



Full length article

Silver nanoparticles enhance survival of white spot syndrome virus infected *Penaeus vannamei* shrimps by activation of its immunological system

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ABSTRACT

The global aquaculture has shown an impressive growth in the last decades contributing with a major part of total food fish supply. However, it also helps in the spread of diseases that in turn, causes great economic losses. The White Spot Syndrome Virus (WSSV) is one of the major viral pathogen for the shrimp aquaculture industry. Several attempts to eliminate the virus in the shrimp have been addressed without achieving a long-term effectiveness. In this work, we determine the capacity of the commercial non-toxic PVP-coated silver nanoparticles to promote the response of the immune system of WSSV-infected shrimps with or without an excess of iron ions. Our results showed that a single dose of metallic silver in the nanomolar range (111 nmol/shrimp), which is equivalent to 12 ng/mL of silver nanoparticles, produces 20% survival of treated infected shrimps. The same concentration administered in healthy shrimps do not show histological evidence of damage. The observed survival rate could be associated with the increase of almost 2-fold of LGBP expression levels compared with non-treated infected shrimps. LGBP is a key gene of shrimp immunological response and its up-regulation is most probably induced by the recognition of silver nanoparticles coating by specific pathogen-associated molecular pattern recognition proteins (PAMPs) of shrimp. Increased LGBP expression levels was observed even with a 10-fold lower dose of silver nanoparticles (1.2 ng/shrimp, 0.011 nmol of metallic silver/shrimp). The increase in LGBP expression levels was also observed even in the presence of iron ion excess, a condition that favors virus proliferation. Those results showed that a single dose of a slight amount of silver nanoparticles were capable to enhance the response of shrimp immune system without toxic effects in healthy shrimps. This response could be enhanced by administration of other doses and might represent an important alternative for the treatment of a disease that has still no cure, white spot syndrome virus.

1. Introduction

Penaeus (Litopenaeus) vannamei is the most common shrimp species grown in the world today. The characteristics of domestication of this species have made it widely accepted [1]. However, its extensive cultivation and management practices have allowed the generation of bacterial and viral diseases that for more than two decades have halted the growth of the cultivated shrimp industry [2]. The white spot

syndrome virus (WSSV) is the causative agent of shrimp white spot disease that has affected seriously the shrimp industry [3–5]. In Mexico, the WSSV was detected in 1999, but it was not until 2004 that it was reported as a disease in the shrimp farms of the northwest coasts of Mexico [6]. During all these years, has not been developed an antiviral or immunostimulant agent capable of decimating the viral infections against this serious shrimp disease, that year after year seem to increase, even though some antivirals have been introduced in the market

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and immunostimulants factors have been reported by other biopharmaceuticals [7–9].

Some of these antiviral agents have given encouraging results such as dsRNA and recombinant protein vaccines [10,11]. Recently, was reported the use of silver nanoparticles (AgNPs) as a promising resource to counteract viral diseases in humans, animals and plants, and more recently the AgNPs against aquatic organisms pathogens [12,13]. Argovit[®] is one of the nanoparticles whose broad-spectrum results as a disinfecting agent has been widely accepted for evaluation. Argovit[®] administration in other animals has achieved remarkable antiviral effects [14]. Dogs positively diagnosed with distemper disease and treated with AgNPs reached more than 85% of recovery rates [15]. On the other hand, these nanoparticles reduce the infectivity of Rift Valley virus on mice administered with a lethal dose of the virus [16]. Previously, our group has used the same AgNPs in Argovit[®] to protect shrimp against a WSSV inoculum, obtaining survival rates up to 85% by the end of treatment [17]. In this work, we are reporting the application of nano molar doses of Argovit[®] in shrimp intramuscularly as a prophylactic activity against WSSV infection. The Argovit[®] has been assessed in a challenge bioassay with juvenile shrimp exposed to WSSV viral inoculum. We have also included the presence of iron ions because this metal has been recognized as one of the factors that trigger epizootics when the WSSV is present under proliferation conditions [18,19].

2. Materials and methods

2.1. Shrimp samples and culture

A batch of shrimp (*P. vannamei*) sizes 8 ± 0.5 g from Acuacultura Mahr, Baja California Sur, Mexico, was transported to a research laboratory in Bámuc, State of Sonora, and placed in 1500 L tanks containing filtered seawater at 26 ± 1 °C. Shrimps were maintained under these conditions until they reached 10 g, fed with a 35% (w/w) protein commercial diet.

2.2. White spot syndrome virus inoculum

Inoculum of WSSV (Isolated-2008, Sonora, Mexico), was provided by the Aquaculture Laboratory from ITVY (Instituto Tecnológico del Valle del Yaqui). The WSSV viral inoculum was prepared from infected shrimp cephalothorax tissue, digestive gland, and eyestalk. Briefly, 500 mg of tissue was homogenized in PBS buffer. Homogenized samples were clarified by centrifugation at $3000 \times g$'s for 20 min at 4 °C. The supernatant was filtered through a 0.45 µm pore size filter and placed on ice until used. It was then diluted (1:10, with PBS buffer); 100 µL (~2500 SID50) of diluted supernatant was injected into the third abdominal segment of the shrimp before treatment with AgNPs.

2.3. Preparation of silver nanoparticle doses

The AgNPs doses were prepared taking 1 mL from Argovit[®] (No. 1324458) stock solution [C = 1.2 wt. % equivalent to 12 mg/mL of metallic silver], diluted in 99 mL with PBS and gently stirred for 30 s. This stock solution was used to carry out serial dilutions until reach final concentrations of 120 ng/mL (1.11 µM) and 12 ng/mL (111 nM) of metallic silver. Physicochemical properties of the Argovit[®] employed in this study are summarized in Table 1.

