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Effect of silver nanoparticles on the metabolic rate, hematological response, and survival of juvenile white shrimp *Litopenaeus vannamei*

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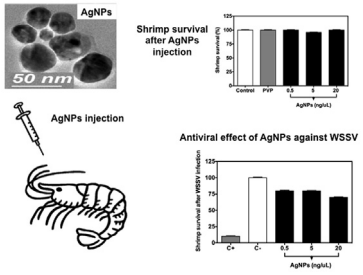
HIGHLIGHTS

- Intramuscular injection of AgNPs and their stabilizer into *Litopenaeus vannamei* shrimps is not toxic.
- Shrimp survival after AgNPs injection was 98.7%.
- AgNPs injection does not affect metabolic rate or total hemocytes count.
- After virus infection, shrimps injected with AgNPs survived 80% for 96 h.
- Proof of principle of AgNPs to act as antiviral against the white spot syndrome virus.

GRAPHICAL ABSTRACT



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ABSTRACT

White spot syndrome virus (WSSV) is highly lethal and contagious in shrimps; its outbreaks causes an economic crisis for aquaculture. Several attempts have been made to treat this disease; however, to date, there is no effective cure. Because of their antimicrobial activities, silver nanoparticles (AgNPs) are the most studied nanomaterial. Although the antiviral properties of AgNPs have been studied, their antiviral effect against viral infection in aquaculture has not been reported. The AgNPs tested herein are coated with polyvinylpyrrolidone (PVP) and possess multiple international certifications for their use in veterinary and human applications.

The aim of this work was to evaluate the survival rate of juvenile white shrimps (*Litopenaeus vannamei*) after the intramuscular administration of AgNPs. For this, different concentrations of metallic

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AgNPs and PVP alone were injected into the organisms. After 96 h of administration, shrimp survival was more than 90% for all treatments. The oxygen consumption routine rate and total hemocyte count remained unaltered after AgNP injection, reflecting no stress caused. We evaluated whether AgNPs had an antiviral effect in shrimps infected with WSSV. The results revealed that the survival rate of WSSV-infected shrimps after AgNP administration was 80%, whereas the survival rate of untreated organisms was only 10% 96 h after infection. These results open up the possibility to explore the potential use of AgNPs as antiviral agents for the treatment of diseases in aquaculture organisms, particularly the WSSV in shrimp culture.

1. Introduction

Although shrimp farming has been growing rapidly in the past decades, viral infections such as white spot syndrome (WSS) have seriously affected this industry. White spot syndrome virus (WSSV) is highly virulent and can cause disease in 100% of organisms within 3–7 days of infection, resulting in huge economic losses for the aquaculture industry every year (Ramos-Carreño et al., 2014).

Different approaches have been explored to treat WSSV infection; however, to date, they have not fulfilled key parameters, e.g., stability and ease of handling. These include virus neutralization in the fleshy prawn shrimp *Penaeus chinensis* by egg yolk antibodies (IgY) (Kim et al., 2004), injection of shrimp lysozyme as a protective agent against WSSV infection in the blue shrimp *Litopenaeus stylirostris* (Mai and Wang, 2010), delivery of double-stranded RNA (ds-RNA) of the main viral proteins VP28 and VP26 against WSSV in *Litopenaeus vannamei* (Mejía-Ruíz et al., 2011), VP28 gene delivery by chitosan nanoparticles (Vimal et al., 2013), and RNA silencing with siRNA from VP28, VP6, or WSSV-DNA polymerase (Wu et al., 2007; Xu et al., 2007).

In the aquaculture industry, nanotechnology has been poorly applied, and it has been mainly restricted to that improvement of the quality of ingredients in food formulations, antifouling coatings, antibacterials for tanks and packaging of sea food products, and environmental remediation systems (Can et al., 2011; Corsi et al., 2014). Recently, the usage of virus-like nanoparticles (VLPs) from *Macrobrachium rosenbergii* nodavirus as nanocarriers to encapsulate ds-RNA from the major WSSV envelope protein VP28 was reported, and this resulted in a survival rate of 44.5% compared with the use of free ds-RNA (Jariyapong et al., 2015). To the best of our knowledge, this was the first approach of using nanotechnology for the treatment of WSSV infection. However, to date, no other nanomaterial has been used as an antiviral agent to overcome the WSSV infection. Therefore, there is an increasing need to find alternative treatments to overcome infections in shrimps and avoid the massive economic losses in the aquaculture field.

Silver nanoparticles (AgNPs) are one of the most widely used nanomaterials in commercial products because of their beneficial antibacterial, antifungal and antiviral properties (Chen and Schluesener, 2008; Marambio-Jones and Hoek, 2010; Galdiero et al., 2011; Ge et al., 2014; Podkopaev et al., 2014; Franci et al., 2015). Taking advantage of the well-known antiviral properties of AgNPs against a wide spectrum of virus, the purpose of this study was to investigate whether the injection of AgNPs affects the oxygen consumption rate, the total hemocyte counts (THCs), and the survival of juvenile white shrimps (*L. vannamei*). It was explored whether the treatment with safe doses of AgNPs may be useful as an antiviral agent against WSSV infection. For this, the percentage of viability in WSSV-infected shrimps untreated and treated with AgNPs was compared as a proof of concept for the evaluation of AgNPs aiming to reduce the infectivity of WSSV disease in juvenile white shrimps.

