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FISH PARASITOLOGY - ORIGINAL PAPER

Efficacy of silver nanoparticles against the adults and eggs of monogenean parasites of fish

Citlalic Altagracia Pimentel-Acosta[1] · Francisco Neptalí Morales-Serna[2] · María Cristina Chávez-Sánchez[1] · Humberto Herman Lara[3] · Alexey Pestryakov[4] · Nina Bogdanchikova[5] · Emma Josefina Fajer-Ávila[1]

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Abstract

Monogeneans are a diverse group of parasites that are commonly found on fish. Some monogenean species are highly pathogenic to cultured fish. The present study aimed to determine the in vitro anthelmintic effect of silver nanoparticles (AgNPs) against adults and eggs of monogeneans in freshwater using *Cichlidogyrus* spp. as a model organism. We tested two types of AgNPs with different synthesis methodologies and size diameters: ARGOVIT (35 nm) and UTSA (1–3 nm) nanoparticles. Damage to the parasite tegument was observed by scanning electron microscopy. UTSA AgNPs were more effective than ARGOVIT; in both cases, there was a concentration-dependent effect. A concentration of 36 μg/L UTSA AgNPs for 1 h was 100% effective against eggs and adult parasites, causing swelling, loss of corrugations, and disruption of the parasite's tegument. This is an interesting result considering that monogenean eggs are typically tolerant to antiparasite drugs and chemical agents. To the best of our knowledge, no previous reports have assessed the effect of AgNPs on any metazoan parasites of fish. Therefore, the present work provides a basis for future research on the control of fish parasite diseases.

Keywords Control disease · Silver nanoparticles · Platyhelminthes · Tegument · Toxicity

Introduction

Monogeneans are ectoparasites commonly found on the gills of marine and freshwater fish. They have a direct life cycle; except for oviparity occurring in the Gyrodactylidae family, the adults of most species lay eggs that hatch into a larva (oncomiracidium), which must find and attach to the definitive host species (Whittington 2005). Heavy infestations may cause excess mucous secretion, damage to the epithelium, hemorrhages, osmotic problems, and atrophy of the gills that leads to failure of the respiratory system (Whittington 2005). In addition, injuries caused by monogeneans may facilitate secondary infections by bacteria, fungi, and viruses, which may lead to host death (Busch et al. 2003). Some species of the Gyrodactylidae, Dactylogyridae, and Capsalidae families are responsible for disease and mortality in freshwater and marine finfish aquaculture (Thoney and Hargis 1991; Cable et al. 2000).

Monogeneans may be notoriously difficult to control in fish farms or aquariums. Among the main treatments to control these parasites are hydrogen peroxide, formalin, potassium permanganate, copper sulfate, salinity (NaCl), freshwater, and praziquantel baths, which can be highly effective against either the juvenile or adult stages of the parasite but not against eggs (Fajer-Ávila et al. 2007; Zhang et al. 2014; Morales-Serna et al. 2018a). The US Food and Drug Administration (FDA) approved formalin to eradicate monogeneans. This

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✉ Emma Josefina Fajer-Ávila
efajer@ciad.mx

1 Centro de Investigación en Alimentación y Desarrollo, A.C. Unidad Mazatlán en Acuicultura y Manejo Ambiental, Mazatlán, Sinaloa, Mexico

2 CONACYT, Centro de Investigación en Alimentación y Desarrollo, A.C. Unidad Mazatlán en Acuicultura y Manejo Ambiental, Mazatlán, Sinaloa, Mexico

3 Department of Biology and South Texas Center for Emerging Infectious Diseases, The University of Texas at San Antonio, San Antonio, TX, USA

4 Tomsk Polytechnic University, Tomsk 634050, Russia

5 Departamento de Fisicoquímica, Centro de Nanociencias y Nanotecnología, Universidad Nacional Autónoma de México, 22860 Ensenada, Baja California, Mexico

chemical may be effective against all life stages of monogeneans (Francis-Floyd 1996; Reed et al. 2012); however, it represents a health risk to the fish and the environment (Kaneko et al. 1988; Rowland et al. 2006; Leal et al. 2018). Therefore, it is necessary to evaluate novel approaches to control monogenean parasites in fish.

Nanobiotechnology has shown a variety of applications in medicine and industry, including the control of infectious diseases in plants and animals (e.g., Resham et al. 2015; Zhao et al. 2017). In particular, the bactericidal, antifungal, and antiviral efficacy of silver nanoparticles (AgNPs) has been suggested to improve human health (Sondi and Salopek-Sondi 2004; Lara et al. 2011, 2015; Franci et al. 2015; Cho et al. 2018). Likewise, AgNPs may be effective against pathogenic bacteria in shrimp (Vaseeharan et al. 2010; Sivaramasamy et al. 2016) and protozoan parasites in fish (Saleh et al. 2017). However, nothing is known about the effect of AgNPs against metazoan parasites of fish.