2.4. Preparation of samples for histological analysis

The cephalothorax of shrimp was fixed with Davidson's solution for histology injecting 10 mL of the solution into each sample and kept in a container of the same solution for 72 h. Samples were transferred to a container with 70% (v/v) ethyl alcohol. A dissection was performed by longitudinal cut into the cephalothorax. A 12 h infiltration and

Table 1
Physicochemical characteristics of Argovit[®] AgNPs (reported in Juarez-Moreno et al., 2017).

Properties	Mean
Content of metallic silver (% wt.)	1.2
Content of PVP (% wt.)	18.8
Content of water (% wt)	80.0
Average diameter of metallic silver particles by TEM data (nm)	35
Morphology of silver nanoparticle	spheroid
Size interval of metallic silver particles by TEM data (nm)	1 to 90
Hydrodynamic diameter: metallic Ag together with PVP (nm)	70
Zeta potential (mV)	-15
Surface plasmon resonance:	420 nm
PVP structure by FTIR	confirmed

embedding of tissue in paraffin was made with a Tissue-Tek Processor (Sakura Americas, Torrance, CA, USA.). Each sample was locked, stained with hematoxylin and eosin in Harris' solution. Samples were mounted on glass slides and studied under a microscope.

2.5. Therapeutic treatment with AgNPs after WSSV infection

Shrimps were acclimated in a tank with filtered seawater at 26 ± 1 °C and constant aeration for 24 h. VIMIFOS[®] commercial diet (35% [w/w] protein) was provided in three daily portions corresponding to 5% of shrimp total mass weight. Six experimental groups were prepared with 3 replicates (18 tanks with 6 individuals each). The first 5 groups (S1-S5) were inoculated with WSSV by injection at the dorsal region between third and fourth abdominal segment. Groups S1 and S3 treated with 100 µL of the previously prepared dilution of 120 ng/mL of AgNPs (metallic silver) while for groups S2 and S4 the dilution used was 12 ng/mL. The final concentration of AgNPs (metallic silver) administered to infected shrimps was 12 ng/shrimp (0.111 nmol/shrimp) in groups S1 and S3 and 1.2 ng/shrimp (0.011 nmol/shrimp) in groups S2 and S4. Group S2 and S4 were kept in presence of 0.1 mM of iron (Fe⁺²) sulfate (Table 2). Mortality data for each group were recorded every 6 h for 4 days.

2.6. Treatment with ferrous sulfate

Ferrous sulfate (FeSO₄·7H₂O) at 0.1 mM (30 mg/L) (Sigma: FeSO₄·7H₂O) was added to each tank containing 56 L of seawater (1.68 g/tank) of groups S3 and S4. FeSO₄ was chosen because it is one of the most available salts found in shrimp ponds. The high extreme concentration was selected according with the acid pH of the shrimp pond with worst possible water found condition [32]. After 24 h, all shrimp received the same treatment: WSSV viral inoculum followed by AgNPs injection. The bioassay concluded when the total number of shrimp in the group inoculated with the WSSV virus (positive control) died.

2.7. WSSV-diagnosis and immune system-related genes expression by qRT-PCR analysis

The VP24 gene of WSSV encodes for the envelope main structural

Table 2
Experimental treatments and Controls for injection shrimps.

Group	Sample name	Treatment	WSSV Inoculum
S1	AgNP-1	120 ng µl ⁻¹	+
S2	AgNP-2	12 ng µl ⁻¹	+
S3	AgNP-3	120 ng µl ⁻¹ + 0.1 mM FeSO ₄	+
S4	AgNP-4	12 ng µl ⁻¹ + 0.1 mM FeSO ₄	+
S5	Positive control	WSSV inoculum 1:10	+
S6	Negative control	PBS 1 at pH 7.0	-

Table 3
Primers used to amplify specific sequences from WSSV and *Penaeus vannamei* genes.

Gene	Primer name	Sequence	PCR product (bp)	Reference
Vp24	VP24 F1	AGGACCCGATCGCTTACTTTG	240	This paper
	VP24 R1	CTCCCTCCCTTGCGAACTT		
Pv β -actin	b-actin F1	GAAGTAGCCGCCCTGGTTG	416	[38]
	b-actin R1	CGGTTAGCCTTGGGGTTGAG		
Cu,Zn-SOD	LvCuSOD F	CGCGGGAGACACAGCTGATTTTC	164	[38]
	LvCuSOD R	GAAATCCAGGTGCCGGAGA		
LGBP	LGBP F1	CGGCAACCAGTACGGAGGAAC	222	[38]
	LGBP R1	GTGGAATCATCGCGGAAGGAG		

protein of the virus, which is located in the mature virion envelope, its expression is early in infected shrimp and is important for its final infection. The WSSV viral load for all samples was determined by qRT-PCR absolute quantification, using VP24 F/R WSSV specific primers (Table 3).

