Evaluation of a new Argovit as an antiviral agent included in feed to protect the shrimp *Litopenaeus vannamei* against White Spot Syndrome Virus infection

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Abstract

In this study, four experimental assays were conducted to evaluate the use of a new silver nanoparticle formulation named Argovit-4, which was prepared with slight modifications to enhance its biological activity against white spot syndrome virus (WSSV) in shrimp culture. The

34   goals of these assays were to (1) determine the protective effect of Argovit-4 against WSSV, (2)

35   determine whether Argovit-4 supplemented in feed exhibits toxicity towards shrimp, (3)

36   determine whether Argovit-4 as antiviral additive in feed can prevent or delay/reduce WSSV-

37   induced shrimp mortality, and (4) determine whether Argovit-4 supplemented in feed alters the

38   early stages of the shrimp immune response. In bioassay 1, several viral inocula calibrated at 7

39   SID_{50} (shrimp infectious doses 50% endpoint) were exposed to 40, 100, 200 and 1000 ng/SID_{50}

40   of Ag^+ and then intramuscularly injected into shrimp for 96 h. In bioassay 2, shrimp were fed

41   Argovit-4 supplemented in feed at different concentrations (10, 100 and 1000 µg per gram of

42   feed) for 192 h. In bioassay 3, shrimp were treated with Argovit-4 supplemented in feed at

43   different concentrations and then challenged against WSSV for 192 h. In bioassay 4, quantitative

44   real-time RT-qPCR was performed to measure the transcriptional responses of five immune-

45   relevant genes in haemocytes of experimental shrimp treated with Argovit-4 supplemented in

46   feed at 0, 6, 12, 24 and 48 h. The intramuscularly injected Argovit-4 showed a dose-dependent

47   effect ($p<0.05$) on the cumulative shrimp mortality from 0-96 h post-infection. In the second

48   bioassay, shrimp fed Argovit-4 supplemented in feed did not show signs of toxicity for the

49   assayed doses over the 192-h experiment. The third and fourth bioassays showed that shrimp

50   challenged with WSSV at 1000 µg/g feed exhibited reduced mortality without altering the

51   expression of some immune system-related genes according to the observed level of

52   transcriptional. This study is the first show that the new Argovit-4 formulation has potential as an

53   antiviral additive in feed against WSSV and demonstrates a practical therapeutic strategy to

54   control WSSV and possibly other invertebrate pathogens in shrimp aquaculture.

55

56   Keywords: Feed additive; AgNPs; Shrimp; WSSV; Gene expression

57

58   **Introduction**

59   The white spot syndrome virus (WSSV) is highly aggressive towards shrimp aquaculture

60   worldwide, for which several studies have attempted to develop a treatment (Álvarez-Sánchez et

61   al., 2018). This pathogen can cause 100% mortality in shrimp culture in just a few days, causing

62   large economic losses, business closures and high unemployment rates (Sánchez-Martínez,

63   Aguirre-Guzmán & Mejía-Ruíz, 2007; Bustillo, Escobedo & Sotelo, 2009). Currently,

64   nanotechnology has attracted a great deal of attention as an emerging field of research and

65   technological development that open the possibility of handling material at the nanometric level,

66   with dimensions between 1 and 100 nm (Meneses-Marquez *et al.*, 2018). In this size range,

67   nanoparticles show new physical and chemical properties that can be used in different scientific

68   areas for the unprecedented study of phenomena that occur at the atomic and molecular level

69   (Parveen, Misra & Sahoo. 2012; Esmaeillou *et al.*, 2017). Among the most interesting and

70   promising nanomaterials are silver nanoparticles (AgNPs), which have shown broad-spectrum

71   antiviral activity (Bello-Bello *et al.*, 2018; Galdiero *et al.*, 2011; Chris, Singh & Agarwal. 2018).

72   Several *in vitro* studies have demonstrated the effect of AgNPs as an antiviral alternative against

73   human viruses, including human immunodeficiency virus (Elechiguerra *et al.*, 2005; Lara *et al.*,

74   2010), H1N1 influenza A virus (Mori *et al.*, 2013), monkeypox virus (Rogers *et* al., 2008),

75   Tacaribe virus (Speshock *et al.*, 2010), hepatitis B virus (Leu *et al.*, 2008) and herpes simplex

76   virus (Baram-Pinto *et al.*, 2009).