The present study aimed to determine the in vitro anthelmintic effect of AgNPs against eggs and adults of *Cichlidogyrus*, a genus of monogenean commonly found on cichlid fish, including the economically important Nile tilapia (*Oreochromis niloticus*). Surface changes to the tegument were observed by scanning electron microscopy (SEM). Given that toxicity may be inversely related with nanoparticle size (Ivask et al. 2014), two types of AgNPs (ARGOVIT and UTSA) synthesized by different methods and with different sizes were tested herein.

Materials and methods

AgNPs

ARGOVIT AgNP solution was donated by Dr. Vasily Burmistrov from the Scientific and Production Center Vector-Vita (Russia), in collaboration with Dr. Nina Bogdanchikova from Centro de Nanociencias y Nanotecnología, Universidad Nacional Autónoma de México. ARGOVIT consists of highly dispersed AgNPs, with an overall concentration of 200 mg/mL (20%) of polyvinylpyrrolidone (PVP)-coated AgNPs in water, a silver metallic concentration of 12 mg/mL stabilized with 188 mg/mL of PVP, and an average silver particle diameter of 35 ± 15 nm (Juarez-Moreno et al. 2017).

AgNP solution, named UTSA, was kindly donated by Dr. Miguel Yacaman from the Department of Physics of the University of Texas at San Antonio. UTSA AgNPs, which were synthesized using microwave irradiation to convert silver nitrate into metallic silver as described by Lara et al. (2015), had a silver metallic concentration of 0.032 mg/mL and size diameter of 1–3 nm.

AgNPs were kept in the dark at 4 °C. For experiments, freshly made stock solutions (ARGOVIT, 2000 μg Ag/mL; UTSA, 269.67 μg Ag/mL) were prepared in distilled water.

Test parasite

For the present study, monogeneans of the genus *Cichlidogyrus* were collected from the gills of Nile tilapia sampled in a fish farm in southern Sinaloa, Mexico. A previous survey revealed that farmed Nile tilapias from this region are commonly infected by two species of *Cichlidogyrus*: *C. sclerosus* and *C. tilapiae* (Morales-Serna et al. 2018b); nonetheless, *C. longicornis* and *C. dossoui* may also be found (E.J. Fajer-Ávila, unpublished data). The identification of species of these parasites typically requires observation of sclerotized structures (e.g., haptor and copulatory complex) after the partial digestion of individuals. Even so, some species may be confused because of their morphological similarity. Therefore, experiments were based on individuals of *Cichlidogyrus* spp. in general, which represents a real situation that occurs in farmed tilapia.

AgNP antiparasitic activity against adult monogeneans in vitro

Fish were killed by spinal severance following the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (AVMA 2013). Gill arches were removed for observation under a stereomicroscope. Gill filaments holding one or more adult individuals of *Cichlidogyrus* spp. were gently removed and placed in six-well plates, with each well containing 5 mL of distilled water at 24.5 °C and 10 parasites. After initial range-finding tests, ten concentrations of both AgNPs types were selected for definitive tests, with ARGOVIT concentrations (6000, 12,000, 18,000, 24,000, 30,000, 36,000, 42,000, 48,000, 54,000, and 60,000 μg/L) being higher than UTSA concentrations (6, 12, 18, 24, 30, 36, 42, 48, 54, and 60 μg/L). To determine the 1-h LC50 of UTSA AgNPs, seven logarithmically separated concentrations between 6 and 30 μg/L were tested. Control wells containing only distilled water were also studied. To account for any possible effect of PVP on the parasites, there were control wells that contained distilled water plus the PVP volume that was used for the highest concentration ARGOVIT AgNPs. For comparative purposes, 250 μL/L formalin (37% formaldehyde) and 36 g/L sodium chloride were tested. Five treatment replicates were performed in all experiments. Parasite mortality was recorded every 30 min for 5 h. Parasites were considered dead if they failed to respond to a gentle touch or external stimuli and did not show any reaction when being transferred to clean distilled water.