For gene expression analysis the following protocol was carried out: TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, U.S.A.) was used to extract total RNA from Gills of shrimps, dead or sacrificed individuals. For qRT-PCR analysis, RNA was treated with DNase I (1 U- μ g-1 DNA, Thermo Fisher Scientific, Waltham, MA, U.S.A.). The absence of DNA was confirmed by performing PCR (40 cycles, like the real-time PCR program) on the DNase I treated RNA using Taq-DNA polymerase. A CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, U.S.A.) was used for real-time PCR quantifications. qRT-PCR was performed according to the standard ImProm-IITM Reverse Transcription System® (Promega, Madison, WI, U.S.A.) kit with the Ssofast™ EVAGreen® Super Mix kit protocol (BioRad, Hercules, CA, U.S.A.). A “no DNA” template control was used in each analysis. The results presented are from four independent ($n = 4$) biological replicates, and statistical significance was determined with a two-way ANOVA followed by Tukey's test. Data were normalized to the Pv β -actin the shrimp reference gene (Table 3). The sequence of the specific primers for immune system-related genes (LGBP and CuSOD) from *P. vannamei* are summarized in Table 3. The method used to analyze the data from real-time PCR experiments corresponds to the relative quantification method, or 2- $\Delta\Delta$ CT method, where the $\Delta\Delta$ CT value = $((CT_{1Target} - CT_{1Reference}) - (CT_{0Target} - CT_{0Reference}))$ [19]. The mean CT values for both the target and the reference genes were determined and the fold change in the target gene normalized to Pv β -actin gene and relative to the expression in the control sample.

3. Results

3.1. Histologic analysis

In order to identify tissue damage produced by the administration of AgNPs on key tissues of shrimp, such as lymphoid organs, gills and stomach, a histopathologic analysis was performed. Results revealed that AgNPs at the concentrations employed in this work, did not produce any significant damage in healthy shrimp (Fig. 1). On the other

hand, WSSV infected shrimp (group S5) showed nuclear hypertrophy or inclusion bodies, among other significant damage. Similar damage it was determined on WSSV-infected shrimp treated with high and low concentrations of iron sulfate in the tank (S3 and S4), but with AgNPs added (Fig. 2). Interestingly, although the lack of differences regarding cellular damage on WSSV-infected organisms treated or no with AgNPs, survival time was definitely different. The shrimp treated only with AgNPs, but without iron sulfate, in its tank died almost 14 hpi after those not-injected with AgNPs.

3.2. WSSV challenge bioassay

The WSSV challenge bioassay was conducted on previously infected shrimp, recording mortality every six hpi after the AgNPs administration until the death of the last individual of the positive control group (S5), which was reached at 96 hpi after the viral infection. WSSV-inoculated shrimp did not show discomfort signs at 24 hpi, but mortality began at 30 h of viral infection (Fig. 3).

Groups S3 and S4 were the first where shrimp died, precisely those groups with iron sulfate added in the tank. At the end of the experiment, groups S5 (positive control), S3 and S4 present 100% mortality. Shrimp of groups S3 and S4 showed yellowing gills (probably due to the excess of iron ions); nonetheless, they showed a normal behavior before they died.

At the end of the experiment, a reduction in shrimp mortality was registered in groups S1 and S2, with 20% and 10%, respectively (Fig. 3). The negative control group S6 (non WSSV-infected and injected with PBS) showed 0% mortality (Fig. 3). Shrimps of group S6 were kept in the same laboratory under the same experimental conditions as the other treatments. No cross-contamination was determined by qRT-PCR absolute quantification analysis. Lifespan enhancement was observed with the AgNPs highest dose administered (group S1) at 48 hpi (Fig. 3).

3.3. qRT-PCR WSSV-diagnosis

The WSSV viral load was determined by qRT-PCR absolute quantification in shrimp through several time (30, 36, 42, 48 and 72 hpi) points, for each sample treated with the concentrations of AgNPs without iron sulfate (S1 and S2); or with iron sulfate added (S3 and S4);

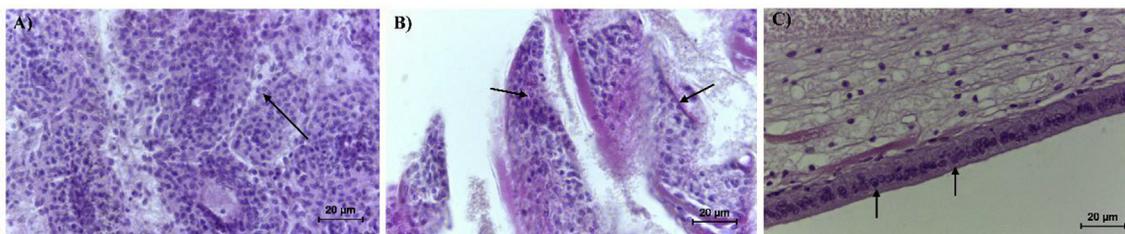


Fig. 1. Healthy tissues treated only with AgNPs, the images correspond to different tissues of shrimp a) lymphoid organ, b) gill and c) stomach to a 40X approach. The apparently healthy cells appear in the image, indicated by yellow arrows showing well-defined nuclei in normal-sized cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

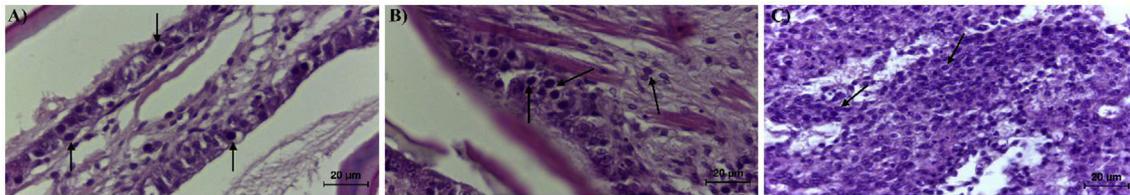


Fig. 2. The tissues treated with AgNP and with WSSV are shown where the inclusion bodies (yellow arrows) are seen characteristic of the virus a) antennal gland b) stomach and c) lymphoid organ, with clearly hypertrophied cells with marginal chromatin. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

