Experimental Investigation of Silver Nanoparticles Dose Response for Larvicidal Activity Against Trichellnolosis *In Vitro*

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Abstract

Trichinellosis is a parasitic disease caused by the nematode *Trichinella spiralis* that affects pigs globally, posing a risk to livestock and to human health. Trichinellosis is acquired by consuming undercooked or raw infected meat containing encysted larvae from pigs and other reservoir hosts. As the disease cannot be effectively treated with known medication, disinfection plays a crucial role in maintaining a healthy, and hygienic environment on farms. Silver nanoparticles (AgNPs) have been proposed as an alternative approach due to their ability to inhibit microorganisms. The purpose of this study was to investigate the effectiveness of AgNPs against *T. spiralis* larvae. AgNPs were applied at concentrations of 100,200, 300, and 400 ppm for 4, 8, 16, and 24 hr to 45 larvae. The number of deaths was recorded, and the morphology of larvae was examined using H&E staining. The results showed that AgNPs with a size of 132.9 nm were effective against *T. spiralis* at a concentration of 100 ppm resulting in the lowest number of deaths (5 larvae or 11.11%) at 16 hr, while a concentration of 400 ppm of AgNPs achieved the highest number of deaths (40 larvae or 88.88%) at 24 hr. Additionally, the study revealed degenerative changes affecting the cuticle of AgNPs-treated *T. spiralis* with the destruction of stichocyte cells in the esophagus and the genital primordium cells which are reproductive cells. The results demonstrate the effective anthelmintic activity of synthesized AgNPs against *Trichinella in vitro* model, offering a promising alternative treatment for the elimination of the parasite.

Keywords: Silver nanoparticles (AgNPs), *Trichinella spiralis*, Larvicidal, Dose-response, Toxicity

Introduction

Trichinellosis, which is caused by the nematode *Trichinella spiralis* (*T. spiralis*), is a parasitic disease that poses a global threat to pigs, and potentially to human health. The transmission of this disease occurs when humans consume undercooked or raw meat containing encysted larvae from infected pigs or other reservoir hosts. After ingestion, *T. spiralis* larvae are released which then mature in the small intestine, reproduce, penetrate the intestinal wall, and enter the bloodstream, leading to muscle tissue encystment. Trichinellosis manifests with muscle pain, fever, gastrointestinal issues, swelling, and, in severe cases, rare cardiac or neurological complications. The economic losses associated with trichinellosis in livestock are significant, making it a pressing concern for the agricultural industry and public health authorities.

Effective disinfection practices play a crucial role in maintaining a healthy, and hygienic environment on farms. Inadequate disinfection can result in the spread of the disease which can have severe consequences for animal health and consequentially meat production. Mebendazole is the current drug of choice for the treatment of *T. spiralis* infection. However, this drug is less effective against encysted larvae than other forms of *T. spiralis* as it is poorly absorbed, and produces cytotoxic effects including anemia, acute liver failure, and

other side effects (Schipper et al., 2000). No vaccines have been developed for trichinellosis, and the emergence of antibiotic resistance has posed challenges in its treatment. Consequently, trichinellosis remains a significant sanitation issue.

To overcome the limitations of traditional treatment methods, alternative approaches such as the use of Silver nanoparticles (AgNPs) have been proposed. AgNPs offer a promising solution to combat trichinellosis, potentially providing an effective means of controlling the disease. By exploring the application of AgNPs, it is hoped that this can offer an alternative treatment strategy that overcomes the drawbacks associated with traditional methods, such as the development of drug resistance, and the presence of chemical residues in meat products. The investigation and development of alternative treatments such as AgNPs offer hope for improved control and prevention of trichinellosis, to mitigate its impact on animal health, human health, and the economic stability of the livestock industry.