AgNP activity against monogenean eggs in vitro

Adults of *Cichlidogyrus* spp. were placed in six-well plates containing 5 mL of distilled water. Monogeneans were allowed to lay eggs for 6 h (Khidr 1989); then, they were removed from the wells. For this experiment, there were ten eggs per well. Three concentrations of both AgNP types (ARGOVIT 6000, 48,000, and 60,000 μg/L; UTSA 6, 36, and 60 μg/L) and 250 μL/L formalin and 36 g/L sodium chloride were applied for 1 h against monogenean eggs. Eggs in control wells received the same manipulation. There were six replicates per concentration. After the exposure time, eggs were transferred to clean water and were examined every 24 h under an inverted microscope (Zeiss Axio Vert. A1) in order to count the number of eggs in the different stages of development (damage, embryonated, developing, and hatched). Water was exchanged (50%) on a daily basis. The temperature varied between 24 and 25 °C throughout the course (7 days) of the experiment.

Ultrastructure observation of the activity of AgNPs against the parasite by SEM

To observe ultrastructural changes to the tegument of adult *Cichlidogyrus* spp., parasites ($n = 10$) were exposed to UTSA AgNPs (6, 36, and 60 μg/L). Control and treated moribund parasites were fixed in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) for 24 h at 4 °C, followed by secondary fixation in 1% osmium tetroxide (OsO_4) in the same buffer for 1 h at 4 °C. They were then dehydrated through a graded ethanol series and critical-point dried with carbon dioxide. Specimens were mounted on metal stubs, coated with gold, and examined in a Hitachi Stereoscan Model SU1510 SEM (Hitachi Ltd., Tokyo, Japan).

Data analyses

Significant differences in mortality and hatching success were detected between treatments with ANOVA and an a posteriori Tukey test in SigmaStat 3.5 (Systat Software, San Jose, CA, USA). Data were normalized by arcsine transformation. The median lethal concentration (LC50) and its confidence intervals were calculated using a Probit analysis with a maximum likelihood regression algorithm (Statgraphics Centurion XVI, version 16.1.03).

Results

AgNPs against adult monogeneans in vitro

Both AgNPs had a concentration-dependent effect. In the case of ARGOVIT, the highest concentrations (48,000, 54,000, and 60,000 μg/L) killed all parasites after 60 min, whereas the lowest (6000 μg/L) did so after 300 min (Fig. 1). In the case of UTSA, the highest concentration (60 μg/L) killed all parasites after 30 min, and the lowest (6 and 12 μg/L) did it after 180 min (Fig. 2). Formalin and sodium chloride were 100% effective after 180 and 60 min, respectively. Mortality was significantly different in all AgNP concentrations ($P < 0.05$) compared to controls, which varied from 0 to 10%. The estimated value of the 1-h LC50 of UTSA AgNPs in adult monogeneans with a 95% confidence interval is shown in Fig. 3.

AgNPs against monogenean eggs in vitro

Both ARGOVIT and UTSA AgNPs completely inhibited egg hatching, except for the lowest UTSA concentration (6 μg/L), which only showed 50% inhibition (Fig. 4). Formalin reduced hatching success to 10%. In contrast, egg hatching (90% successful) was significantly higher ($P < 0.05$) in both control and sodium chloride treatment after 4 days (Fig. 4). Unhatched eggs showed no signs of embryonic development and were dark brown in appearance (Fig. 5a). Embryonated eggs were green-yellow, developing into larvae with eyespots, which escaped from the capsules through a hole at the apex (Fig. 5b-d).

Ultrastructural activity of AgNPs against adult parasites

Parasites treated with UTSA AgNPs showed significant changes compared to the control. The surface architecture, including anterior and posterior (haptor) parts and annular corrugations of the control parasites, were normal (Fig. 6). Parasites exposed to AgNPs at 6 μg/L did not show apparent damage in morphology, but they showed a physiological response that included formation of vacuoles and slight swelling of the tegument (Fig. 7). Parasites exposed to 36 and 60 μg/L AgNPs showed swelling, loss of corrugations, and disruption of the tegument (Fig. 8).

Discussion

To the best of our knowledge, this is the first work to examine the efficacy of AgNPs against metazoan parasites of fish, particularly against the adults and eggs of *Cichlidogyrus*. Numerous studies have shown the antimicrobial effect of AgNPs against bacteria, fungi, viruses, and insects (Gorth et al. 2011; Ivask et al. 2014; Franci et al. 2015; Govindarajan and Benelli 2015; Lara et al. 2015; Aderibigbe 2017). However, few studies have evaluated the effectiveness of AgNPs against pathogens of fish. Saleh et al. (2017) determined that 10 μg/L AgNPs with an average diameter of 21 nm