2. Materials and methods

2.1. Reagents

Polyvinylpyrrolidone (PVP) and all components to prepare phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.0) were purchased from Sigma-Aldrich (St. Louis MO, USA). All other chemicals were obtained from commercial sources and were of analytical grade.

2.2. AgNPs suspension

An AgNPs solution named Argovit™ was kindly donated by Dr. Vasily Burmistrov from the Scientific and Production Center Vector-Vita (Russia). Argovit is a preparation of highly dispersed AgNPs with an overall concentration of 200 mg/mL (20%) of PVP-coated AgNPs in water. The content of metallic Ag in Argovit preparation is 12 mg/mL, stabilized with 188 mg/mL of PVP. AgNPs concentrations were calculated according to the metallic Ag content in Argovit preparation. Solutions were prepared in PBS and were kept at 4 °C in darkness.

After a comparison of different AgNPs commercially available, it was concluded that only Argovit preparation had multiple certificates for its usage in veterinary and human applications (Borrego et al., 2016).

2.3. AgNPs characterization

Size distribution and morphology of AgNPs were determined on the basis of the results obtained by high-resolution transmission electron microscopy (HRTEM) using a JEOL-JEM-2010 microscope. Hydrodynamic radius and zeta potential were measured by using dynamic light scattering (Malvern Instruments Zetasizer Nano NS model DTS 1060, UK) equipped with a green laser operating at $\lambda = 532$ nm at 25 °C. AgNPs were characterized by UV–Vis spectroscopy in the range of 200–900 nm using a Cary 60 UV–Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Further characterization of lyophilized Argovit was performed with Fourier transform infrared spectroscopy-attenuated total reflectance (FTIR-ATR) analysis in the range of 400–4000/cm with a resolution of 2/cm[1] on a universal diamond ATR top plate accessory (Perkin Elmer, USA); the sample spectrum was compared with that of standard solid PVP (Mw 100 kDa).

2.4. Shrimp culture

Juvenile white shrimps (*L. vannamei*) obtained from a farm (Aquasoles S.A. de C.V, Obregón Sonora, Mexico) were cultivated as previously described (Re et al., 2012). Briefly, postlarvae weighing 2 g were placed in 2000-L reservoirs with continuous flow of seawater at 35 psμ and maintained at a temperature of 28 ± 1 °C. Shrimps were maintained in these conditions until they reached 10

g of weight. They were provided daily with commercial food (Rangen Inc.) with 40% protein at 8% of their wet weight.

2.5. AgNPs administration to juvenile white shrimps

Different control and experimental groups were evaluated, and every group included 50 organisms as given in Table 1. Treatments were administered in a final volume of 100 μL by injection to the arthrodial membrane of the fifth pereiopod of each organism. The control group was treated with PBS, whereas the PVP group was treated with a solution of PVP (the main stabilizer of Argovit) at 188 ng/μL diluted in PBS.

AgNPs dissolutions were prepared in PBS solution and kept at 4 °C in darkness. Injections of 100 μL containing different concentrations of AgNPs were injected to each group, as given in Table 1. AgNPs dilutions were calculated from the metallic Ag content of Argovit preparation (12 mg/mL). After injection, juvenile shrimps were placed in water tanks. Mortality and behavior of each group were monitored for 96 h after AgNPs injection.

2.6. Oxygen consumption

The oxygen consumption routine rate (OCRR) was measured in an intermittent respirometer flow system, as described by Díaz et al. (2007), consisting of 10 acrylic chambers of 2500 mL each. An optic fiber oxygen sensor (precision ± 0.005% O_2, detection limit 0.03% O_2) was introduced at the bottom of the chamber and was connected to an OXY-10 mini-amplifier (PreSens GmbH, PreSens©, Germany). Software from the same company recorded the dissolved oxygen concentration of the respirometric chamber on a laptop hard drive. Oxygen consumption was recorded at 6, 12, 24, 48, 72, and 96 h after the injection of AgNPs. All measurements were taken between 09:00 and 13:00. To minimize the possible influence of body weight on oxygen consumption, shrimps within a narrow weight range (mean ± SD wet weight, 10.0 ± 2.6 g) were used.

Nine shrimps and one chamber without organism (control) from each experimental condition were individually introduced into a respirometric chamber. After the initial oxygen concentration was recorded, the water flow was interrupted for 30 min to avoid a decline in dissolved oxygen to 25% as this constitutes a stress factor according to Stern et al. (1984). During this time, the software recorded the oxygen concentration every 5 min.

The OCRR was calculated according to Equation (1) (Cerezo Valverde et al., 2006; Zheng et al., 2008).

$$OCRR = (C_t - C_0)V/(W \times T) \quad (1)$$

where C_t is the change in the oxygen content in the respirometric chambers before and after the test, C_0 is the change in the oxygen content in the blank (control), V is the volume of the chamber, W is the weight of the shrimps in kg wet biomass, and T is the time duration in hours.

The 10th acrylic chamber was used as a control to measure oxygen consumption of the microorganisms present in water, and the necessary corrections were made. Each test was performed in duplicates. The results of the OCRR are given in mg of O_2/h kg wet weight.