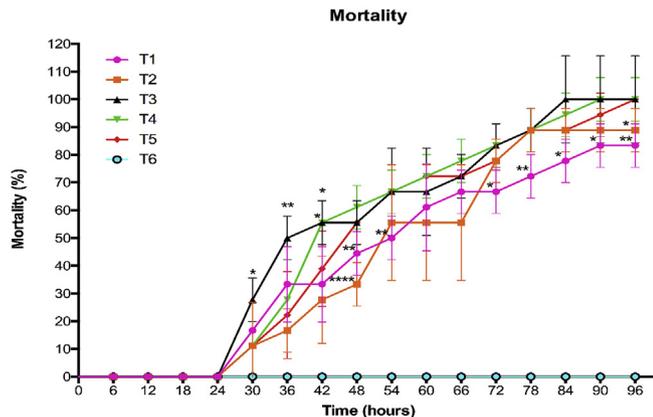


Fig. 3. Mortality kinetic between treatments. Treatments: S1 (solid circle, pink line, WSSV + 12 ng/shrimp AgNPs), S2 (solid square, orange line, WSSV + 1.2 ng/shrimp AgNPs), S3 (solid triangle, black line, FeSO₄ 100 nM + WSSV + 12 ng/shrimp AgNPs), S4 (inverted solid triangle, green line, FeSO₄ 100 nM + WSSV + 1.2 ng/shrimp AgNPs), S5 (solid diamond, red line, WSSV virus [positive control]) and S6 (cyan solid circle, cyan line, No WSSV virus [negative control]). The results presented are means \pm SD from three independent ($n = 3$) experiments, and statistical significance for every time-point was determined with an unpaired two-tailed Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

or only infected with WSSV (S5), and non WSSV-infected and non-treated (S6). WSSV-infection was confirmed for shrimp of S1–S5 treatments (Fig. 4C), and viral load absence on S6 group (Fig. 4C). The WSSV viral load of shrimp for groups S1–S4 was significantly higher (> two orders of magnitude) than the determined for the infected shrimps without any treatment (S5) (Fig. 4C).

3.4. Immune system-related genes expression by qRT-PCR analysis

Immune system-related (LGBP and Cu,Zn-SOD) genes expression analysis of shrimp was performed through time (30, 36, 42, 48 and 72 hpi) by qRT-PCR. The LGBP expression levels reaches its maximum at 36 hpi in groups S1 and S2 (Fig. 4A). And after 36 h, the expression levels were decreased until reaching values comparable with those found at the initial time (Fig. 4A). For those groups with added iron (S3 and S4), a similar behavior to that described for groups S1 and S2 was observed. However, the expression levels of LGBP at 30 and 36 hpi seems to be lower for group S4, which has the lower concentration of AgNPs in the presence of iron sulfate (group S4). After that, LGBP expression levels were quite similar for S1–S4 groups. For all groups treated with AgNPs, either in the presence or absence of iron sulfate, their LGBP expression levels were 2-fold higher than that determined for the infected shrimp without treatment (positive control, group S5). In the case of non WSSV-infected shrimps (S6, negative control), the expression levels of this gene were always lower compared with the other groups and without significant changes through time (Fig. 4A).

On the other hand, Cu,Zn-SOD expression levels showed a continuous decrease respect to time (Fig. 4B). The most important difference in the expression levels for this gene was observed between 30 and 36 hpi, with an even lower expression in those groups with added iron (S3 and S4) (Fig. 4B). At 42 hpi, groups S1, S2, S5 and S6 showed similar expression levels meanwhile groups S3 and S4 showed lower values compared with the other groups. For this gene, its expression levels were 2-fold than that found in non WSSV-infected shrimps at the first time of measurement (30 hpi). Altogether, this gene had shown similar expression levels for all treatments associated with WSSV infection, excepting 36 and 48 hpi with higher expression levels of groups S1 and S2 (Fig. 4B).

4. Discussion

The widespread applications of different AgNPs are present in aquaculture [12,13]. Recently, several research groups focused their efforts on the knowledge of toxicological effects of metal and metal oxides nanoparticles in marine organisms [16]. Despite this growth in the field and with the remarkable antiviral and/or immunostimulant activity observed for AgNPs in other organisms [14,15,17,22], it is surprising the scarce investigation regarding their use in the treatment of other shrimp diseases, such as white spot syndrome virus [17]; whilst has been widely assessed for diseases from bacterial origin [23]. In this study, we determined the potential antiviral activity of previously infected shrimps with WSSV, determining their defensive response against viral infection [24]. We follow the expression of LGBP and Cu,Zn-SOD, two key enzymes of the innate immune system of arthropods, that play an important role for growth, development, survival, and adaptation to the environment [33]. LGBP and Cu,Zn-SOD gene expression analysis of WSSV-infected shrimp was determined in two experimental conditions: (i) with only AgNPs treatment, and (ii) with ferrous sulfate addition to facilitate the WSSV viral proliferation. The LGBP gene encode for a lipopolysaccharide and β -1,3-glucan binding protein (LGBP) that is part of the pathogen-associated molecular patterns (PAMPs) recognition proteins, to recognize molecules associated to bacterial and fungal pathogens, and WSSV viral infection, and plays an essential role for crustacean innate immune system [21,25,26]. Furthermore, iron ions availability in shrimp ponds is indispensable for WSSV viral proliferation [19]. For this reason, the virus ensures its availability inhibiting the iron sequester capacity of ferritin and apoferritin. This is achieved by a direct protein-protein interaction between the viral protein kinase 1 (PK1) and the host ferritin [19,20].