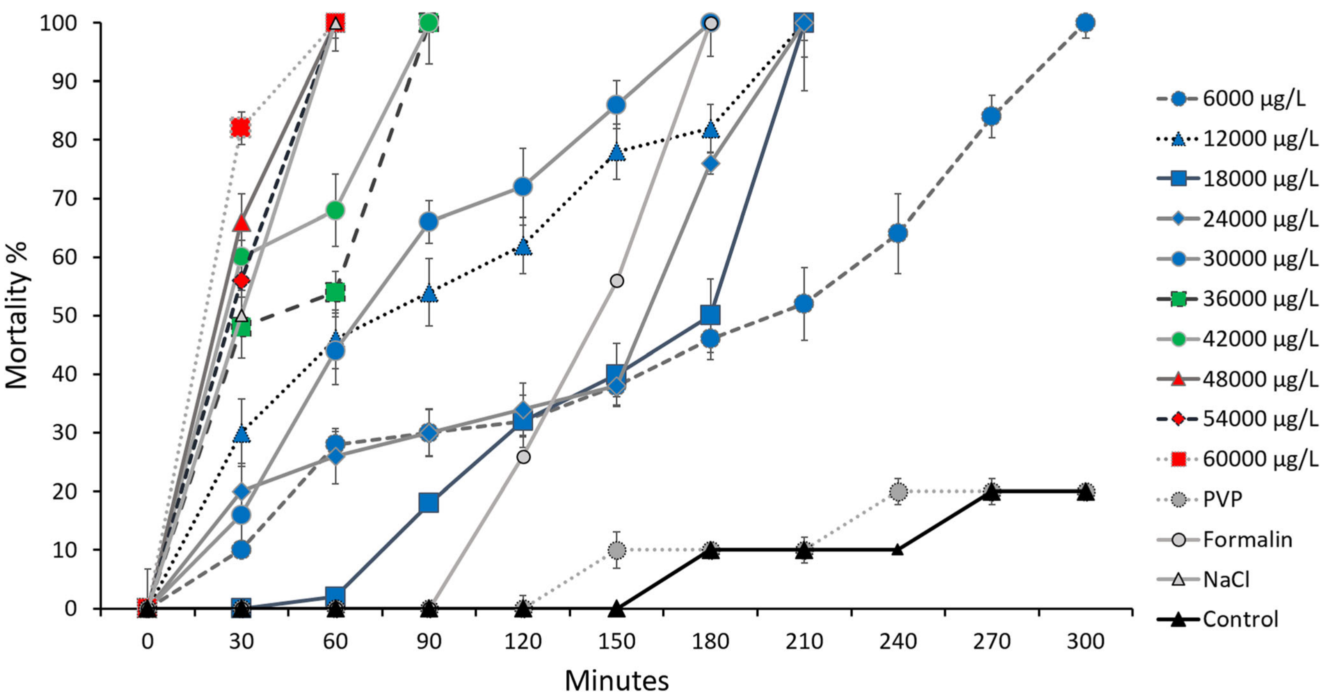

Fig. 1 Cumulative mortality of adult *Cichlidogyrus* spp. that were exposed to different concentrations of ARGOVIT AgNPs. Error bars indicate SE

were 100% effective after 2 h against the ciliated *Ichthyophthirius multifiliis*, which is the causal agent of "white spot disease" in fish; given that no mortalities were recorded in treated fish, they recommended AgNPs for the development of effective antiprotozoal agents for fish aquaculture.

In the present study, AgNPs UTSA at 6 µg/L were effective after 3 h, which is comparable to the aforementioned results of Saleh et al. (2017). Furthermore, we observed that such total efficacy may be achieved in much less time (30 min) by applying 60 µg/L of UTSA AgNPs.