2.7. Total hemocyte count

The hemolymph from each shrimp was individually sampled using a prechilled syringe from the thoracic-abdominal membrane (previously dried with an absorbent paper). The samples were obtained at 6, 12, 24, 48, 72, and 96 h after the injection of AgNPs. To perform the THC, 10 μL of hemolymph was loaded onto a cell counting slide, and cells were counted in a TC10 Automated Cell Counter from Biorad (CA, USA). The results from THC were expressed as the total number of hemocytes per organism tested.

2.8. White spot syndrome virus stock

The WSSV (Sonora isolate, 2006) was kindly provided by Dr. Claudio Humberto Mejía Ruíz (CIBNOR, Baja California Sur, Mexico). The WSSV inoculum was prepared using infected gill tissues. Briefly, 50 mg of tissue was homogenized in PBS. The homogenized sample was clarified by centrifugation at 3000×*g* for 20 min at 4 °C. The supernatant was filtered through a 0.45-μm pore diameter filter and placed on ice until use.

2.9. Treatment with AgNPs after WSSV infection

Each experimental group consists of 10 organisms distributed as 5 individuals per 14-L tank. The organisms were kept under constants parameters of temperature and salinity (28 °C and 35 psμ). The animals were fed twice *ad libitum*, as described above. A negative control group was mock-treated with PBS, whereas the positive control group received no AgNPs treatment. The experimental groups were infected with WSSV. After 24 h of virus infection, shrimps were injected with different amounts of AgNPs, as given in Table 1. Animals were observed for 96 h post infection; the survival rate was monitored daily.

2.10. WSSV inoculum and detection of infection by PCR

All animals between 10 ± 1 g were intramuscularly inoculated with a shrimp infectious dose 50% endpoint (SID_{50}) of 2500 in a volume of 50 μL with an *in vivo*-titrated WSSV inoculum. The infection of shrimps with WSSV and further treatment with AgNPs was confirmed with the IQ Plus™ WSSV Kit POCKIT system (GeneReach Biotechnology Co., Taiwan) using the VP28 and VP26 WSSV primers as previously reported (Mejía-Ruíz et al., 2011). Briefly, tissue/hemolymph was obtained from each organism, and genomic DNA was isolated. The amplification reactions were performed according to the manufacturer's instructions in a final volume of 50 μL of premix buffer containing dNTPs, WSSV-specific primers, fluorescent probes, DNA Taq polymerase enzyme, and 0.05 μg of total DNA. The PCR reaction was run for one cycle at 92 °C for 4 min; 30 amplification cycles at 92 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; and one final extension cycle at 72 °C for 5 min. The PCR

Table 1
Control and experimental treatments for shrimp injection.

Sample name	AgNPs concentration (ng/μL)	Total volume injected into shrimps	Treatment
Control	0	100 μL	PBS
PVP	0	100 μL	18,800 ng of PVP
AgNP-1	0.5	100 μL	50 ng of AgNPs
AgNP-2	5	100 μL	500 ng of AgNPs
AgNP-3	20	100 μL	2000 ng of AgNPs

products were analyzed according to the manufacturer's instructions. An internal control of a housekeeping gene of *Penaied* shrimps provided by the supplier was used as an endogenous reporter gene. The positive control for WSSV infection was also provided by the supplier and was used as the positive control for viral infection. The results were plotted as the average of triplicates, and standard errors were calculated using the Graph Pad Prism v6.0 software. The folds of WSSV DNA levels were calculated relative to WSSV positive control supplied within the IQ Plus™ WSSV kit. Statistical differences between the groups were determined by Student *t*-test.

2.11. Statistical analysis

The results from experiments were expressed either as mean ± standard deviation of three independent experiments or as the mean ± standard error. The data were evaluated by the student *t*-test or analysis of variance (ANOVA) and Tukey's multiple comparison test to determine significant differences using the GraphPad Prism version 6.0c software. The results were considered statistically significant when $p < 0.05$.

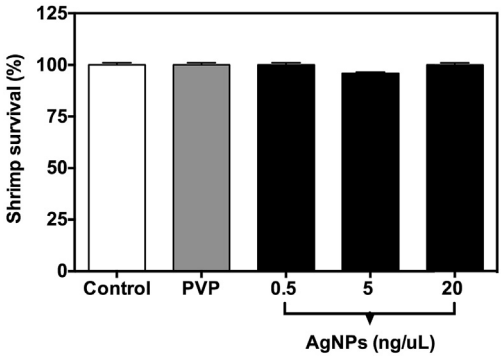

Fig. 2. Effect of AgNPs injection on the survival of juvenile white shrimps. Each group contained 50 shrimps. Organisms were injected with 100 μL of each treatment, as given in Table 1. Control organisms were injected with PBS solution. PVP was used at 188 ng/μL and 0.5, 5, and 20 ng/μL of AgNPs were injected into each shrimp. Survival was recorded 96 h after AgNPs injection. Data represent mean ± standard deviation. No statistically significant differences (ns) were found between control group and experimental treatments and in multiple comparisons between treatment groups by ANOVA and Tukey's multiple comparison test ($p^{ns} = 0.557$).

