the percentage of 12.5 µg/mL of nano-chitosan (85.33%) in 24 hours and the same as the percentage of inhibition for 1000 µg/mL of nano-curcumin (89%) in 48 hours.

The results indicated that chitosan NPs has a higher SI compared to other drugs. The SI of chitosan NPs is extremely different from that of silver NPs, curcumin NPs, and metronidazole (Tables 1-6).

Discussion

Many years after the identification and reporting of *blastocystis* species, the pathogenicity of this fungus-sister parasite for humans is still unclear.[12] After its initial

Table 1. The Growth Inhibition Percentage of Silver NPs on *Blastocystis* (µg/mL)

Time	Concentration			
	10	25	50	Control
24 h	55	57	64	0
48 h	56.66	59	75	0

Note. NP: Nanoparticle.

Table 2. The Growth Inhibition Percentage of Chitosan NPs on *Blastocystis* (µg/mL)

Time	Concentration				
	12.5	25	50	75	Control
24 h	85.33	94.33	97.66	100	0
48 h	93	96.33	98.66	100	0

Note. NPs: Nanoparticles.

Table 3. The Growth Inhibition Percentage of Curcumin NPs on *Blastocystis* (µg/mL)

Time	Concentration			
	250	500	1000	Control
24 h	21.66	29.66	34.33	0
48 h	53.33	69	73	0
72 h	83.33	88.33	89	0

Note. NPs: Nanoparticles.

Table 4. IC50 Value of Silver, Chitosan, Curcumin NPs, and Metronidazole

Composition	Time	
	24-hour IC50 (µg/mL)	48-hour IC50 (µg/mL)
Metronidazole	66.17	2
Silver NPs	225	71.57
Chitosan NPs	3.4	0.37
Curcumin NPs	230	174.68

Note. NPs: Nanoparticles.

Table 5. IC50 Value of Silver, Chitosan, Curcumin NPs, and Metronidazole

Composition	24-h IC50 (µg/mL)
Metronidazole	10.22
Silver NPs	32.18
Chitosan NPs	44.73
Curcumin NPs	470.77

Note. NPs: Nanoparticles.

Table 6. SI Index Value of Metronidazole, Silver, Chitosan, and Curcumin NPs

Composition	24-h Cells IC50 (µg/mL)	24-h Parasites IC50 (µg/mL)	SI
Metronidazole	10.22	66.17	0.154
Silver NPs	32.18	225	0.143
Chitosan NPs	44.73	3.4	13.15
Curcumin NPs	470.77	230	2.04

Note. NPs: Nanoparticles; SI: Selectivity index.

identification in the previous decades, it was described as a non-pathogenic organism, but in recent years, it has been isolated and reported from the clinical samples of symptomatic individuals and has attracted a great deal of attention.[8] Currently, *blastocystis* is one of the most plentiful (emerging) gastrointestinal protozoa in the world.[13] Considering the *blastocystis* as a gastrointestinal health problem, metronidazole as the choice drug has many clinical complications such as renal failure due to long-term use. Furthermore, efforts to use natural substances to substitute metronidazole are still ongoing.[14] In this regard, the use of NPs for the treatment of infectious diseases, including parasitic diseases has attracted considerable attention.[15] In the present study, chitosan and curcumin NPs (liposomal curcumin) had a better inhibitory effect on blastocystis compared to metronidazole, but silver NP did not perform better than metronidazole.

The effects of these three NPs have been demonstrated on viruses, bacteria, fungi, as well as parasites such as *Leishmania* spp., malaria, and filers, and the results have been promising.[16-18] Ahmad et al investigated the effect of silver NPs (1-100 nm) in concentrations of 50, 75, and 100 µg/mL on *Entamoeba histolytica* trophozoites under laboratory conditions and compared it with metronidazole, finding a significant decrease in the number of trophozoites.[19] In another study, Said et al investigated the effect of silver, chitosan, and curcumin NPs on *Giardia lamblia* cysts. After comparing the results, silver NPs had the greatest effect, but the combined treatment yielded better results, and the best effect was observed in the combination of silver and chitosan NPs.[20] Saad et al investigated the effect of silver and copper oxide NPs (9-29 nm) against the cysts of *E. histolytica* and *Cryptosporidium parvum* in Egypt. 1C50-3 h of copper oxide NPs for *E. histolytica* and *C. parvum* were 0.13 and 0.72 mg/L, respectively, while this index for silver NPs was 0.34 and 0.54 mg/L, respectively. Therefore, the applied NPs for treatment were safe and effective.[21]

In 2018, Oyeyemi et al performed the ovicidal activity and toxicity of curcumin-nisin NPs against *Fasciola* spp. eggs. The result of the test confirmed the ovicidal activity of these NPs at concentrations of 5-0.3125 mg/mL against *Fasciola* spp., and it was the most ovicidal at the concentration of 5 mg/mL. This formulation did not show any toxicity on sperm cells, but the authors recommended more studies in *in vitro* and *in vivo* conditions.[22]

So far, there has been no study on the effect of chitosan and curcumin NPs (liposomal curcumin) as well as silver NP with a size of 5-8 nm on *blastocystis*. Therefore, the use of these drugs to treat *blastocystis* requires further investigations. According to the achieved IC50 results, chitosan NPs have a better effect on inhibiting parasite growth compared to silver and curcumin NPs.

The effectiveness of each drug combination depends on its SI, which is calculated according to the ratio of IC50 cell to IC50 *blastocystis* of each drug, and this value was 0.143 for silver NPs, 13.15 for chitosan NPs, and 2.04 for curcumin NPs, but it was 0.154 for metronidazole. The effect of all these three drugs was better than that of metronidazole, but the performance of chitosan NPs was better than that of other drugs. According to the results obtained in all three studied drugs, an increase in the concentration and duration of the proximity of the parasite and the drug led to a significant decrease in the number of parasites.

Conclusion

The use of natural substances, especially in nano size, is promising against *blastocystis*. Based on the present research results, chitosan and curcumin NPs (liposomal curcumin) had a better inhibitory effect on *blastocystis* compared to metronidazole, but silver NPs did not perform better than metronidazole. The optimization

of natural materials and further studies on their anti-parasitic effects seem necessary.

Authors' Contribution

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Formal analysis: Saeed Bahadory.
Funding acquisition: Javid Sadraei.
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Methodology: Raheleh Taji and Majid Pirestani.
Project administration: Javid Sadraei.
Resources: Javid Sadraei.
Supervision: Javid Sadraei.
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Writing–original draft: All Authors.
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Competing Interests

No conflict of interests is declared.

Ethical Approval

Not applicable.

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