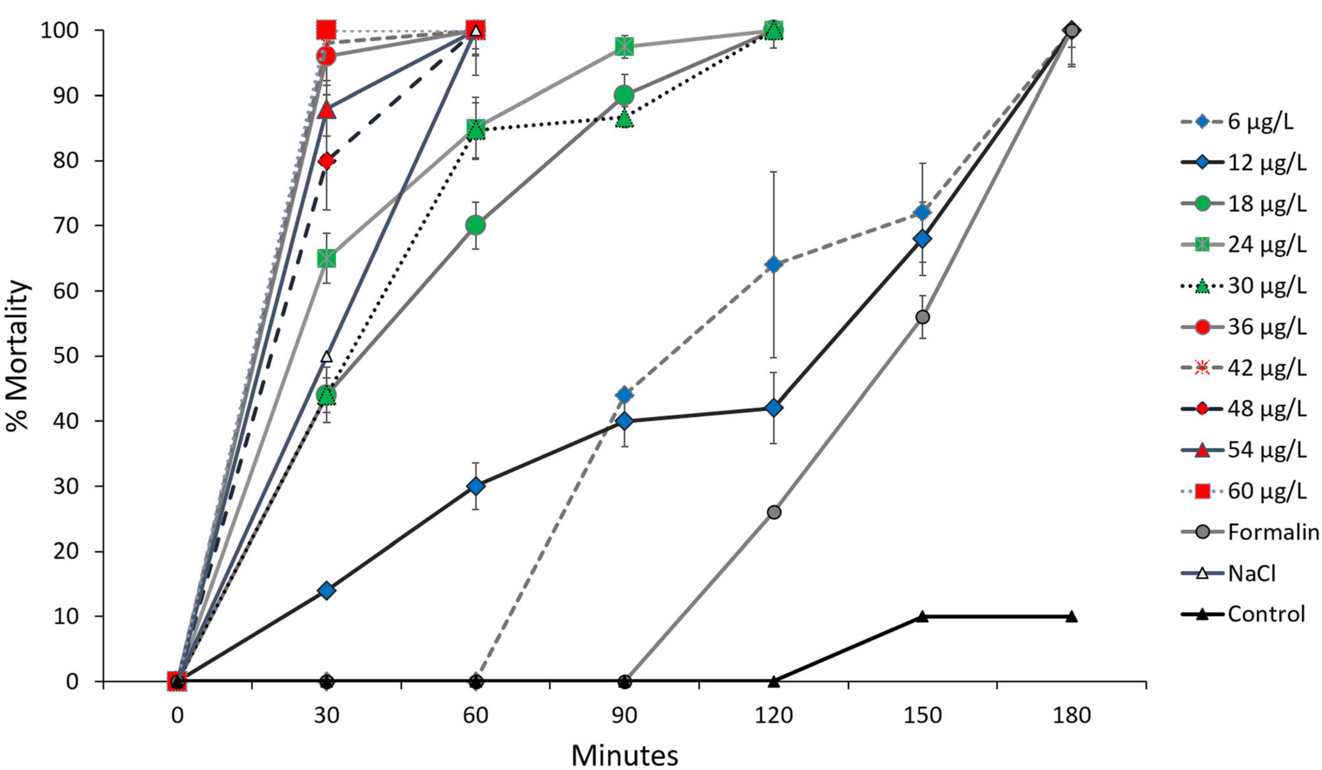

Fig. 2 Cumulative mortality of adult *Cichlidogyrus* spp. that were exposed to different concentrations of UTSA AgNPs. Error bars indicate SE

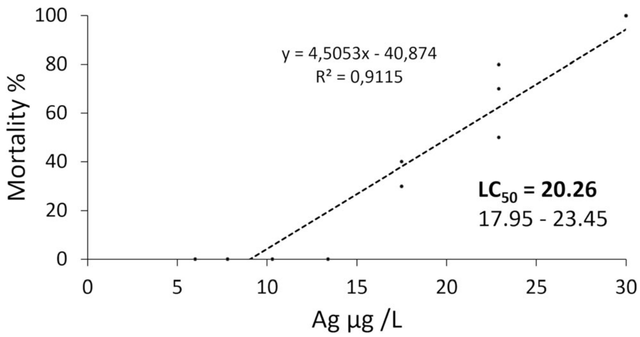

Fig. 3 Linear equation of the concentration–response relationship in determining the 1-h LC50 of UTSA AgNPs for adult *Cichlidogyrus* spp.

However, these results are not comparable with the much higher concentrations of ARGOVIT AgNPs required to kill all monogeneans. As expected, such differences are possibly due to nanoparticle sizes. Our results agree with other authors who observed that smaller nanoparticles have higher toxicity. For instance, using different test organisms/cells, Ivask et al. (2014) showed that 10 nm AgNPs were more toxic than 20–80 nm and argued that 10 nm AgNPs have additional non-dissolution-driven toxic properties compared to 20–80 nm AgNPs, in which toxicity was induced by released Ag^{+} ions. In addition, Ivask et al. hypothesized that, due to their small size, 10 nm AgNPs may induce ROS and/or increase the bioavailability of silver. Likewise, Ayala-Núñez et al. (2009) observed that the relatively high toxicity of small AgNPs may improve the therapeutic index and pointed out that small AgNPs (< 10 nm) are more toxic given their ability to reach structures that are otherwise not available to bigger nanoparticles. In addition, it has been shown that PVP-coated AgNPs may be less toxic than uncoated ones (Zhao and Wang 2012; Nguyen et al. 2013).