3. Results and discussion

3.1. AgNPs characterization

The UV–Vis spectra of AgNPs at different concentrations were measured, as shown in Fig. 1. All the samples exhibited the characteristic peak for AgNPs at 420 nm because of the surface plasmon resonance characteristic of metallic Ag of nanometric size (Fig. 1A). AgNPs were analyzed by HRTEM (Figs. 1B and 1C); they were spheroid in shape with a mean diameter of 35 ± 15 nm, as depicted in the histogram of size distribution of AgNPs.

To avoid their agglomeration and increase their stability, AgNPs are covered with PVP of low molecular weight. As shown in Fig. 1D, the spectra of both AgNPs and PVP standard showed the main characteristic peaks corresponding to the vibration of unbound water (ν OH) at 3406/cm, symmetric C–H stretching at 2948/cm, and asymmetric C–H stretching at 2915/cm (ν_s C–H and ν_{as} C–H, respectively), carbonyl stretching at 1650/cm (ν C=O), and a stretching of the nitrogen–carbon bound in the ring at 1269/cm.

Thus, FTIR-ATR spectrum of AgNPs confirmed that they are covered by PVP, as described by the supplier. The peaks assigned to the vibrations of the pyrrole heterocycle typical of PVP remained

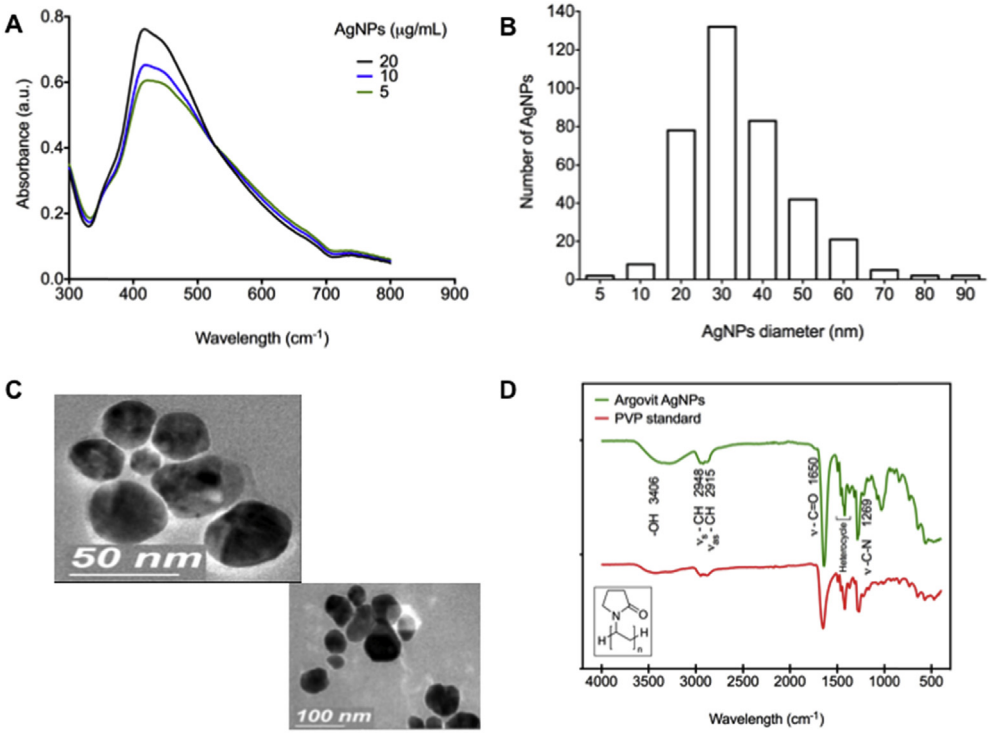

Fig. 1. Physicochemical characterization of AgNPs. **(A)** Characterization of AgNPs by UV–Vis spectrophotometry. Absorption spectra of AgNPs from Argovit preparation diluted in deionized water. Concentrations of metallic AgNPs are given in μg/mL. **(B)** Size distribution of metallic AgNPs in Argovit formulation. **(C)** TEM image of metallic AgNPs using different magnifications. **(D)** Characterization of AgNPs by ATR-FTIR. FTIR spectrum of AgNPs was compared with solid PVP standard. Structure of PVP is shown at the bottom of the graphic.

unchanged compared with the standard PVP such that all the chemical features of PVP functionalizing the metallic AgNPs are maintained providing an important biocompatible character to the AgNPs studied here (Fig. 1D).

It has been reported that uncoated metallic AgNPs tend to agglomerate and their size enlarges. This has been reported to cause a significant damage to the cell membrane integrity of hepatocyte primary cultures from the rainbow trout *Oncorhynchus mykiss* (Farkas et al., 2010). Thus, surface chemistry of AgNPs plays a key role in their interaction with biological systems (Soenen et al., 2015). The presence of a biocompatible polymer, such as PVP, allows the use of nontoxic concentrations of AgNPs for the host cells while executing their activity as bactericidal, antiviral and antifungal agents. It is worth mentioning that metallic AgNPs functionalized with PVP have shown a better performance in several biological assays than non-capped AgNPs or those capped with other agents (Borrego et al., 2016; Durán et al., 2015).

Because of the multidisciplinary nature of this work, it is vital to indicate the importance of a complete physicochemical characterization of AgNPs. This must include data such as metallic silver and capping agent concentrations, nanoparticle size and size-distribution, hydrodynamic diameter, and zeta potential.

