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Research Article

Antibacterial effect of biosynthesized silver nanoparticles in Pacific white shrimp *Litopenaeus vannamei* (Boone) infected with necrotizing hepatopancreatitis bacterium (NHP-B)

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ABSTRACT. The shrimp necrotizing hepatopancreatitis is caused by a pleomorphic Gram-negative *Rickettsia*-like bacteria known as NHP-B. It is well known that silver is an effective bactericidal, but experiments with aquatic organisms are scarce, even more at nanoparticle level. The objective of this study was to determine the antibacterial effect of silver nanoparticles (AgNP) against NHP-B in the Pacific white shrimp *Litopenaeus vannamei*. For this, $AgNO_3$ was used as source of silver, and extracts of dried leaves of green tea *Camellia sinensis* and neem tree *Azadirachta indica* as reducing agents. Different lots of infected shrimp received 0,5 and 35 µg of AgNP by forced feeding. The differences between the number of bacterial nodules in shrimp hepatopancreas and shrimp mortality relative to the amount of AgNP demonstrated to be effective against this pathogen.

Keywords: *Litopenaeus vannamei*, *Azadirachta indica*, *Camellia sinensis*, necrotizing hepatopancreatitis bacterium, Pacific white shrimp, silver nanoparticles.

INTRODUCTION

Several diseases, caused by a wide variety of pathogens such as viruses, fungi, protozoa, and bacteria, have negatively affected the production of farmed shrimp in major producing countries such as China, Thailand, Taiwan and Ecuador (Lightner & Pantoja, 2003). The intensive farming conditions, stock densities, or administration of low and unbalanced nutritional quality feeds, may result in stress, which leads to the invasion of pathogenic microorganisms. Therefore, research for developing efficient techniques and methods that allow early detection and control of diseases is very important. Disease control is more effective in broodstock and larval culture, than during the nursery phase, where it is much more difficult to implement.

The necrotizing hepatopancreatitis bacterium (NHP-B), a Gram-negative *Rickettsia*-like intracellular bacterium, has emerged as one of the major diseases impacting the shrimp farming industry (Krol *et al.*, 1991). During the acute phase of the disease, the shrimp hepatopancreas is atrophied while during the chronic phase the hepatopancreas is melanized, the tubules are necrotized, among many other anomalies. This disease causes mortalities of up to 40%, and its diagnosis is based on the use of histological and molecular tools (Morales-Covarrubias, 2004; Avila-Villa *et al*., 2012; Nunan *et al*., 2013; Varela-Mejías & Peña-Navarro, 2016).

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Nanotechnology, the engineering of functional systems at the molecular scale, has been focused on the manufacturing industry, but it has also been applied in medicine as agents for *in vitro* detection, *in vivo* diagnostics, multimodal imagining, chemotherapy, phototherapy, and immunotherapy (Giasuddin *et al.*, 2013; Lin, 2015). Moreover, in agriculture activities it has been pointed out that nanometric biological pesticides poses higher efficiency and present lower cost than regular pesticides, offering a reliable choice (Huang *et al*., 2015). On the other hand, in the application of nanosciences in aquaculture production Khalafalla *et al*. (2011) reported that selenium nanoparticles could improve reproduction, growth, and survival of *Oreochormis niloticus*. In addition, nanotechnology might be applied on food to prevent microbial contamination or decomposition. Some other applications involve nano-vehicles as carrying agents for nutrients, drugs, enzymes or food and antimicrobial additives (Can *et al*., 2011; Handy, 2012; Giasuddin *et al*., 2013).

Some nanoparticles may have antibacterial activity, and affect many species of bacteria (Lu *et al*., 2013; Huang *et al*., 2015: Bakare *et al*., 2016). For example, copper nanoparticles have been proved as promising antibacterial agents against the intestinal bacteria *Aeromonas hudrophila*, *Vibrio parahaemolyticus*, and *Pseudomonas fluorescens* in silver and grass carp (Huang *et al*., 2015), and Kim *et al*. (2007) reported that the use of silver nanoparticles (AgNPs) inhibited the growth of yeast, *Escherichia coli* (Escherich), and *Staphylococcus aureus* (Rosenbach).