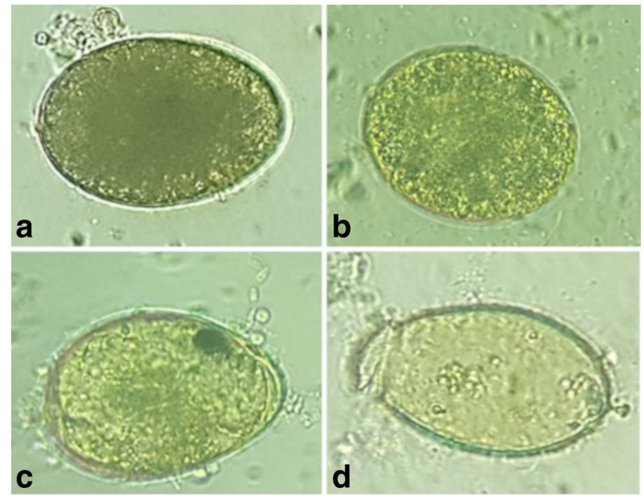

Fig. 5 *Cichlidogyrus* egg development. **a** Damage. **b** Embryonated. **c** Developing. **d** Hatched

Prior to the present study, the efficacy of AgNPs against helminths had only been proven in non-aquatic species. For instance, Tomar and Preet (2017) found that 25 µg/mL (= 25,000 µg/L) AgNPs with a diameter of 15–25 nm induced 87% mortality in adult *Haemonchus contortus* nematodes after 24 h of treatment. This concentration is much higher than that of UTSA or ARGOVIT AgNPs that is required to kill all monogeneans after 5 h

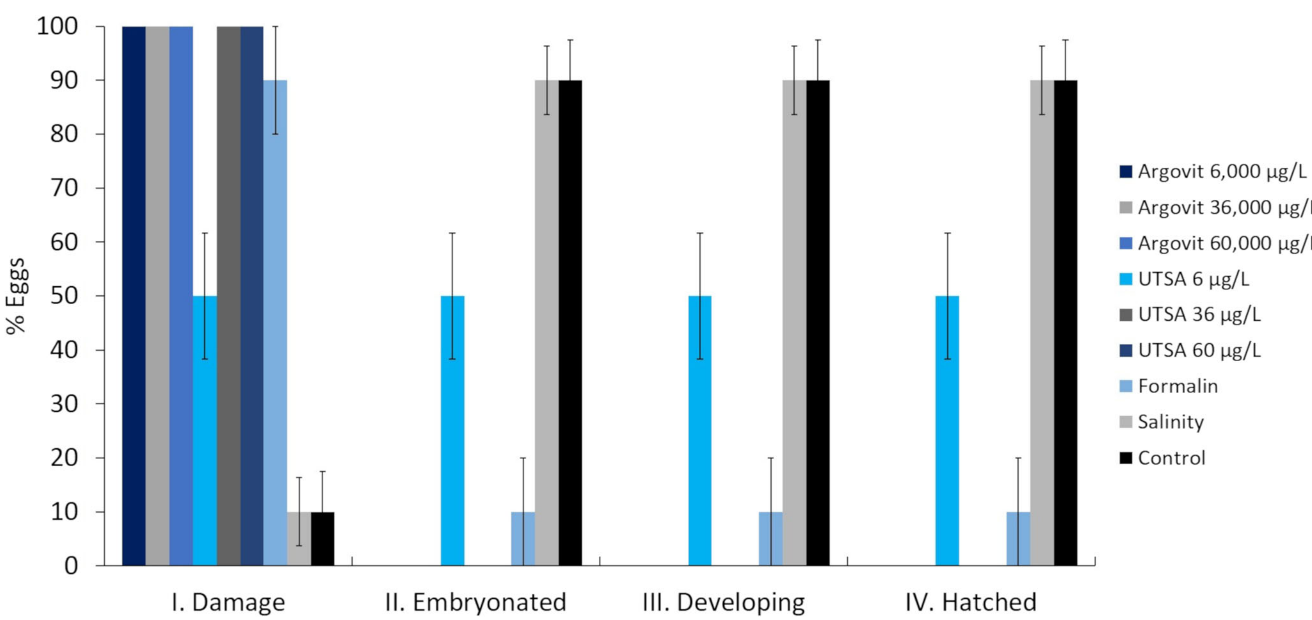

Fig. 4 Cumulative developmental stages of *Cichlidogyrus* eggs following immersion in different treatments (AgNPs, formalin, and sodium chloride). Error bars indicate SE