The average hydrodynamic diameter of Argovit AgNPs is 70 nm, whereas the zeta potential is −15 mV. This confers them stability in solution for up to 2 years at 4 °C (Parveen et al., 2012). It is essential to compare these parameters with other studies and correlate the effects of different AgNPs on the basis of their physicochemical characteristics, which are summarized in Table 2.

3.2. Effects of AgNPs on the survival of juvenile white shrimps

There are few studies that have evaluated the toxicological effect of nanomaterials in marine organisms, particularly metal and metal-oxide NPs (Baker et al., 2014). The antimicrobial properties of AgNPs are the main reason for their broad commercialization and technological applications (Piccinno et al., 2012).

As the first step to use of AgNPs for the treatment of marine organism diseases, it is important to evaluate the potential toxicity of these NPs. Herein juvenile white shrimps were injected with each treatment as described in Table 1. The results revealed that AgNPs injection did not affect the survival of the shrimps, given that the overall survival rate was 98.7% 96 h after injection. The remaining organisms died because they did not overcome the ecdysis process (Fig. 2).

In addition, the toxic effect of PVP reagent was evaluated because it is the main stabilizer in Argovit AgNPs. As shown in Fig. 2, the percentage of shrimp survival was not affected by the injection of PVP, corroborating that this reagent does not affect the survival rate 96 h after injecting into the organisms. Moreover, PVP has been approved and considered safe by the FDA since 2010 for many applications including medical and food technologies (FDA/Center for Drug Evaluation and Research, Office of Generic Drugs, 2010; Kramer, 1999).

Although AgNPs have been widely used because of their beneficial properties as antibacterial, antifungal and antiviral agents (Chen and Schluesener, 2008; Marambio-Jones and Hoek, 2010; Galdiero et al., 2011; Ge et al., 2014; Podkopaev et al., 2014; Franci et al., 2015), little is known about their effectiveness in the alleviation of health problems in marine fauna for further human consumption, such as *L. vannamei*.

Therefore, to the best of our knowledge, this study represents the first attempt to test the effect of AgNPs in the survival of marine organisms of high economic impact, and moreover, our results showed that no evident effects in behavioral activity were recorded (swimming and feeding). We can explain this by the fact that Argovit AgNPs have been approved by sanitary international organizations to be safe for their use in veterinary and medical applications (Bogdanchikova et al., 2016; Borrego et al., 2016).

Although the injection of AgNPs into shrimps did not affect their survival, it is important to measure if treated animals experienced stress after being injected with AgNPs. This approach includes the measurement of metabolic rate and the TCH, which are described below.

3.3. Effects of AgNPs on the oxygen consumption of juvenile white shrimps

The measurement of metabolic rate has been a useful tool to determine the energetic costs of environmental factors such as temperature, salinity, and exposure to pollutants in marine organisms (González et al., 2010). Similarly, the consumption of oxygen is closely associated with the metabolic work and energy flow that the organism can use for the regulation of homeostatic mechanisms.

A recent study correlated the thermal preferentiality in the pink shrimp *Farfantepenaeus paulensis* to be between 15 °C and 20 °C with unchanged values of oxygen consumption. This result implies that within this temperature range, pink shrimps do not need to allocate additional energy to compensate the stress caused by temperature changes (Barbieri et al., 2016).

Here, the OCRR in juvenile shrimps was measured to determine whether the injection of PVP or different amounts of AgNPs caused them stress. The total amount of PVP injected into each shrimp was 18,800 ng, which represents a 1000-fold higher concentration of those found in Argovit AgNPs. This was done to ensure that PVP, the main stabilizer of AgNPs preparation used here, was not toxic. The metabolic rate measured through the oxygen consumption of injected shrimps was compared with the data of oxygen consumption of organisms in the control group that were injected only with PBS (767.5 ± 176.3 mg O_2/h Kg of wet weight), as shown in Fig. 3.

It is evident that although no statistical significance has been found after ANOVA, followed by Tukey's multiple comparison test and *a posteriori* analysis with Newman-Keuls test, there is a tendency for a slight increase in oxygen consumption at 48 and 72 h in the AgNPs- and PVP-treated groups. However, as there are no statistically significant differences between those values and the values of the control group, it is possible that the tendency of the increase in the metabolic rate could be solely attributed to the effect of PVP or to the stress caused by manipulation and injection.

These results contribute to the evidence that the injection with neither PVP (at 1000 times higher dose than that in Argovit AgNPs) nor AgNPs caused a stress response in the juvenile shrimps because the levels of oxygen consumption did not increase along the time of the length of the experiment. The unchanged parameter of oxygen

Table 2
Physicochemical characteristics of Argovit AgNPs.