Silver nanoparticles (AgNPs) have generated attention for their potent disinfectant properties, particularly in targeting a wide range of pathogens and contaminants, including bacteria. It has been observed in previous studies that the antibacterial activity of AgNPs is influenced by their size and shape, with smaller-sized AgNPs exhibiting a greater inhibitory effect on bacteria growth (Jung et al., 2008). Additionally, AgNPs have shown efficacy in controlling the growth of other microorganisms such as *Caenorhabditis elegans* (Meyer et al., 2010). In the context of *T. spiralis*, the use of AgNPs as a larvicidal agent has been explored in previous studies where it has demonstrated a potent larvicidal effect of myrrh-synthesized AgNPs against *T. spiralis in vitro*. However, there is a need to establish an appropriate high-dose response relationship, ranging from 1 μg/ml to 20 μg/ml, size 10-25 nm, and long incubation time for effective control of *T. spiralis*. (Abd-ELrahman et al., 2021). These results highlight the potential of AgNPs as a promising tool in combating *T. spiralis*, and they suggest the necessity for further research to determine the optimal dosage, and application methods for achieving effective control of trichinellosis.

The primary objective of this study, therefore, was to bridge the existing knowledge gap regarding the dose-response relationship of purifying and synthesized AgNPs for larvicidal activity against *T. spiralis*. In this study, we have experimented with the dose-response relationship using lower quantities of AgNPs. This approach involves testing the dilution of the substance at levels lower than before, with different-sized particles, and a short time of incubation, to determine the optimal dosage for achieving the desired response in effectively controlling trichinellosis and enhancing the overall efficiency of disease management. By exploring the cytotoxic effects of AgNPs on *T. spiralis*, including the assessment of larvicidal numbers, and the examination of morphological structures using light and electron microscopy, this research will provide valuable insights into the potential use of AgNPs as an alternative treatment for trichinellosis which holds significant implications for farmers and veterinarians. By contributing to the development of effective, sustainable, and safe control strategies for this parasitic disease, the findings in this study will enhance the well-being of livestock, minimize economic losses, and ensure the safety of meat products intended for human consumption.

The potential safety concerns of AgNPs in livestock, and for meat consumption, include the accumulation of AgNPs in meat, the development of antibiotic resistance in both animals and humans, long-term human health effects, and environmental impacts. In contrast to previous studies that primarily focused on cuticle damage, this experiment also investigates the disruption of cells within *T. spiralis*. We designed the experiment

using the H&E staining method, which provides insights into how AgNPs may impact internal organs, particularly the reproductive system. These findings contribute to our understanding of how AgNPs can interfere with the control of infection spread, potentially inhibiting further transmission. The findings of this study will not only shed light on the potential of AgNPs as a viable option for managing trichinellosis but also address the current limitations associated with conventional treatments, such as the development of drug resistance, and the presence of chemical residues in meat products. Ultimately, by providing a comprehensive understanding of the dose-response relationship, and elucidating the cytotoxic effects of AgNPs on *T. spiralis*, this study will further inform the practical implementation of AgNPs as a promising solution in the fight against trichinellosis.

Methods and Materials

Materials

- **The Synthesis and Characterization of Silver Nanoparticles**

The synthesis and characterization of silver nanoparticles using the Solomon method (Solomon et al., 2007) was undertaken. The process involved capping the silver nanoparticles with polyvinyl alcohol by adding 0.001 Molar of $AgNO_3$, followed by the addition of 0.3% PVA under constant stirring at room temperature for 2 hr. The characterization of the silver nanoparticles was determined using a Malvern Zetasizer Nano Series and a Hitachi, ST7700 transmission electron microscope. The study provides insight into the synthesis and characterization of silver nanoparticles.

Method

- **The Preparation of *T. spiralis* larvae**

Mice were purchased from the National Animal Laboratory Center, Mahidol University with ethics approval obtained from the Laboratory Animal Science Center, Faculty of Tropical Medicine, Mahidol University. The study was conducted over 8 weeks, and Ethical Guidelines were consistently followed. Initially, 100 *T. spiralis* larvae were orally fed to mice using a stomach tube, and after 10 weeks, the infected mice were slaughtered. The viscera, feet, tail, head, and skin were removed, and the *T. spiralis* larvae were examined under the stereomicroscope. The muscle samples were chopped, and subjected to 1% Pepsin in 1% HCl at 37°C for 1-2 hr, and the third-stage larvae were collected using Baermann's technique. The protocol provides a method for the preparation of *T. spiralis* larvae for further studies.