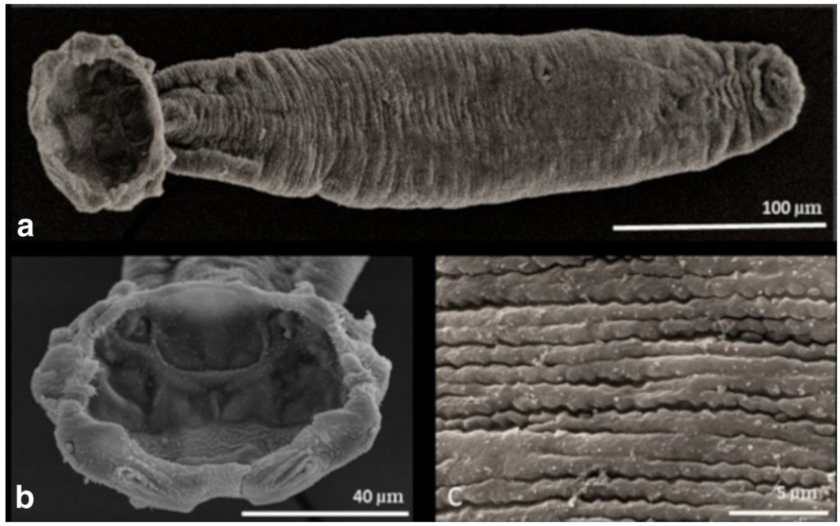

Fig. 6 Scanning electron micrographs of the tegumental surface of *Cichlidogyrus* sp. (control). **a** Whole body. **b** Haptor. **c** Middle region with normal corrugations

of treatment. Such differences could be due to the possibly more resistant cuticle of nematodes, which has a more complex and varied structure including three main layers (cortical, medial, and basal), with the outer layer usually being toughened or strengthened in some way (Lee 1967; Davies and Curtis 2011), which is a characteristic that could give nematodes greater protection against the entry of external agents (Johnstone 1993; Page and Johnstone 2007). In contrast, monogeneans have a thinner outer membrane with a cytoplasmic epidermis (syncytium) and a basal lamina, followed by a layer of circular and longitudinal muscle fibers (Lee 1967; El-Naggar et al. 1991).

Ultrastructural changes of the tegument of monogeneans induced by UTSA AgNP treatment, particularly at the highest concentration, were evidenced through our SEM micrographs. The tegument of monogeneans fulfills functions that are essential to their ectoparasitic lifestyle, such as absorption and secretion of substances, osmoregulation, mechanical support, and protection against harmful agents (Dalton et al. 2004; Hodová et al. 2018). Thus, severe tegumental damage may result in parasite death. In the present study, major ultrastructural changes in the tegument were observed after exposures to 36 and 60 µg/L UTSA AgNPs. Similar results were obtained by Kar et al. (2014) when 1 mg/mL of gold nanoparticles was applied to cestodes (*Raillietina* sp.). These authors argued that ultrastructural changes in the tegument were linked to possible inhibition of protein synthesis as a result of the treatment. Nevertheless, when schistosome cercariae were exposed to 15–1000 µg/mL AgNPs, Cheng et al. (2013) did not observe damage to the tegument. According to these authors, this may have occurred because, given the hydrophobic and unstable properties of AgNPs in aquatic suspension, only a small proportion is truly functional. In our study, even at the lowest AgNP concentration, monogeneans exhibited a physiological response due to the presence of vesicles or "blebs." This drug-induced vacuolization in the tegument has also been observed in other helminths as a response to stress (Toner et al. 2008; O'Neill et al. 2015; De la Torre-Escudero et al. 2016). Future research is needed to understand the mechanism of action of AgNPs in the tegument of monogeneans as well as to observe internal damage to the parasitic ultrastructure and molecular changes leading to parasite death.

We also observed that, contrary to formalin and sodium chloride, AgNPs were completely effective at inhibiting the egg hatching of monogeneans. This is an interesting

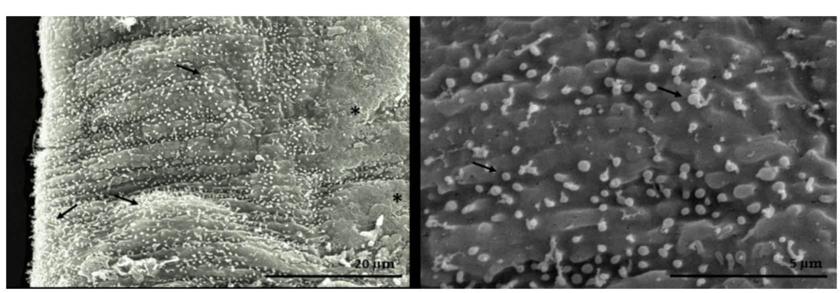

Fig. 7 Scanning electron micrographs of the tegumental surface of *Cichlidogyrus* sp. after exposure to 6 µg/L UTSA AgNPs. Arrows indicate vesicles or bubbles, and asterisks indicate swelling