Properties	Mean
Content of metallic silver (% wt)	1.2
Content of PVP (% wt)	18.8
Average diameter of metallic silver particles by TEM data (nm)	35
Morphology of silver nanoparticle	spheroidal
Hydrodynamic diameter: metallic silver with PVP (nm)	70
Size interval of metallic silver particles by TEM data (nm)	1 to 90
Zeta potential (mV)	−15
Surface plasmon resonance	420 nm
PVP structure by FTIR	confirmed

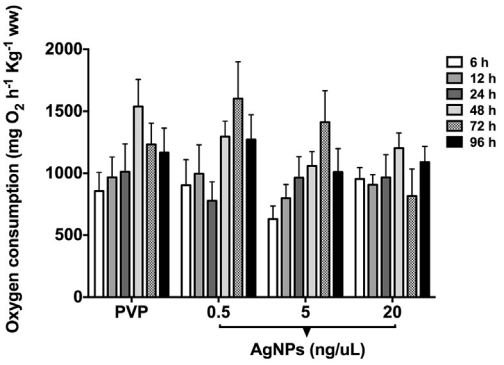

Fig. 3. OCRR in juvenile white shrimps treated with AgNPs. Shrimps were injected with a final volume of 100 μL of each treatment. Control organisms were injected with PBS solution. PVP was used at 188 ng/μL and 0.5, 5, and 20 ng/μL of AgNPs were injected into each shrimp. Oxygen consumption was measured for 6, 12, 24, 48, 72, and 96 h after nanoparticles injection. Data represent the mean ± error standard. No statistically significant differences (ns) were found between oxygen consumption in control group (767.5 ± 176.3) and experimental groups and in multiple comparisons between treatment groups by ANOVA and Tukey's multiple comparison test ($p^{ns} = 0.86$).

consumption in shrimps injected with AgNPs revealed that the administration of these NPs into the organisms did not affect their metabolic rate. Thus, there no energy is applied for stress compensation after the administration of AgNPs injection.

3.4. Effect of AgNPs on the total number of hemocytes in juvenile white shrimps

Hemocytes in crustaceans are analogous to vertebrate white blood cells. Their functions are to eliminate foreign particles and old cells through phagocytosis and participate in the cicatrization through cellular aggregation and liberation of clotting factors (Sánchez Campos et al., 2014). Thus, hemocytes are considered to be the most important cells in the crustacean defense system. In this sense, the THC reflects the immune response status of the shrimps (Ji et al., 2009).

For example, the role of hemocytes against pathogen-associated molecular patterns (PAMPs) has been studied by injecting lipopolysaccharides or laminarin into shrimps. Both molecules were administered intramuscularly into white shrimps. A significant increase in the THC was induced by PAMPs, indicating the importance of hemocytes as cellular response against stress, pathogens, and infections (Ji et al., 2009; Sánchez Campos et al., 2010).

Because the THC in the hemolymph is increased when the organisms are subjected to stress or infections, herein as the first step to use AgNPs for further antiviral experiments, we investigated whether the injection of AgNPs at different amounts causes stress and modifies the THC of juvenile shrimps. As shown in Fig. 4, shrimps injected with different concentrations of AgNPs, namely (A) 0.5, (B) 5, and (C) 20 ng/μL, with the exception of one single measurement at 24 h for 5 ng/μL AgNPs, did not show any statistically significant difference compared with the control group during the course of the experiments.

Unchanged THC values were also observed when the juvenile shrimps were injected with PVP at 188 ng/μL, a 1000-fold higher concentration than that found in Argovit formulation. As the number of hemocytes has not significantly changed after the administration of AgNPs, it can be concluded that after injection of PVP or AgNPs, shrimps were not affected by any stress. In addition, it may be plausible that no hemocyte-mediated cellular defense response against any of the treatments had been initiated.

3.5. Antiviral activity of AgNPs against WSSV in juvenile white shrimps

There is an urgent need to find alternative treatments to overcome bacterial and viral infections and avoid the massive economic losses in the aquaculture field. It is surprising that although the antiviral properties of AgNPs are well known (Borrego et al., 2016; de Lima et al., 2012; Franci et al., 2015; Galdiero et al., 2011; Ge et al., 2014; Rai et al., 2016), there are no studies reporting the antiviral effects of AgNPs against WSSV infections in shrimps.

Taking advantage of the broad spectrum of antiviral properties of AgNPs, it is important to test whether their injection into white shrimps would lead to any antiviral effect against WSSV that directly affects the survival rate of an already infected organism.

Interestingly, as shown in Fig. 5A, when juvenile shrimps were infected with WSSV, organisms treated with AgNPs at 0.5 or 5 ng/μL showed a survival rate of 80% in both treatments. The WSSV-infected shrimps treated with 20 ng/μL of AgNPs exhibited a 70% survival rate after 96 h.

To corroborate the specific presence of genetic material from WSSV in the WSSV-infected shrimps treated with AgNPs, a PCR analysis with their total DNA was performed, the results of which are shown in Fig. 5B. The IQ Plus™ WSSV Kit provides a negative control of viral infection (WSSV− Kit) and an internal positive control for DNA amplification of WSSV (WSSV+ Kit); thus, the relative fold of WSSV DNA amplified within the control and experimental treatments were compared with WSSV− Kit and WSSV+ Kit.

According to the Student *t*-test results, the viral DNA amplification in the samples of shrimps treated with PBS (C−) did not show any difference compared with the negative control of viral infection (WSSV− Kit), but it showed a significant difference with WSSV+ Kit.

However, shrimps infected with WSSV (C+) showed a significant difference in viral DNA amplification, which is even higher than the internal positive control provided in the kit. This confirms that shrimps in fact were infected with WSSV. In addition, they developed clear symptoms of the WSS such as erratic swimming and lack of appetite.