Some studies had been demonstrated the antibacterial effect of AgNPs against common pathogenic bacteria infecting species of aquaculture interest. For example, the antibacterial effect of AgNPs synthesized using *Prosopis chilensis* extract has been tested in *Penaeus monodon* infected with *Vibrio cholerae*, *V. harveyi*, and *V. parahaemolyticus* (Kandasamy *et al*., 2013). Moreover, the effect of AgNPs, synthetized using green tea extract was tested in *Fenneropenaeus indicus* infected with *V. harveyi* (Vaseeharan *et al*., 2010). Recently, Juárez-Moreno *et al.* (2017) demonstrated the antiviral properties of AgNPs against white spot syndrome virus infecting juvenile *Litopenaeus vannamei.* Therefore, the use of nanoparticles against pathogens is a promising field in aquaculture (Can *et al.*, 2011; Rather *et al.*, 2011; Selvaraj *et al.*, 2014).

The mechanisms on the biocidal action of silver are mainly related to the sulfhydryl groups of enzymes and proteins like glucose-6-phosphate dehydrogenase and glutathione reductase (Shukla & Chandra, 1977), by interfering with the protein normal functions. Silver also inhibits DNA replication, and in bacteria it induces oxidative stress in the cell wall, where important cellular functions occur, affecting the maintenance of the internal ion balance. In this way, bacteria exposed to silver showed growth inhibition, massive loss of potassium, and suppresses the chemical transportation from the cell wall (Hwang *et al.*, 2007; Luoma, 2008).

It is well recognized that certain bacteria inhabiting a vacuole or phagosome, are able to synthesize some proteins and toxins to form holes in the vacuole membrane of their hosts to escape from these organelles (Van der Meer-Janssen *et al.*, 2010). The disrupted cellular membranes could facilitate the entry of nanoparticles into the host cell and several reports have demonstrated that Gram-negative bacteria show an increased susceptibility to AgNPs (Sondi & Salopek-Sondi, 2004; Radzig *et al*., 2013), which makes these vehicles suitable candidates as an antimicrobial agent against NHP-B. The application of nanoparticles to prevent bacterial colonization in human cells has been explored, but no progress has been reported against NHP-B in aquaculture species.

Despite the fact that the effective antimicrobial activity of AgNPs has been proven, there have been very few experiments conducted on aquatic organisms. Thus, the objective of this study was to determine the antibacterial effect of AgNPs against NHP-B in the Pacific white shrimp, *Litopenaeus vannamei*.

MATERIALS AND METHODOS

Synthesis of AgNPs

Dry leaves of green tea (*Camellia sinensis*, Kuntze) and fresh leaves of neem (*Azadirachta indica*, Jussieu) were used as reducing agents to determine which of the two species favored the production of monodisperse AgNPs. Neem leaves were dried in the dark at room temperature and stored in paper bags for three days. Nanoparticles synthesis was carried out based on the methodology described by Vasseharan *et al.* (2010) with some modifications in quantities and times as follows: For both plants, 6 g of dry leaves were added to 60 mL of boiling deionized water for 2 min, stirring occasionally. Then, after 5 min, the infusion was filtered through 4 μm filters three times to separate the suspended solids. The infusions were made just before starting the synthesis of nanoparticles.

The reaction was conducted in a 50 mL test tube with 1.0 mL of 0.01 M silver nitrate, 3.0 mL of deionized water and 30 mL of plant infusion. The absorbance of the reaction was evaluated in a Shimadzu UV-2450 spectrophotometer every 5 min during 1 h. To assess the form and dispersion of nanoparticles, a sample of 3 μL of the reaction solution was placed and

analyzed in a JEOL 2000 transmission electron microscope using a copper grid of 200 mesh dried at room temperature for 6-12 h. Images were analyzed using the ImageJ 1.44 p (NIH) software.

NHP-B inoculum preparation

To have a storage of NHP-B infective shrimp tissue, 60 live specimens were collected from two shrimp farms (30 per farm). The farms remained anonymous at the request of the farmers, and were identified as Farm A and Farm B. The whole hepatopancreas of each shrimp was dissected with flame-sterilized surgical material at the laboratory, and hepatopancreas was divided into three sections that were distributed as follows: a piece was placed in a 1.5 mL tubes with absolute ethanol for further PCR analysis to confirm the bacterial and WSSV presence. Another piece was immersed on Davidson solution for histological analysis. To inoculate the bacteria in healthy shrimp if positive PCR diagnostic results were obtained, a third piece was transferred into a cryoprotectant solution (50% glycerol), according to Gracia-Valenzuela *et al.* (2011). Samples in alcohol and glycerol were stored at -20°C, and Davidson samples were transferred to 70% ethanol for histological analysis.