Recently, we have shown the non-toxic effects of PVP-coated AgNPs in the concentration range of 0.5–20 mg/mL and the prophylactic effect of these concentrations inhibiting the WSSV infection of shrimp [17]. The absence of cellular damage (Fig. 1), confirms the lack of toxicity previously determined by the evaluation of biochemical parameters (oxygen consumption and total hemocyte count) in healthy shrimps [16]. Moreover, differences in the cellular damage observed on WSSV infected shrimp treated with AgNPs with iron ions (Group S3 and S4) and those of the positive control (group S5), suggest a systemic response of the shrimp, probably triggered by the activation of their immunologic system (Fig. 2). In spite of adverse effects that has been

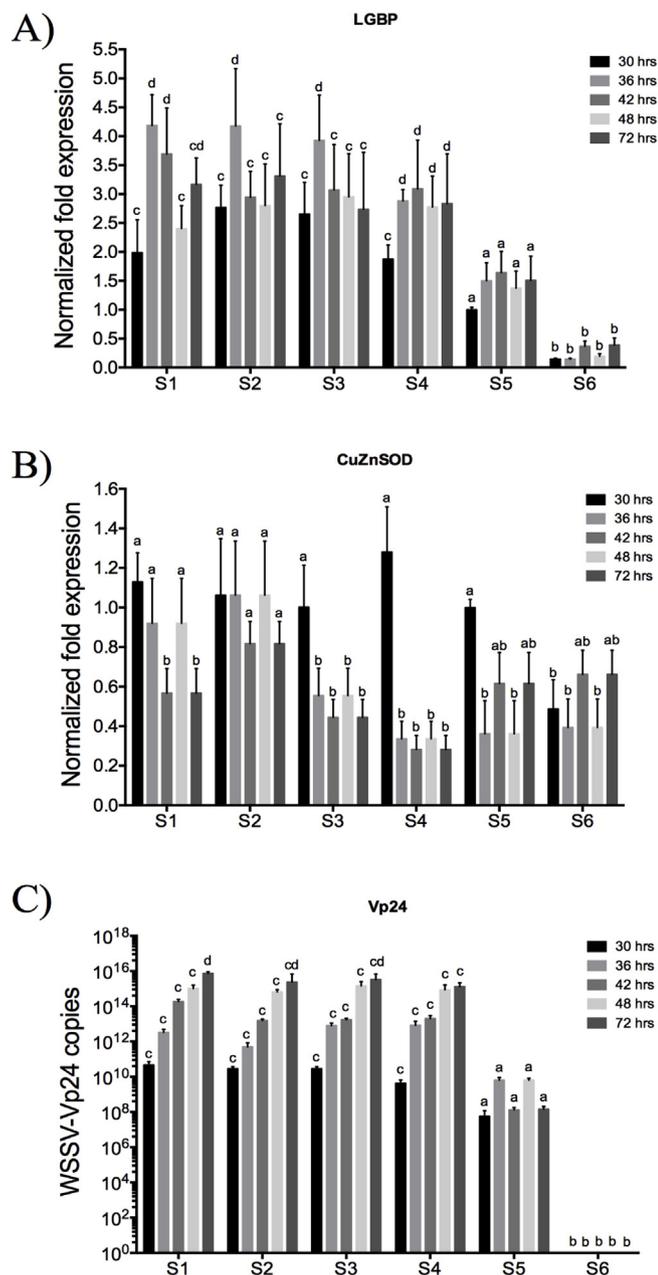


Fig. 4. Expression levels analysis for immune system-related 577 genes LGBP and CuZn-SOD from *P. vannamei* and presence of WSSV in shrimp subjected to different treatments (S1–S6) and at different time-points (30, 36, 42, 48 and 72 hpi). Treatments: S1 (WSSV + 12 ng/shrimp AgNPs), S2 (WSSV + 1.2 ng/shrimp AgNPs), S3 (FeSO₄ 100 nM + WSSV + 12 ng/shrimp AgNPs), S4 (FeSO₄ 100 nM + WSSV + 1.2 ng/shrimp AgNPs), S5 (WSSV virus [positive control]) and S6 (No WSSV virus [negative control]). The results presented are means \pm SD from four independent ($n = 4$) biological replicates, and statistical significance was determined with a two-way ANOVA followed by Tukey's test. Means with the same letter are not significantly different.

reported on shrimps by exposure to heavy metals [29] and other transition metals such as copper and nickel [30,31], in this work, high iron concentrations did not contribute to the oxidative stress damage. Unfortunately, this response was not enough to avoid the proliferation of the virus that leads to the death of the host. This is consistent with the highest mortality ratio observed on infected shrimp, which were treated with AgNPs but in presence of high concentrations of iron ions (Fig. 3). Similar survival effects have been recorded in other WSSV challenge experiments with low initial viral loads [28]. And also, is

worth noting that iron concentration in a shrimp pond can reach more than 0.5 g/L [32], while in this work the concentration used was 0.03 g/L.

Our results have shown that, as expected, the LGBP expression on WSSV-infected shrimp (S1–S5) was significantly higher than non WSSV-infected shrimp (S6). Interestingly, in the four groups of WSSV-infected shrimp treated with AgNPs with (S3, S4) or without (S1, S2) an excess of iron ions, the expression levels of this gene was 2-fold higher compared with WSSV-infected shrimp without AgNPs treatment (S5). This outstanding behavior with very small amounts of injected metallic silver suggests a possible interaction of AgNPs with recognition proteins present in the cellular membrane. Both the virus and silver nanoparticles activate the immune response of shrimp leading to a higher production of PAMPs recognition proteins, such as LGBP. We could suggest a possible mechanism, which AgNPs might compete or even interfere with the virus attachment to the cell, occupying the interaction sites of PAMPs recognition proteins and triggering immune response to cope against virus proliferation, or through an interaction with specific proteins of the virus, and even with those proteins essential for viral infection [33–35].