The PCR analysis of WSSV DNA amplification from the shrimps infected with WSSV and treated with AgNPs revealed that the amplification of viral DNA in shrimps treated with the smaller concentration of AgNPs (0.5 ng/μL) is similar to that of the WSSV+ Kit. However, the infected shrimps treated with higher concentrations of AgNPs (5 and 20 ng/μL) harbored amounts of viral DNA that were to both negative controls (WSSV− Kit) and C−. After the injection of AgNPs into infected shrimps, they did not develop any behavioral symptoms of viral infection; moreover, feeding and swimming behavior remained unaltered.

This indicates that the shrimps were infected with WSSV; however, after treatment with AgNPs, 80% of the animals survived 96 h post infection. Although WSSV DNA was detected in the samples, the lack of WSS symptoms in the infected shrimps and the sustainability of their viability suggests that the virions present in the hemolymph and tissues of the infected shrimps are rendered uninfectious because of their interactions with AgNPs. The antiviral activity of AgNPs against a wide variety of unrelated families of viruses, such as influenza (A/H1N1), HIV-1, monkey pox virus, murine norovirus, vaccinia, respiratory syncytial virus, hepatitis B virus, and Tacaribe virus, suggests that the interaction of AgNPs depends more on their ability to directly bind to the surface viral proteins rather than to specific proteins expressed on every different cell type that hosts such a broad spectrum of viral families (Wei et al., 2014). This phenomenon has been reported for the interaction of AgNPs with influenza virus, monkey pox virus, and

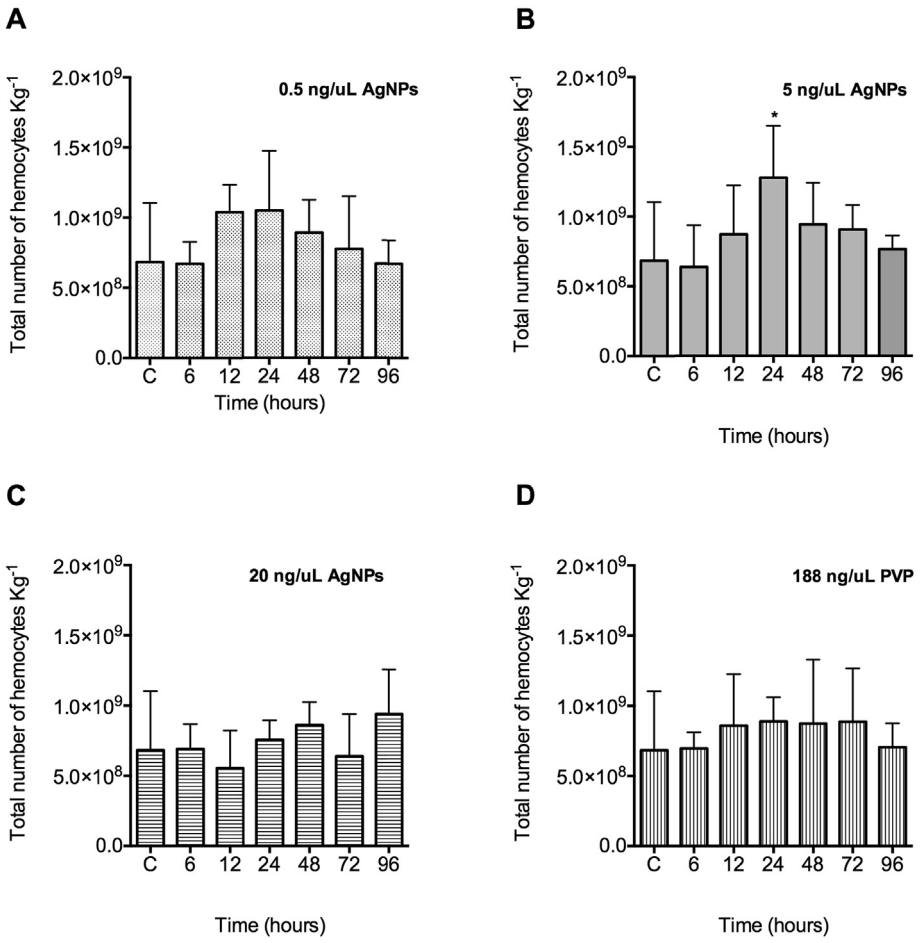

Fig. 4. THC in juvenile white shrimps injected with AgNPs. Organisms were injected with **(A)** 0.5, **(B)** 5, and **(C)** 20 ng/μL of AgNPs (concentrations are given according to the metallic silver content in AgNPs), whereas organisms in the negative control **(D)** were injected with a solution of PVP at 188 ng/μL in PBS. THC was measured for 96 h during the course of the experiment and compared with the initial THC values. Data represent the mean ± standard deviation of 10 measurements for each treatment. Statistical analysis in comparison with untreated organisms was performed and significance was $^{*}p < 0.05$.