To confirm bacterial infection, DNA was isolated from the previously dissected hepatopancreas by using the QIAamp DNA Mini Kit (QIAGEN), according to the manufacturer's instructions. DNA quantification and verification of A260/A280 ratio were done in a NanoDrop 1000 spectrophotometer. PCR reactions were carried out using Illustra PuRe Taq Ready-To-Go PCR beads (GE Healthcare). According to the results of the DNA quantification, the PCR reaction was performed using 1 µL of DNA if the concentration was greater than 100 ng μL^{-1} and 2 µL if the DNA concentration was lower than 100 ng μL^{-1}. One µL of the oligonucleotides described by Loy *et al*. (1996) for diagnosis of NHP-B (pf-1: 5'-ACGTTGGAGGT TCGTCCTTCAG-3' and pr-1: 5'-TCACCCCCTTGC TTCTCATTGT-3') were added into the reaction mix. Thermal cycling conditions were as described by Loy *et al*. (1996).

Due to the white spot syndrome virus (WSSV) has shown high incidence in shrimp farms in northwestern Mexico, a PCR analysis was also conducted to exclude samples that tested positive for the virus. The oligos WSSVVP28-F1: 5'-CTCGCTTGCCAATTGTCCTGT TA-3' and WSSVVP28-R1: 5'-ATTTCCACCGGC GGTAGCTGC-3' reported by Ramos-Paredes *et al.* (2012) were used, as well as thermal cycling conditions as recommended by these authors. A negative control without DNA was used in all cases.

PCR products were examined on 2% agarose gels, stained with ethidium bromide and were photographed under UV light. The expected amplicon sizes for NHP-B and WSSV were 441 and 702 bp, respectively. To verify the identity of the NHP-B amplicons, 3 PCR products, randomly selected, were sequenced in both directions (Macrogen). The sequences were edited with ChromasPro 1.7.4, and analyzed with the BLAST tool of the National Center for Biotechnology Information (Altschul *et al*., 1990).

Samples for histological analysis were dehydrated through 70-100% alcohol and xylene. Subsequently, the tissues were embedded in paraffin and histological sections of 5 µm were obtained using a rotary microtome. Tissue sections were stained with Hematoxylin-Eosin and the infection was assessed according to Del Río-Rodríguez *et al*. (2006).

Reactivation of NHP-B inoculum

Ten live shrimp from an experimental culture in the Universidad de Sonora facilities in Bahía de Kino, Sonora, were transported to our inland laboratory. The specimens were placed in a tank with 15 L of filtered seawater and were fed with a commercial shrimp feed twice daily *ad libitum* for 5 days. No food was provided subsequently for 24 h to clean the gut and then organism were force-fed with macerated hepatopancreas from NHP-B positive/WSSV-negative shrimp from stored tissue bank mentioned above, using a catheter adapted to a 20-100 µL micropipette, and the procedure was repeated every third day for three times. After 30 days, whole hepatopancreas was dissected, most was preserved in glycerol and a small section was used to extract DNA and analyzed by PCR looking for NHP-B presence according to the method described above. The organisms which tested NHP-B positive joined the infected tissue bank to conduct bioassay infection and treatment with AgNPs.

NHP-B infection bioassay

After one week from the reactivation of the NHP-B inoculum, one hundred and ninety live shrimp weighing 6 g from our facilities at Bahía de Kino, Sonora, were transported to our inland facilities. This number of shrimp was considered because the possibilities of mortalities due to handling, stress, cannibalism, or illness, and so made sure to have enough survivors to carry out the bioassay. To assess that those shrimp were free from both NHP-B and WSSV, 11 shrimp were chosen randomly and analyzed by PCR using DNA extracted from a pleopod, with the methodology previously described. All shrimp were transferred into 16 aquaria (10 to 12 shrimp per aquarium) containing 40 L of filtered and UV-sterilized

seawater and maintained at 28-29°C. The shrimp were kept under observation for three months with constant aeration and pelletized commercial food, provided twice a day at doses of 5% of their biomass. In that time, several shrimp died because some of them were cannibalized or because they jumped out of the aquarium, but none sick. Water changes of 80% were done every 3-4 days with filtered and UV-sterilized seawater.