- **The Toxicity of metal nanoparticles test**

A test was conducted to determine the toxicity of metal nanoparticles on *T. spiralis*. A total of 45 larvae were prepared in a 96-well microplate, and 150 uL of Roswell Park Memorial Institute (RPMI) media was added to each well. The samples were divided into four groups, including a negative control group containing only RPMI media, a positive control group containing Mebendazole with RPMI media, and two experimental groups containing AgNPs with RPMI media. The concentrations of AgNPs used were 100, 200, 300, and 400 ppm, and the treatment period varied between 4, 8, 12, 16, and 24 hr (Fahmy et al., 2020). The potential toxicity of metal nanoparticles on *T. spiralis* larvae was evaluated and a method for assessing their toxicity levels was developed.

Larvicidal rate % = the number of dead parasites/total parasites × 100

- **Hematoxylin and eosin (H&E) staining**

H&E staining was used to examine the morphology of the *T. spiralis* larvae. The larvae were first fixed with buffered neutral formalin solution for one day, dehydrated with ethanol and embedded in paraffin. Individual tissue sections fixed in paraffin (4-6 μm thick) were placed on microscopic slides and deparaffinized by placing the slides into a xylene solution for 2-4 min. The slides were washed thoroughly by dipping them into an absolute ethanol solution and rehydrated with 95% and 70% ethanol. The preparations were then placed into a Lugol's iodine solution for 15 minutes, rinsed with tap water, and placed in a 5% aqueous sodium thiosulfate solution for 3 min. The tissue sections were stained by placing them into a Mayer's hematoxylin solution for 15 min then rinsed under running tap water for 20 minutes, and then counter-stained by placing them into an eosin solution for 1 min. The slides were then dehydrated with gradients of ethanol, and the rehydrated steps previously performed were reversed. Finally, the slides were dipped in a xylene solution, mounted with a mounting medium, and covered with coverslips. The morphological changes of the parasite were examined under a light microscope to identify the structure and characteristics of the *T. spiralis* larvae.

- **Statistical analysis**

The number of *T. spiralis* larvae in the negative control, and the Mebendazole and AgNPs groups that died after the various treatments including at 4, 8, 12, 16, and 24 hr were counted, and the larvae were collected and analyzed to determine the percentage difference in larvicidal activity. A comparison was made between the negative control, and Mebendazole groups to identify the larvicidal activity of *T. spiralis* treated with AgNPs at different for various periods. The standard deviation (SD) of the larvicidal activity was calculated and analyzed using ANOVA to determine the differences in the SDs. Graph Pad was used for the statistical analysis.

Results

Characterization of Silver Nanoparticles (AgNPs)

The achieve the purpose of this study, which was to investigate the effectiveness of AgNPs against *T. spiralis* larvae, we successfully synthesized silver nanoparticles that formed stable colloids in Milli-Q water. The AgNPs were characterized using a zetasizer which showed that the nanoparticles were spherical with an average size of approximately 132.9 nm, as shown in Figures 1A, and 1B. Furthermore, Figure 1C demonstrated a relativity uniform particle size distribution of the dispersed AgNPs.

The results obtained from the zetasizer analysis were consistent with the observations made using transmission electron microscopy (TEM), where the TEM image displayed AgNPs with a diameter of 100 nm, as indicated by the scale bar (Fig. 1). The AgNPs size and zeta potential were evaluated (Fig. 1C). The result showed that AgNPs were spherical and the average hydrodynamic diameter was 132.9 nm.

These findings provide valuable insights into the characterization, and physical properties of the synthesized AgNPs about their interaction with *T. spiralis* larvae.

The effective concentration of AgNPs varied (Table 1), with the lowest concentration of 100 ppm resulting in the lowest number of larval deaths: 10 larvae (22.22%) after 16 hr of treatment whereas the highest concentration of 400 ppm led to the highest number of larval death (40 larvae, 88.88%) after 24 hr.

When comparing the different treatment groups, the number of larval deaths varied. In the negative control group, only one larva (2.22%) died. In the Mebendazole group, three larvae (6.66%) died. In the 100 ppm AgNPs group, 26 larvae (57.77%) died. In the 200 ppm AgNPs group, 33 larvae (73.33%) died. Lastly, in the 300 ppm AgNPs group, 34 larvae (75.55%) died, all after 24 hr of treatment.

These findings provide valuable insights into the effectiveness of different concentrations of AgNPs in causing larval death in comparison to the negative control and Mebendazole treatment.