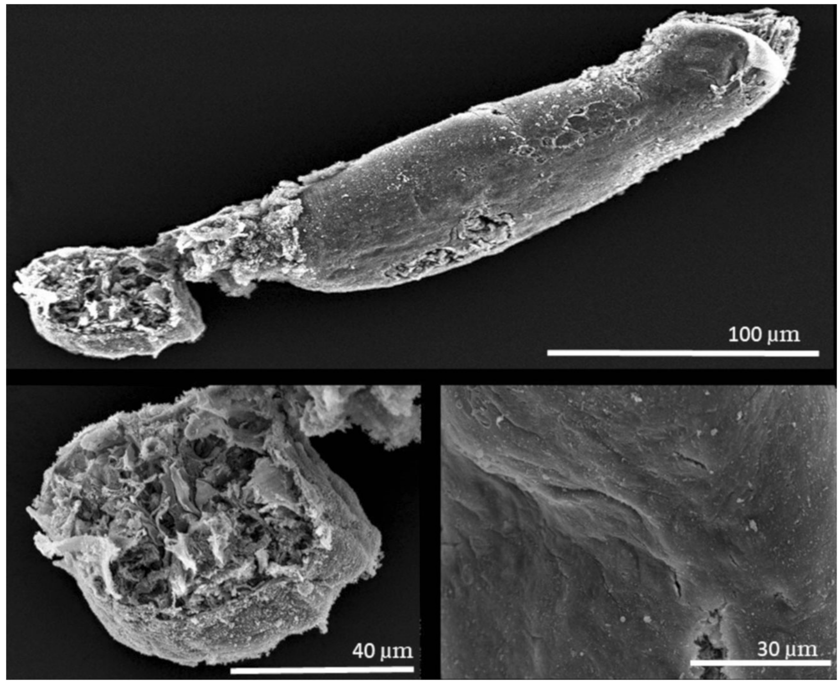

Fig. 8 Scanning electron micrographs of the tegumental surface of *Cichlidogyrus* sp. after exposure to 36 and 60 µg/L UTSA AgNPs. Swelling and destruction of the tegument

finding given that monogenean eggs are typically tolerant to antiparasitic drugs (Zhang et al. 2014; Morales-Serna et al. 2018a). There have been a few studies on the effect of AgNPs on eggs of other helminth parasites. Gherbawy et al. (2013) observed that triclabendazole in combination with AgNPs had an efficacy (90%) higher than triclabendazole alone (70%) in inhibiting egg hatching of the liver fluke *Fasciola hepatica*. In another study, Tomar and Preet (2017) found that 1 µg/mL of green synthesized AgNPs inhibited egg hatching of *H. contortus* by 85%. In these studies, SEM micrographs uncovered damage to the egg surface and distortion, perforation, shrinkage, and disintegration of the embryo (Gherbawy et al. 2013; Tomar and Preet 2017). Lara et al. (2015) suggested that disruption and permeabilization caused by AgNPs in *Candida albicans* eggs allowed the entry of AgNPs into the cell wall and consequently the death of yeast, despite the rigidity of the cell wall. Something similar could happen in monogenean eggs exposed to AgNPs. The monogenean eggshell is formed by lipids, proteins, and carbohydrates from vitelline cells and undergoes an enzymatic process (sclerotization) that protects the developing embryo from chemical and physical agents (Kearn 1986; Whittington and Kearn 2011). These characteristics are similar to the structural components of the bacteria cell wall, for which it has been identified that AgNPs adhere to the surface of the cell wall and membrane and then penetrate inside the cell, where they damage intracellular structures (Dakal et al. 2016).

Despite the potential of AgNPs as anthelminthics in fish, concerns remain regarding the possible negative impact to the aquatic environment. To date, there have not been clear predictions, as the effects depend on AgNP concentration and particle size and on the amount and species of products yielded from chemical interactions between AgNPs and other variables (Sharma et al. 2019). In any case, some strategies should be investigated to minimize the possible negative impact of AgNPs, such as bioremediation using plants (see Zeng et al. 2019) or other remediation techniques (e.g., coagulation–ultrafiltration; Wang et al. 2018) in an aquacultural context.

In conclusion, the present study shows that UTSA AgNPs at a concentration of 36 µg/L for 1 h may be completely effective against adults and eggs of *Cichlidogyrus* monogeneans. Nonetheless, additional research is necessary to evaluate the in vivo the efficacy of this treatment as well as its toxicity on fish and the surrounding environment.

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Compliance with ethical standards This work was conducted using a tilapia–monogenean model system, and all procedures were performed in accordance with the ethical standards of the CIAD-Mazatlán following the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals.

Conflict of interest The authors declare that they have no conflicts of interest.

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