One hundred and twenty shrimp of the mentioned in the preceding paragraph, weighing 16 g, were infected with NHP-B by forced feed using the infective inoculum obtained before. To ensure infection and an advanced stage of the disease, shrimp were maintained for a period of 50 days. This was done because, in the previous propagation assays, clinical signs of disease on shrimp were observed after 50 days. The control treatment consisted of uninfected shrimp.

To detect the NHP-B infection in shrimp, feces were collected daily. For this, shrimp of each aquarium were placed in cylindrical nets and let a span of 30-40 min to allow time for the feces to be deposited. The feces were preserved in 1.5 mL tubes with >95% ethanol. Once the NHP-B infection was established in all shrimp, we proceeded to apply treatments based on AgNPs.

AgNPs treatments

From the shrimp that were infected, 45 were selected and three groups were formed, with three aquariums per group (n = 5), for AgNPs treatments explained as follows. The AgNPs synthesized with *A. indica* were used in two different treatments, 5 µg and 35 µg of AgNPs in aqueous solution, and were administrated by forced feed to 15 shrimp per treatment, distributed in five shrimp per aquarium. This was decided by the extreme concentrations used by Vaseeharan *et al*. (2010) against *Vibrio harveyi*. The third group of infected shrimp was inoculated with an aqueous solution without nanoparticles. The fourth group of 15 uninfected shrimp without nanoparticles remained as a control group. One shrimp per tank (three shrimp per treatment) at days 12 and 24 was collected, and sacrificed by hypothermia to reduce its metabolism, and were immediately processed.

Cephalothorax sections (3-5 mm thick) from sampled organisms were placed in cassettes for histology in Davidson solution. A small section of hepatopancreas was taken and placed in 1.5 mL tubes with >95% ethanol for PCR analysis as explained above. The hepatopancreas tissue infection was assessed by histology, based on Lightner & Pantoja (2003) and Del Río-Rodríguez *et al*. (2006).

Determination of hepatopancreatic metabolites

The hepatopancreatic glucose, total protein, glycogen, total lipid, acylglycerides, and sterols were extracted and measured according to Sánchez-Paz *et al*. (2007) in a Synergy microplate reader (BioTek Instrumets), using commercial kits (Randox Laboratories). Briefly, a weighed portion of hepatopancreas was macerated with one volume of buffer A (100 mM potassium phosphate buffer, pH 7.2 and 1.0 mM EDTA, 10 µM PMSF) and one volume of chloroform-methanol-water (2:2:1) and centrifuged at 15,000×*g* for 15 min. The aqueous phase was used to quantify the levels of glucose, total protein, and glycogen. Determination of glycogen was made by hydrolysis and measured as glucose according to Passonneau & Lauderdale (1974). The solvent phase containing the chloroform was collected and air-dried for 12 h in complete darkness at room temperature (25°C), then homogenized with distilled water and used for quantification of total lipid, acylglycerides, and sterols. Metabolites data, represented as mg g^{-1} of hepatopancreas, were statistically analyzed by Kruskal-Wallis one-way analysis of variance with Dunn's multiple comparison test as *post-hoc* analysis using SigmaPlot v.12.0.

RESULTS

Characterization of AgNPs nanoparticles

The reaction solution of *Azadirachta indica* in contact with $AgNO_3$ showed a single absorption peak at 440 nm, while the reaction solution of *Camelia sinensis* showed a small absorption peak at 390 nm and a more pronounced peak at 440 nm. The control solution of $AgNO_3$ presented no absorption peaks in either case.

AgNPs synthesized with *A. indica* showed both polyhedral semi-hemispherical and flattened shapes after 120 min of reaction time. The AgNPs size was between 5 and 45 nm with an average of 14.84 ± 5.81 nm. The 84.65% of nanoparticles was found between 5 and 21 nm with an average of 13.01 ± 3.71 nm. AgNPs obtained with *A. indica* were monodisperse due to the high percentage of nanoparticles in the same size class (Fig. 1a), and this was the criterion for its use in the treatment bioassay. On the other hand, for *C. sinensis*, after 120 min of reaction time, polyhedral, hemispherical, and flattened shapes and some forms of nanowires were observed. The size range was between 7.4 and 68.8 nm with an average of 29.67 ± 14.17 nm, 29% was from 13.1 to 20.8 nm with a mean of 16.60 ± 2.02 nm, and 20% was from 37.05 to 44.03 nm with an average of 40.56 ± 1.88 nm (Fig. 1b). This was enough reason to dismiss these nanoparticles from bioassays.