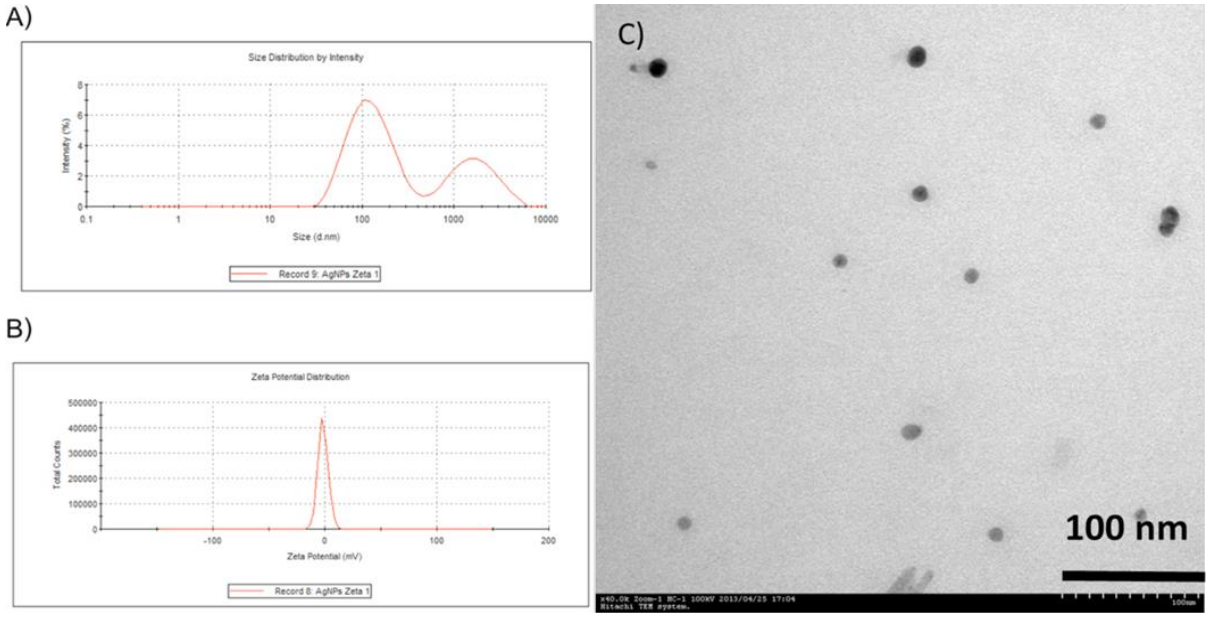

Figure 1 Characterization of AgNPs (size and zeta potential) were evaluated using A) and B) zetasizer® and C) Transmission Electron Microscopy (TEM). Scale bars were 100 nm

Table 1 The number of dead *T. spiralis* larvae with silver nanoparticles at 4, 8, 16, and 24 hr

Test	4 hr n=45 (%)	8 hr n=45 (%)	16 hr n=45 (%)	24 hr n=45 (%)
Negative control	0 (0%)	0 (0%)	0 (0%)	1 (2.22%)
Mebendazole	0 (0%)	1 (2.22%)	3 (6.66%)	3 (6.66%)
AgNPs 100 ppm.	0 (0%)	0 (0%)	10 (22.22%)	26 (57.77%)
AgNPs 200 ppm.	0 (0%)	0 (0%)	12 (26.66%)	33 (73.33%)
AgNPs 300 ppm.	2 (4.44%)	3 (6.66%)	15 (33.33%)	34 (75.55%)
AgNPs 400 ppm.	2 (4.44%)	3 (6.66%)	16 (35.55%)	40 (88.88%)

Toxicity of silver nanoparticles

The correlation analysis involved plotting the group tested on the X-axis, and the number of larvae deaths on the Y-axis (Fig. 2).

Concentrations of AgNPs at 100 ppm, 200 ppm, and 300 ppm were separately administered to 45 larvae after 16 hr. The toxicity of AgNPs was evaluated based on the number of larval deaths observed.

The analysis revealed that the concentration of AgNPs with the highest toxicity was 300 ppm, resulting in the greatest number of larval deaths (15 larvae, 33.33%) after 16 hr. This finding indicates that higher concentrations of AgNPs have a more pronounced impact on larval mortality.