According to Beer and coworkers [36], free silver ions in AgNPs preparations could play a considerable role in the toxicity of AgNPs suspensions. However, we previously demonstrated that release of silver ions from our nanoparticles was not toxic [18] and then, did not contribute in the redox unbalance to induce the expression of Cu,Zn-SOD gene. Therefore, further studies have to be done to explore the proposed interaction of AgNPs with membrane proteins, such as LGBP. Interestingly, we determined a decrease in Cu,Zn-SOD gene expression at 36 hpi of WSSV-infected shrimp without AgNPs and without iron ions (S5). These results can be comparable with the data reported by Parrilla-Taylor and coworkers [37], which shows that shrimp infected with WSSV after 24 to 48 hpi showed a dramatic enzymatic activity decrease of the redox system (SOD, CAT, GPX, GR, and GST) that eventually led to shrimp death [38]. Altogether, these data might suggest that the slight amounts of metallic silver administered as AgNPs may induce the shrimp immunological response, possibly through their molecular interaction with recognition of specific membrane proteins of shrimp, such as LGBP and even others, or blocking epitopes of viral proteins [24–26]. Our experimental results also suggest that these nanoparticles could fulfill the requirements to improve the immune system of the shrimp.

It is very important to highlight the survival rate reached on WSSV-infected shrimp with a very small amount of metallic silver administered (nanomolar range/shrimp), compared with results obtained with other strategies against to WSSV infections that include feed supplementation, vaccination with an inactive virus, immunomodulators, and antiviral agents [27,39,40]. Vaccination with inactive WSSV produces 30–33% of mortality decrease, while 20% decrease was obtained with the antiviral agent Cidofovir[®] with a concentration of 200 mg/kg (5.73 μ mol Cidofovir[®]/shrimp) [41]. Immunostimulant agents such as GSH needs from 30 to 95-fold higher concentration to obtain the efficient activation of the immunological system of *P. vannamei* compared with AgNPs [42]. Additionally, feed supplementation with a commercial formulation Viusid[®] administered for seventeen days prior the WSSV infection or 30-day feeding prior infection with the probiotic *Bacillus* PC465 isolated from the gut of *Fenneropenaeus chinensis* [43], had a decrease mortality of 20% for the former and 26–29% by the latter.

Therefore, those results have shown that Argovit[®] is capable to induce the shrimp innate immune system response to decrease the mortality rate compared to that found in WSSV-infected non-treated organisms. No toxicity was observed with the concentration employed. Thus, further experiments with higher AgNPs concentrations must be done to determine whether these nanoparticles are capable to eliminate the virus conserving their biocompatibility or not [44]. This study represents the first attempt to test the effect of non-toxic AgNPs in the

survival of shrimp for treatment of WSSV viral infection.

5. Conclusions

AgNPs treatment in WSSV-infected *Penaeus vannamei* had an immunostimulant effect, using non-toxic concentrations, by the induce of shrimp innate immune response. Our results have shown that AgNPs promotes the expression increase of LGBP, a key component in the innate immune system of arthropods. We suggest that these occurs through the recognition of metallic silver of AgNPs or by their interaction with the WSSV viral envelope to trigger the PAMPs recognition proteins activation. Both suggested processes, acting independently or concomitantly, decrease mortality of infected shrimps.

LGBP up-regulation was observed both with or without available iron ions in solution, therefore, it could be possible that increasing the amount of AgNPs reduces the mortality of infected shrimp even in conditions where the proliferation of the virus is favored. Further experiments with higher concentrations of AgNPs to determine its capacity to eliminate the virus is needed. Finally, the non-toxic effects and its capacity to induce the innate immune system of WSSV-infected shrimps, make these AgNPs an excellent alternative for the treatment of white spot disease of shrimp, a disease that causes enormous economic losses and that still has no cure.