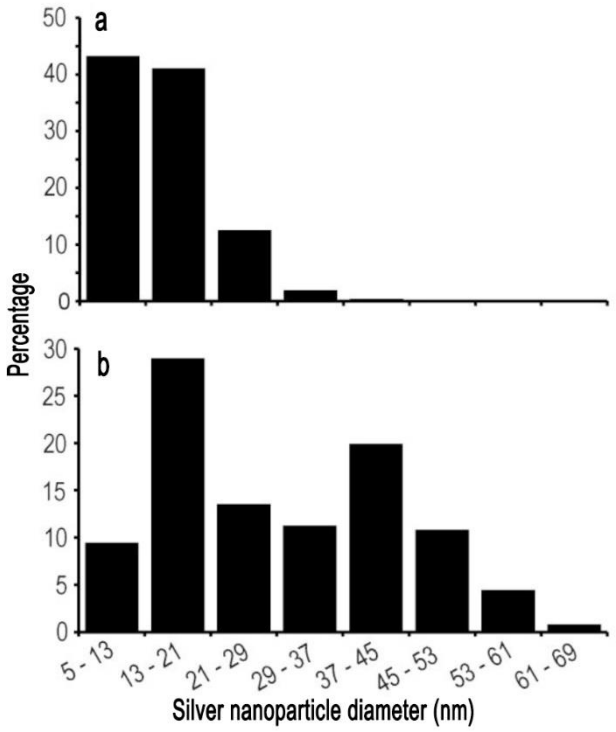

Figure 1. Histogram of size frequencies of AgNPs synthesized at 120 min of reaction by infusion of a) *Azadiractha indica* (n = 189), and b) *Camellia sinensis* (n = 220).

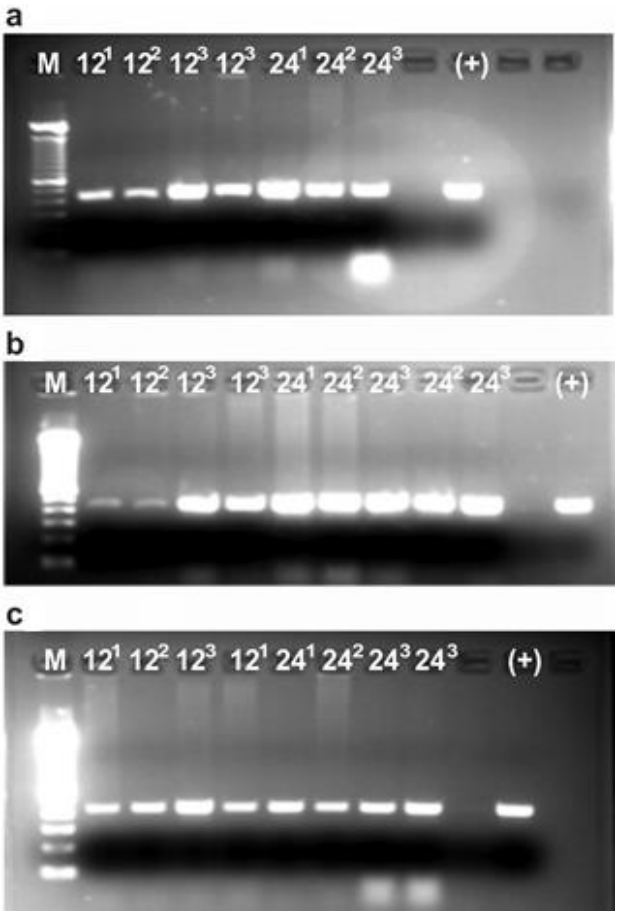

Figure 2. PCR Analysis of NHP-B infected organisms after 12 and 24 days of treatment. The superscript indicates the replica of the bioassay. Some replicate PCR analyzes are shown. a) shrimp with 0 µg of AgNPs, b) shrimp with 5 µg of AgNPs, c) shrimp with 35 µg AgNPs. AgNPs were synthesized with neem leaves. M: 100 bp ladder. (+): NHP-B positive control.

Detection of pathogens

The organisms from farm A indicated that 17% were positive for NHP-B, 23% positive for WSSV, 3% with both infections and 57% were free of these pathogens. Moreover, samples from the farm B indicated that 63% were WSSV infected, 3% showed a dual infection and in 33% of the samples no presence of these pathogens was detected. The NHP-B sequence showed a 100% identity with the NHP-B sequence U65509 of GenBank and was further registered with the accession number KM305771.