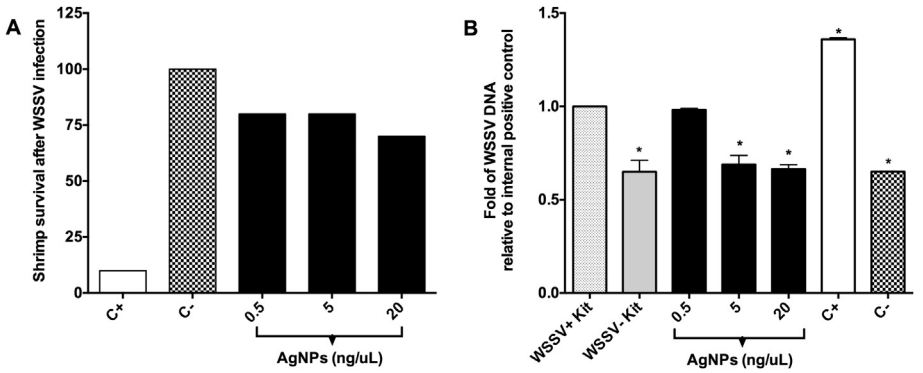

Fig. 5. Antiviral effect of AgNPs injection into juvenile white shrimps infected with WSSV. (**A**) Survival of WSSV-infected shrimps after AgNPs treatment. Positive control organisms (C+) were injected with an inoculum of WSSV. Negative control organisms (C−) were injected with PBS solution. Experimental groups of shrimps were infected with WSSV, and 24 h later, they were treated with 0.5, 5 or 20 ng/μL of AgNPs. Survival was monitored 96 h after WSSV infection. (**B**) PCR analysis of total DNA isolated from WSSV-infected shrimps and treated with AgNPs. Statistical analysis was achieved between groups by comparing with an internal positive control of WSSV infection (WSSV+ Kit); significance was $^{*}p < 0.05$.

Rift Valley fever virus (Borrego et al., 2016; Rogers et al., 2008; Xiang et al., 2013). Therefore, further nanostructure characterization of the interaction of AgNPs with viral particles is needed to determine the broad spectrum of antiviral activity of AgNPs.

Because of the economic impact of viral infections in shrimps, several efforts have been made to treat and overcome WSS. These include the injection of recombinant proteins such as lysozyme or egg yolk antibodies, which act as the protective agents, inducing

between 70% and 85% of shrimp survival after WSSV infection (Kim et al., 2004; Mai and Wang, 2010). The delivery of ds-RNA of two WSSV viral proteins (VP28 and VP26) resulted in a cumulative survival of 37% and 20%, respectively (Mejía-Ruíz et al., 2011). RNA silencing of viral proteins resulted in a shrimp survival rate between 33% and 60% (Wu et al., 2007; Xu et al., 2007), and VLPs with ds-RNA of major WSSV protein envelope induced a 44.5% survival rate after WSSV infection (Jariyapong et al., 2015). In comparison, in the present study the overall survival rate of WSSV-infected shrimps treated with AgNPs was 80%. Although this value is comparable with those obtained in other studies, none of those approaches fulfill the essential requirements such as stability, easy handling and preparation, low production cost, and, more importantly, having license to be used in veterinary applications, such as the AgNPs used here (Borrego et al., 2016).

We have previously reported the successful application of Argovit AgNPs for the treatment of the canine distemper virus (CDV) in dogs. The oral administration of AgNPs to dogs with non-neurological symptoms resulted in a survival rate of more than 90%; conversely, untreated animals did not survive the CDV infection (Bogdanchikova et al., 2016). Recently, our group published that Argovit AgNPs act as an effective antiviral agent to control the infectivity of the Rift Valley fever virus *in vitro* and *in vivo* in rats (Borrego et al., 2016). A manuscript related to the antiviral properties of AgNPs against WSSV in shrimps is currently being processed by our group (Ochoa-Meza, Alba Rocío et al. Evaluation of antiviral therapy with low doses of silver nanoparticles on the survival of *Litopenaeus vannamei* inoculated with white spot syndrome virus and exposed to ferrous ions in solution).

To the best of our knowledge, before this work, there are no studies describing the antiviral properties of AgNPs to overcome viral infections in shrimps. It is worth mentioning that different formulations of AgNPs act as effective antimicrobial and antiviral agents against a wide variety of bacteria and virus (Borrego et al., 2016; de Lima et al., 2012; Franci et al., 2015; Galdiero et al., 2011). Therefore, the results presented here open up the possibilities to investigate further applications of Argovit AgNPs to treat not only viral but a number of microbial infections that affect animals reared for human consumption and of highly alimentary and economic impact.

4. Conclusions

To our knowledge, this is the first study where the ability of AgNPs as antiviral agent was evaluated against the WSSV. The results of biological experiments were supported by a detailed characterization of the physicochemical properties of applied AgNPs. These include the content of metallic Ag and the amount of nanoparticle coating, average nanoparticle size and size distribution, hydrodynamic diameter and zeta potential, nanoparticle morphology, and UV–Vis spectra of AgNPs.

The intramuscular administration of AgNPs at concentrations of 0.5, 5, and 20 ng/μL and their stabilizer (PVP) at 188 ng/μL into the juvenile white shrimp *L. vannamei* proved to be nontoxic because it did not affect the viability or the behavior of the organisms. The injection of AgNPs did not alter the OCRR and TCH in the hemolymph of treated shrimps, indicating that the animals were not under stress. Injection of AgNPs into WSSV-infected shrimps resulted in a survival rate of 80% of the animals 96 h after infection. Thus, this study paves the way to test the potential antiviral effect of AgNPs as a safe and promising treatment or as a preventive agent for viral infections in aquaculture farms, which is expected to reduce the huge economic losses in the shrimp culture industry caused by this disease.

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