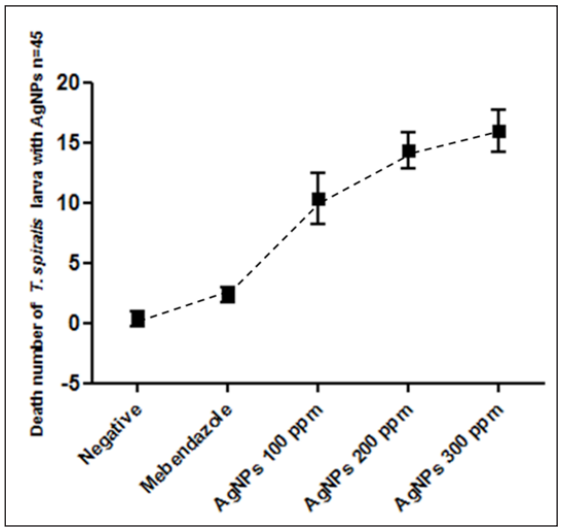

Figure 2 Lethal effect of AgNPs on *T. spiralis* after being treated with AgNPs 100, 200, and 300 ppm (Negative control; no AgNPs treated, Mebendazole; treated with Mebendazole) after 16 hr

Morphology with AgNPs nanoparticles by light microscopy

In addition to toxicity analysis, the morphology of *T. spiralis* larvae in the presence of AgNPs was examined using light microscopy (Fig. 3).

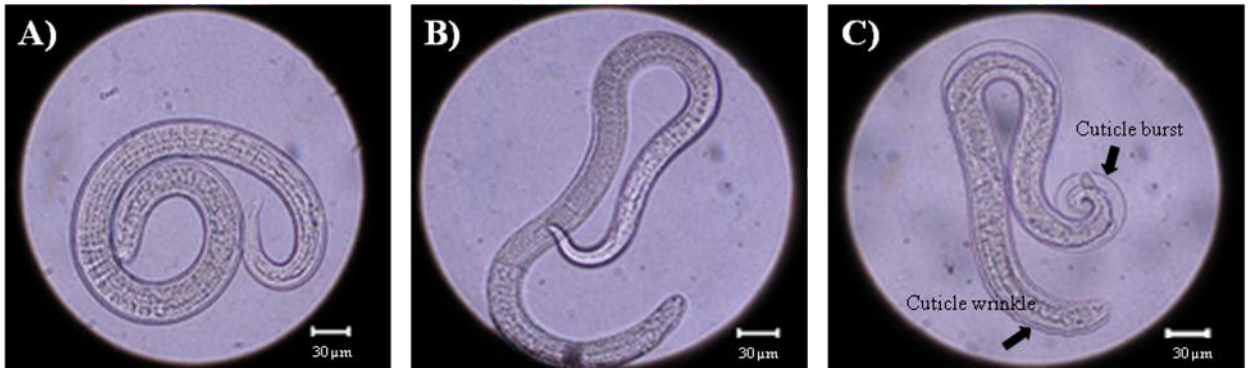

Figure 3 The morphology of *T. spiralis* larvae with silver nanoparticles (AgNPs) by light microscopy. A) normal of typical coiled larvae; B) active motile larvae; C) muscular larvae of *T. spiralis* after complete death

In Fig. 3C, the *T. spiralis* larvae were subjected to treatment with 400 ppm AgNPs for 24 hr. The image depicts the presence of cuticle burst and wrinkles in the larvae, as indicated by the black arrow. This observation suggests that the AgNPs treatment at the concentration, and duration had a significant impact on the morphology and integrity of the cuticle.

H&E staining with AgNPs

In Fig. 4A, the typical morphology of untreated *T. spiralis* larvae is depicted. The image reveals the presence of column-shaped stichocyte cells originating from the esophagus of the *T. spiralis* larvae. The midgut system and genital primordium cells are also visible within the body. Additionally, the cuticle of the epidermis can be observed, indicated by the black arrow marked (c) that formed the external structure of the *T. spiralis* larvae. This image provides insights into the natural anatomy and cellular composition of the untreated larvae.