Experimental NHP-B Infection Bioassay

Experimentally NHP-B infected shrimp displayed typical gross clinical signs of the disease as soft exoskeleton, hepatopancreas reduction, erratic swimming and lethargy (Vincent *et al.*, 2004). In addition, this results show that shrimp exposed per os to infective material were successfully infected since 100% of the diagnostic tests resulted positive (Fig. 2).

It is noteworthy that the mortality rate of NHP-B infected shrimp treated with 35 µg of AgNPs was 0%, while the shrimp with the treatment of 5 µg of AgNPs presented a mortality of 40% by the end of the experiment. Organisms without nanoparticles, but infected showed a 41% mortality. After 12 days of treatment numerous bacterial nodules were observed in hepatopancreatic tissue in shrimp with 5 µg of AgNPs and tubules were slightly retracted and with bacterial nodules (Fig. 3a). After 24 days, severe hepatopancreas hypertrophy and bacterial nodules were observed in (Fig. 3b). the epithelial tissue of the hepatopancreas tubules (Fig. 3b).

In the treatment of 35 µg of AgNPs, and after 12 and 24 days of treatment, the shrimp hepatopancreas showed signs of infection with damaged hepatopancreas and severe vacuolization of the epithelium (Figs. 3c-3d). Hepatopancreatic tissue of infected shrimp but without AgNPs showed a severe infection level evident since the first sample at day 12, with many bacterial nodules and hepatopancreas tubules severely atrophied (Fig. 3e).

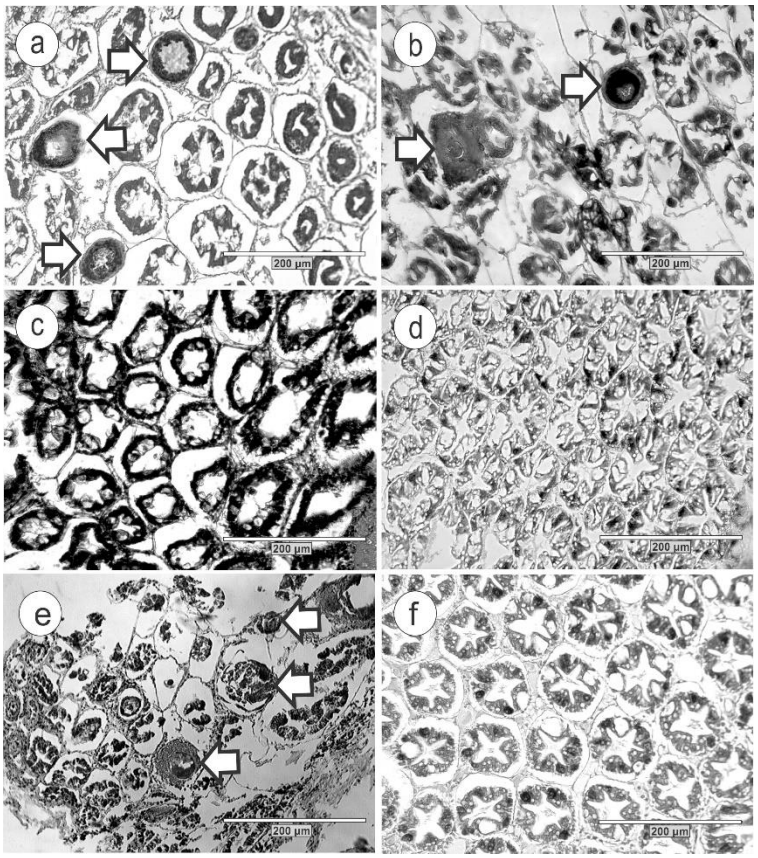

Figure 3. Hepatopancreas of NHP-B infected shrimp exposed to a) 5 µg of AgNPs during 12 days, b) 5 µg of AgNPs during 24 days, c) 35 µg of AgNPs during 12 days, d) 35 µg of AgNPs during 24 days. Control shrimp without AgNPs: e) NHP-B infected, f) uninfected. The arrows indicates bacterial nodules.