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References

- [1] T.W. Flegel, Kallaya Sritunyalucksana, Shrimp molecular responses to viral pathogens, *Mar. Biotech.* 13 (2010) 587–607.
- [2] P.J. Walker, C.V. Mohan, Viral disease emergence in shrimp aquaculture: origins, impact and the effectiveness of health management strategies, *Rev. Aquac.* 1 (2009) 125–154.
- [3] J. Rodríguez, B. Bayot, Y. Amano, F. Panchana, I. Blas, V. Alday, J. Calderon, White spot syndrome virus infection in cultured *Penaeus vannamei* (Boone) in Ecuador with emphasis on histopathology and ultrastructure, *J. Fish. Dis.* 26 (2003) 439–450.
- [4] K.W. Hasson, Y. Fan, T. Reisinger, J. Venuti, P.W. Varner, White-spot syndrome virus (WSSV) introduction into the Gulf of Mexico and Texas freshwater system through imported, frozen bait-shrimp, *Dis. Aquat. Org.* 71 (2006) 91–100.
- [5] M. Maldonado, J. Rodríguez, I. de Blas, El camarón de cultivo frente al WSSV, su principal patógeno, *Rev. Aquatic.* 21 (2004) 78–91.
- [6] L. Galaviz-Silva, Z.J. Molina-Garza, J.M. Alcocer-González, J.L. Rosales-Encinas, C. Ibarra-Gámez, White spot syndrome virus genetic variants detected in Mexico by a new multiplex PCR method, *Aquaculture* 242 (2004) 53–68.
- [7] D.V. Lightner, R.M. Redman, C.R. Pantoja, K.F. Tang, B.L. Noble, P. Schofield, L.L. Mohny, L.M. Nunan, S.A. Navarro, Historic emergence, impact and current status of shrimp pathogens in the Americas, *J. Invertebr. Pathol.* 110 (2012) 174–183.
- [8] J. Wyban, J.N. Sweeney, Tecnología intensiva de producción de camarón: Manual del camarón del Oceanic Institute, Oceanic Institute Honolulu, 1991.
- [9] J. Cock, T. Gitterle, M. Salazar, M. Rye, Breeding for disease resistance of Penaeid shrimps, *Aquaculture* 286 (2009) 1–11.
- [10] J.M.J. Ruiz-Velazco, A. Hernández-Llamas, V.M. Gomez-Muñoz, F.J. Magallón, Dynamics of intensive production of shrimp *Litopenaeus vannamei* affected by white spot disease, *Aquaculture* 300 (2010) 113–119.
- [11] P. Álvarez-Ruiz, A. Luna-González, C.H. Mejía-Ruiz, F.J. Magallón-Barajas, D. Galván-Álvarez, Long-lasting effect against white spot syndrome virus in shrimp broodstock, *Litopenaeus vannamei*, by LvRab7 silencing, *J. WAS.* 46 (2015) 571–582.
- [12] T.J. Baker, C.R. Tyler, T.S. Galloway, Impacts of metal and metal oxide nanoparticles on marine organism, *Environ. Pollut.* 186 (2014) 257–271.
- [13] J.C. Meneses-Márquez, A. Hamdan-Partida, M. del C. Monroy-Dosta, J. Castro-Mejía, J.A. Bustos-Martínez, Silver nanoparticles applications (AgNPS) in aquaculture, *Int. J. Fish. Aquat. Stu.* 6 (2018) 5–11.
- [14] N. Bogdanchikova, R. Vázquez-Muñoz, A. Huerta-Saquero, A. Peña-Jasso, G. Aguilar-Uzcanga, P.L. Picos-Díaz, A. Pstryakov, V. Burmistrov, O. Martynyuk, R. Luna-Vázquez-Gómez, H. Almanza, Silver nanoparticles composition for treatment of distemper in dogs, *Int. J. Nanotechnol.* 13 (2016) 227–237.
- [15] B. Borrego, G. Lorenzo, J.D. Mota-Morales, H. Almanza-Reyes, F. Mateos, E. Lopez-Gil, N. Bogdanchikova, Potential application of silver nanoparticles to control the infectivity of Rift Valley fever virus in vitro and in vivo, *Nanomed. Nanotechnol.* 12 (2016) 1185–1192.
- [16] H. Wang, K.T. Ho, K.G. Scheckel, F. Wu, M.G. Cantwell, D.R. Katz, D.B. Horowitz, W.S. Boothman, R.M. Burgess, Toxicity, bioaccumulation, and biotransformation of silver nanoparticles in marine organisms, *Environ. Sci. Technol.* 48 (2014) 13711–13717.
- [17] K.O. Juárez-Moreno, H. Mejía-Ruiz, E. Vázquez-Félix, A. Pstryakov, N. Bogdanchikova, H. Reyna-Verdugo, Antiviral effect of AgNP in white leg shrimp *Litopenaeus vannamei* protect to WSSV infection, *Chemosphere* 169 (2017) 716–724.
- [18] S.T. Ong, J.Z. Shan Ho, B. Ho, J.L. Ding, Iron-withholding strategy in innate immunity, *Immunobiology* (2001) 295–314 (2006).
- [19] S.J. Lin, D.Y. Lee, H.C. Wang, S.T. Kang, P.P. Hwang, G.H. Kou, M.F. Huang, G.D. Chang, C.F. Lo, White spot syndrome virus protein kinase 1 defeats the host cell's iron withholding defense mechanism by interacting with host ferritin, *J. Virol.* 89 (2015) 1083–1093.
- [20] T. Ye, X. Wu, W. Wu, C. Dai, J. Yuan, Ferritin protect shrimp *Litopenaeus vannamei* from WSSV infection by inhibiting virus replication, *Fish Shellfish Immunol.* 42 (2015) 138–143.
- [21] T. Itami, M. Asano, K. Tokushige, K. Kubonu, A. Nakagawa, N. Takeno, H. Nishimura, M. Maeda, M. Kondo, Y. Takahashi, Enhancement of disease resistance of kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*, *Aquaculture* 164 (1998) 277–288.
- [22] S. Galdiero, A. Falanga, M. Vitiello, M. Cantisani, V. Marra, M. Galdiero, Silver nanoparticles as potential antiviral agents, *Molecules* 16 (2011) 8894–8918.
- [23] K. Kandasamy, N. Aliunhi, Synthesis of silver nanoparticles by coastal plant *Prosopis chilensis* (L.) and their efficacy in controlling vibriosis in shrimp *Penaeus monodon*, *Appl. Nanosci.* 3 (2013) 65–73.
- [24] H.H. Lara, N.V. Ayala-Nuñez, L. Ixtepan-Turrent, C. Rodríguez-Padilla, Mode of antiviral action of silver nanoparticles against HIV-1, *J. Nanobiotechnol.* 8 (2010) 1–10.
- [25] M.M. Roux, A. Pain, K.R. Klimpel, A.K. Dhar, The lipopolysaccharide and β -1,3-glucan binding protein gene is upregulated in white spot virus-infected shrimp (*Penaeus stylirostris*), *J. Virol.* 76 (2002) 7140–7149.
- [26] Y.Y. Chen, J.C. Chen, Y.H. Kuo, Y.C. Lin, Y.H. Chang, H.Y. Gong, C.L. Huang, Lipopolysaccharide and β -1,3-glucan-binding protein (LGBP) bind to seaweed polysaccharides and activate the prophenoloxidase system in white shrimp *Litopenaeus vannamei*, *Dev. Comp. Imm.* 55 (2016) 144–151.
- [27] B. Verbruggen, L.K. Bickley, R. van Aerle, K.S. Bateman, G.D. Stentiford, E.M. Santos, C.R. Tyler, Molecular mechanisms of white spot syndrome virus infection and perspectives on treatments, *Virus* 8 (2016) 23.
- [28] C.H. Mejía-Ruiz, S. Vega-Peña, C.M. Escobedo-Bonilla, Double-stranded RNA against white spot syndrome virus (WSSV) vp28 or vp26 reduced susceptibility of *Litopenaeus vannamei* to WSSV, and survivors exhibited decreased susceptibility in subsequent re-infections, *J. Invertebr. Pathol.* 107 (2011) 65–68.
- [29] G. Kaya, S. Turkoglu, Bioaccumulation of heavy metals in various tissues of some fish species and green tiger shrimp (*Penaeus semisulcatus*) from İskenderun bay, Turkey, and risk assessment for human health, *Biol. Trace Elem. Res.* 180 (2017) 314–326.
- [30] R. Mendoza-Rodríguez, Acute toxicity of copper (Cu²⁺) on postlarvae of river prawn *Cryphiops caementarius* (Natantia, Palaemonidae), *Rev. Peru. Biol.* 14 (2007) 53–54.
- [31] E.M. Leonard, I. Barcarolli, K.R. Silva, W. Wasieleski, C.M. Wood, A. Bianchini, The effects of salinity on acute and chronic nickel toxicity and bioaccumulation in two euryhaline crustaceans: *Litopenaeus vannamei* and *Excitrolana armata*, *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 154 (2011) 409–419 Ref. 27.
- [32] F. Páez-Osuna, A.C. Ruiz-Fernández, Environmental load of nitrogen and phosphorus from extensive, semi-intensive and intensive shrimp farms in the Gulf of California ecoregion, *Bull. Environ. Contam. Toxicol.* 74 (2005) 681–688.
- [33] L. Sun, A.K. Singh, K. Vig, S. Pillai, R. Shreekrumar, S.R. Singh, Silver nanoparticles inhibit replication of respiratory syncytial virus, *J. Biomed. Biotechnol.* 4 (2008) 149–158.
- [34] J.V. Rogers, C.V. Parkinson, Y.W. Choi, J.L. Speshock, S.M.A. Hussain, Preliminary assessment of silver nanoparticles inhibition of monkeypox virus plaque formation, *Nanoscale Res. Lett.* 3 (2008) 129–133.
- [35] G. Le Moullac, M. Le Gromellec, D. Anquer, S. Froissard, P. Levy, AQUACOP, Haematological and phenoloxidase activity changes in the shrimp *Penaeus stylirostris* in relation with the moult cycle: protection against vibriosis, *Fish Shellfish Immunol.* 7 (1997) 227–234.
- [36] C. Beer, R. Foldbjerg, Y. Hayashi, D.S. Sutherland, H. Autrup, Toxicity of silver nanoparticles - nanoparticle or silver ion? *Toxicol. Lett.* 208 (2012) 286–292.
- [37] D.P. Parrilla-Taylor, T. Zenteno-Savin, F.J. Magallón-Barajas, Antioxidant enzyme activity in pacific whiteleg shrimp (*Litopenaeus vannamei*) in response to infection with white spot syndrome virus, *Aquaculture* 380–383 (2013) 41–46.
- [38] Z. Xia, S. Wu, Effects of glutathione on the survival, growth performance and non-specific immunity of white shrimps (*Litopenaeus vannamei*), *Fish Shellfish Immun.* 73 (2018) 141–144.
- [39] D. Muñoz-Naranjo, J. Gilbert-Jaramillo, E. Marcillo-Gallino, F. Marcillo-Morla,