In Fig. 4B, *T. spiralis* larvae were subjected to treatment with 200 ppm AgNPs for 16 hr. The image shows significant changes in the morphology of the larvae. Most of the stichocyte cells (indicated by the black arrow labelled (stc)) have disappeared from the esophagus. The midgut system and the genital primordium cells have degraded within the body. The cuticle of the worm (indicated by the black arrow, c) has fissured and is exhibited from the body, indicating damage to the epidermal layer of the *T. spiralis* larvae. This image demonstrates the detrimental effects of the AgNPs treatment on the structure and integrity of the larvae.

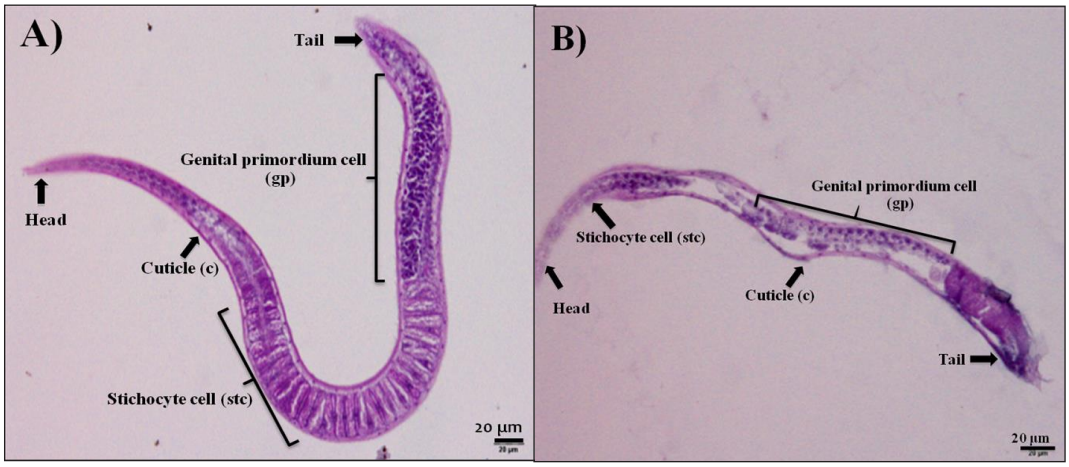

Figure 4 Morphologic of *T. spiralis* by H&E staining, results showed the histological; A) no AgNPs treated B) treated with 200 ppm AgNPs

Our study focused on investigating the toxicity of silver nanoparticles (AgNPs) for *T. spiralis* larvae. The size of the AgNPs was determined to be 132.9 nm using Zetasizer analysis. We observed that the effective concentration of AgNPs was 100 ppm, resulting in the lowest number of larval deaths: 10 larvae (22.22%) after 16 hr of exposure. On the other hand, the concentration of 400 ppm led to the highest number of larval deaths: 40 larvae (88.88%) after 24 hr of exposure. AgNPs had a detrimental effect on the genital primordium cells.

Discussions

Silver nanoparticles are widely recognized for their ability to inhibit various infection agents, making them a valuable alternative treatment option in the face of growing microbial resistance. In the prior studies, AgNPs using Myrrh extract with an average particle size of 20 nm, to test their inhibitory effects on *T. spiralis* (Abd-ELrahman et al., 2021). In contrast, in the current study, only AgNPs with an average particle size of

132.9 nm were used, and the findings indicated that using AgNPs effectively inhibited *T. spiralis*, and reduced the need for additional chemical agents. Selecting a lower concentration at 100-400 ppm of AgNPs, and using a shorter duration of exposure time were deliberate choices in this study. However, when compared to another study (Taha et al., 2022) using larger-sized AgNPs ranging from 450 to 850 nm. The experiment results indicated that the time required for the elimination of *T. spiralis* can be reduced. Therefore, the size of AgNPs is essential in the selection of testing parameters. These are factors that influence the concentration, and duration time chosen for the elimination of *T. spiralis.*

These results are correlated with a previous study where AgNPs at a concentration of 1 ppm for 30 min reduced the viability of *Cryptosporidium* oocysts by 97.2% (Hassan et al., 2010). Myrrh-biosynthesized AgNPs showed that concentrations of 1-20 μg/ml after 2 days reduced the viability of *Trichinella spiralis*, and *Haemonchus contortus*. Also, AgNPs exhibited a lethal effect on the L3 stage of *Ancylostoma caninum* and cercariae of *Schistosoma mansoni*. (Abd-ELrahman et al., 2021).