Hepatopancreatic metabolites

The statistical analysis of the biochemical components analyzed at 12 and 24 days of bacterial challenge, showed no significant differences in total protein (P = 0.225), acylglycerides (P = 0.174), total lipids (P = 0.166), glucose (P = 0.225) and glycogen (P = 0.270). However, there were differences in the concentration of sterol (P = 0.017), with higher values in infected shrimp that did not receive AgNPs (Fig. 4).

DISCUSSION

Silver nanoparticles from extracts of green tea and neem leaves were successfully synthesized in a fast, practical and economical way compared to other chemical and physical techniques (Poole & Owens, 2007; Guzmán *et al.*, 2009). However, differences in the nanoparticules shape and dispersion were obtained as compared to Vaseeharan *et al.* (2010) and Gavhane *et al.* (2012). The green tea extract, as a reducing agent, showed two peaks in the absorption spectrum, suggesting an increase in the polydispersity (Hsu & Wu, 2010; Kamal *et al.*, 2010), which was confirmed in the transmission electron microscope where sizes of AgNPs were widely dispersed.

In this study different shapes of nanoparticles were observed, and although it is not well determined whether the antibacterial activity depends on the shape of the nanoparticle, it seems certainly plausible that all of the forms found may have led to an antibacterial effect. However, more studies are needed to confirm that both the form and size of nanoparticles are not relevant for a therapeutic use.

The histological results of NHP-B infected shrimp are in agreement with those reported by Del Río-Rodríguez *et al.* (2006). The shrimp treated with 5 µg of AgNPs showed similar tissue damage as that of infected shrimp without nanoparticles, which also presented tissue necrosis as well as the presence of bacterial nodules and hemocyte infiltration. Moreover, while in the shrimp treated with 35 µg of AgNPs some tissue damage was observed, no bacteria or hemocyte

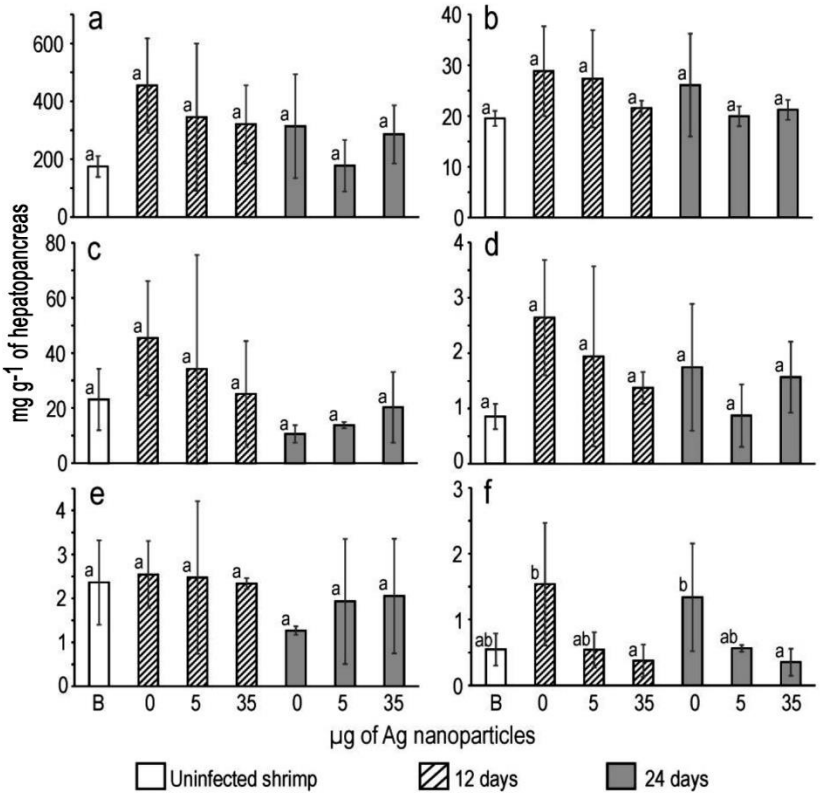

Figure 4. Average concentration of a) total protein, b) acylglycerides, c) total lipids, d) glucose, e) glycogen, and f) sterols in shrimp hepatopancreas with different AgNPs treatments synthetized with *Azadirachta indica*. B: Control uninfected shrimp. Letters in bars correspond to *post-hoc* statistical analysis: different letters indicate statistically significant differences.

infiltration was found as evidence of NHP-B. In addition, the epithelia of the tubules were slightly thinned, and the tissue integrity was modified. This could indicate that the treatment with AgNPs eliminates or drastically reduces the NHP-B in shrimp cells. In this case, the positive PCR analysis shows the presence of bacteria, presumably in low abundance, but not obvious to microscopic analysis, or residual bacterial DNA from dead bacteria still present in shrimp.