- M. Muñoz-Naranjo, Survival in juvenile shrimps (*Penaeus vannamei*) exposed to inactive against active white spot virus: a challenge bioassay perspective, *Lat. Am. J. Aquat. Res.* 46 (2018) 225–229.
- [40] A. Namikoshi, J.L. Wu, T. Yamashita, T. Nishizawa, T. Nishioka, M. Arimoto, K. Muroga, Vaccination trials with *Penaeus japonicus* to induce resistance to white spot syndrome virus, *Aquaculture* 229 (2004) 25–35.
- [41] M.M. Rahman, C.M. Escobedo-Bonilla, M. Wille, V. Alday Sanz, L. Audoorn, J. Neyts, M.B. Pensaert, P. Sorgeloos, H.J. Nauwynck, Clinical effect of cidofovir and a diet supplemented with *Spirulina platensis* in white spot syndrome virus (WSSV) infected specific pathogen-free *Litopenaeus vannamei* juveniles, *Aquaculture* 255 (2006) 600–605.
- [42] C.F. Lo, J.H. Leu, C.H. Ho, C.H. Chen, S.E. Peng, Y.T. Chen, C.M. Chou, P.Y. Yeh, C.J. Huang, H.Y. Chou, C.H. Wang, G.H. Kou, Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction, *Dis. Aquat. Org.* 25 (1996) 133–141.
- [43] P.C. Chai, X.L. Song, G.F. Chen, H. Xu, J. Huang, Dietary supplementation of probiotic *Bacillus* PC465 isolated from the gut of *Fenneropenaeus chinensis* improves the health status and resistance of *Litopenaeus vannamei* against white spot syndrome virus, *Fish Shellfish Immunol.* 54 (2016) 602–611.
- [44] C. Zhang, Z. Hu, B. Deng, Silver nanoparticles in aquatic environments: physiochemical behavior and antimicrobial mechanisms, *Water Res.* 88 (2016) 403–427.