We hypothesize that the concentration, and types of metal nanoparticles used in the test are significant factors contributing to the mortality of *T. spiralis* larvae. Furthermore, the concentration of 100 mgL, and 10 mgL of AgNPs as being toxic to *Caenorhabditis elegans*, affecting their digestive system (Kim et al., 2012). These observations suggest that the effects of metal nanoparticles can vary depending on the concentration specific to a species, and the stage of growth of the species being studied. It can be surmised that genetic diversity within species can result in different responses to AgNPs. Some individuals or populations may be more resistant or susceptible due to genetic factors. Including, the metabolic, and physiological processes of species can differ, affecting how they interact with, and respond to AgNPs. This can include differences in silver ion uptake, detoxification pathways, and cellular mechanisms.

An outcome of the current study was the design of the experiment utilizing the H&E staining method to investigate the morphology effects of AgNPs on *T. spiralis*. Our findings revealed that AgNPs can damage the genital primordium cells present in the nematode, which significantly impacts their reproductive system. As well, the considerable destruction of stichocyte cells within the internal structure of *the T. spiralis* was also detected. These observations align with a previous study highlighting the impact of AgNPs on the digestive system of *C. elegans* (Kim et al., 2012). Additionally, we observed the exfoliation of cuticles from the body of the *T. spiralis*. Based on the damage observed in the esophagus, a vital component of their digestive system, and the dispersion of AgNPs within their hypodermis, we can assume that *T. spiralis* likely ingested AgNPs through their oral route, and absorbed them through their cuticle. This suggests that AgNPs can enter the body of *T. spiralis* through multiple routes of exposure. We observed that AgNPs had a detrimental effect on the genital primordium cells, which play a crucial role in reproduction. This suggests that AgNPs may directly contribute to the destruction of the digestive system, and the exfoliation of cuticles from the body of *T. spiralis*. The phenomenon facilitates the movement of AgNPs through various structures within the body of the parasite. The destruction of cuticles that was observed can be attributed to the binding of released positive silver ions to the negatively charged cell membranes, thereby disrupting their integrity. Furthermore, these ions may adhere to the membrane walls, creating openings that allow penetration into the organism. This aligns with the findings of (Abd-ELrahman et al., 2021) that demonstrated that silver nanoparticles caused cuticle damage through their interaction with cell membranes and subsequent disruption of their structure. These results indicate that AgNPs not only affect the digestive system, and cuticles but also have potential

implications for reproductive functions. The mechanism of action involves the binding, and interaction of silver ions, ultimately leading to cell damage, and compromising the integrity of the *T. spiralis* larvae. In summary, the application of this newly developed AgNPs formula in this study has shown that AgNPs processes are capable of inducing irreversible damage to the cuticle, gastrointestinal system, and reproductive system of the nematode. Currently, an *in vivo* study is in process, using data from this *in vitro* study to assess the potential of AgNPs as an effective parasitical material.

Conclusion and Suggestions

It can be concluded that AgNPs have an impact on *T. spiralis* parasites through various routes, including the cuticle, gastrointestinal system, and reproductive system. These findings show the potential for the development of alternative methods to eliminate *T. spiralis*. However, it is crucial to consider the cost-effectiveness of this alternative approach in comparison to existing methods. To ensure the safe implementation of AgNPs, further experiments are required to investigate the potential environmental harm posed by AgNPs and assess their impact on the infective stage of *T. spiralis*. This information is essential to evaluate the overall effectiveness and potential risks associated with the use of AgNPs as a control measure for *T. spiralis*.

Additional studies should be conducted to gather more comprehensive data before considering the implementation of AgNPs as a viable solution. It is essential to weigh the benefits, costs, and potential environmental implications to make an informed decision about their practical use in controlling *T. spiralis* infections.

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Author Contributions

Author 1 (Jaruwan Jankong): Conceptualization of the research, Development or design of methodology, Data analysis and interpretation, Investigation, Manuscript writing, Manuscript review and editing

Author 2 (Suphasit Aroonrungsawasdi): Collection of data, Manuscript review and editing

Author 3 (Thanit Songsumud): Providing of materials subjects, Data analysis and interpretation

Conflict of Interests

The authors declare no conflicts of interest.

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