The probability that AgNPs remained in the tissue is high. Bianchini *et al.* (2007) reported that in *Penaeus duorarum* (Burkenroad), silver accumulation occurs mainly in the hepatopancreas followed by hemolymph, however, in gill, muscle and eyestalks there was very little silver accumulation. Presumably, it is of great benefit that silver accumulates in the hepatopancreas as it is the target organ to NHP-B (Lightner *et al.*, 1992), so most of the silver will be available as an antibacterial agent. In addition, due to the great concern about the effects of AgNPs on human health (Korani *et al.*, 2015), and because the consumer market largely considers the abdomen and not the cephalothorax, the fact that the muscle of shrimp accumulates few nanoparticles is an advantage.

According to Vincent *et al.* (2004), the course of the NHP disease indicates that during the first 20 days the disease is in the development phase. After that time, the disease enters an acute phase and mortality begins, and from 35 days post-infection the chronic phase begins. In the current study, normal behavior and clinical signs were observed on shrimp until just before the 20 days, while mortalities occurred during the experiment. However, the chronic phase began at 56 days of challenge, beyond the end of our experiment. This difference with the Vincent *et al.* (2004) study needs to be assessed, either looking for NHP-B genetic variants or the condition of the immune health of shrimp.

The slight difference in mortality between treatments of 0 µg and 5 µg of AgNPs may indicate that the concentration of silver nanoparticles was not enough against the pathogen, which is consistent with the findings in the histological analysis. Moreover, organisms that were administered 35 µg of AgNPs had a survival rate of 100%, and although the molecular

analysis was positive at the end of the experiment, the tissue was almost anatomically recovered when compared to the first samples. Morales-Covarrubias *et al*. (2012) tested florfenicol and oxytetracycline in NHP-B infected shrimp, and observed survival of 56 to 83%, with antibiotic residues up to 10 days in shrimp muscle. It is important to note that the use of antibiotics in aquaculture involves generation of resistance in bacteria. Bacteria are able to withstand certain silver concentrations and reduce them to form nanocrystals in the periplasmic space as a defense (Klaus-Joerger *et al*., 2001). However, the vast majority of pathogenic bacteria succumb to AgNPs (Parameswari *et al*., 2010; Saxena *et al*., 2010; Vaseeharan *et al*., 2010). Furthermore, it has been found that antibiotic-resistant organisms, compared to non-resistant, are equally susceptible to AgNPs. For example, Lara *et al*. (2010) found that strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Schroeter) resistant to antibiotics were susceptible to AgNPs.

The application of AgNPs synthesized using tea extracts involves inexpensive and readily available ingredients compared to other reducing agents. This implies a considerable reduction in costs as well as the application of simple methodologies. In addition, the profitability of applying nanoparticles synthetized in an eco-friendly way, and the low volume of the dose may be a great candidate as an antibacterial agent in certain branches of shrimp aquaculture, for example in brood stock maintaining. Although it was found that the nanoparticles had an antibacterial effect against the intracellular bacteria causing NHP, it is necessary to understand the mechanisms of action of these nanoparticles against these bacteria. The absence of statistical differences in five biochemical components analyzed indicate that there was insufficient evidence to prove that the levels of that components were affected by the effect of infection and different concentrations of nanoparticles in infected and healthy shrimp. Future studies should take into account a larger sample size to avoid large dispersion values like those observed in the analysis of metabolites, since high values of dispersion mask any differences that might exist. It is also important to experiment wider ranges of concentrations of nanoparticles and to test the feasibility in the application of nanoparticles in large aquaculture systems, perhaps adding the nanoparticles as food additives. An adverse effect of AgNP on coastal biota has been recently shown (Burić *et al*., 2015; Degger *et al*., 2015; Gambardella *et al*., 2015; Rocha *et al*., 2015), but fortunately Juárez-Moreno *et al*. (2017) had demonstrated that AgNPs at therapeutic doses are not toxic, and does not affect the metabolic rate or total hemocytes count in juvenile *Litopenaeus vannamei*. However, given the high variety of metals and metalloids used as nanoparticles, it is important to study the impact of their use on aquaculture species, human health, and the coastal environment, to implement future strategies to develop a sustainable and responsible aquaculture.

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