77   The mechanism of action of AgNPs as an antiviral agent has been studied against several

78   enveloped viruses (Lara *et al.*, 2011; Bogdanchikova *et al.*, 2016), and it has suggested that

79   AgNPs undergo preferential binding with viral envelope glycoproteins to inhibit viruses from

80   binding to host cells (Elichiguerra *et al.*, 2005; Lara *et al.*, 2010). In addition, multiple studies

81   have shown that nanoparticles can be recognized by the immune system and modulate the

82   induction of immunostimulatory effects (Dacoba *et al.*, 2017; Boraschi *et al.*, 2017).

83   Recent advances have been made in aquaculture using AgNP-based formulations. In one study,

84   Argovit® that was administered intramuscularly in shrimp *Litopenaeus vannamei* showed

85   antiviral activity against WSSV (Juarez-Moreno *et al.*, 2017), a highly lethal and contagious

86   pathogen. This result indicated that a single dose of silver nanoparticles was able to increase

87   shrimp survival by up to 80% without toxic effects. In another study, Ochoa-Meza *et al.* (2019)

88   evaluated the ability of silver nanoparticles coated with commercial non-toxic PVP (Argovit®)

89   to promote the immune system response of shrimp infected with WSSV and observed a late

90   immunostimulatory activity of two immune-related genes. In the same report, authors showed

91   that a single dose of AgNP was able to increase the survival of the shrimp against severe

92   infections of WSSV, even under adverse conditions in the presence of a high concentration of

93   ferrous ions.

94   Crustaceans have an innate immune system, similar to other invertebrates, however, they lack a

95   developed adaptive immune system. The innate immune system of shrimp consists of several

96 natural defence mechanisms: a microbial recognition system, a prophenoloxidase system (proPO

97 system), a coagulation system, phagocytosis, encapsulation and the formation of nodules and

98 oxygen reactive compounds (Kurtz, 2005; Ji, Xiao & Wang, 2009; Hirono, *et al.*, 2011). Several

99 genes that participate in the shrimp immune system have been described in the species *L.*

100 *vannamei*, some of which have been described in previous reports from our group (Flegel &

101 Sritunyalucksana, 2010). To identify a potential mechanism of the antiviral and

102 immunostimulatory activities of silver nanoparticles in shrimp, we selected five genes to study

103 that are representative of different immunological routes. Two of the genes encode proteins

104 (Rab6 and PAP) that participate in endocytosis and phagocytosis (Ye, Tang & Zhang 2012;

105 Khimmakthong *et al.*, 2013). The genes encoding Crustin and PEN4 have been identified as

106 participating in antiviral immunity against WSSV infections in *L. vannamei* (Sun *et al.*, 2017;

107 Xiao *et al.*, 2018). Finally, the proPO gene activates the proPO system, which is involved in

108 melanin formation in arthropods and other invertebrates (Sritunyalucksana & Soderhall, 2000)

109 and results in the production of the melanin pigment that can often be seen as dark spots in the

110 cuticle of arthropods and two antimicrobial peptides from defence system also in WSSV

111 infections (Abad-Rosales *et al.*, 2019). These advances have demonstrated that AgNPs are

112 effective against a wide spectrum of viruses, especially against the disease-causing WSSV, for

113 which there have been no effective treatments until now (Verbruggen *et al.*, 2016). However,

114 intramuscular administration of AgNPs is impractical, considering the large volumes of

115 organisms to that would need to be treated. A practical alternative is the inclusion of AgNPs in

116 balanced shrimp feed (Dananjaya *et al.*, 2016), guaranteeing a greater usefulness of AgNPs

117 therapy.

118 Although the Argovit® formulation is no longer commercially available, new formulations have

119 been developed with unique features (N. Bogdanchikova, Personal communication, December

120 2017). For instance, the Argovit-4 formulation retains essentially same the silver nanoparticle

121 design of Argovit®. Thus, in this study, we investigated whether the AgNPs-based Argovit-4

122 could be used as an antiviral additive in feed to interfere with WSSV infection in shrimp.

123

124 **Materials and Methods**

125 **Argovit-4 formulation**

126 The silver nanoparticles Argovit-4, which replaced Argovit® (Novosibirsk, Russia; patent

127 2427380) was kindly donated by Dr. Nina Bogdanchikova from CNyN-UNAM, and this can be

128   requested by contacting Dr. Bodganchikova, (or it can be purchased at Bionag SAPI de CV,

129   Tijuana, México. MC Martha Alvarado, (52) 664-317-99-21). The supplier's specifications

130   indicate that Argovit-4 consists of spherical AgNPs with an average size of 35 ± 15 nm that are

131   functionalized with non-toxic PVP (K30) in water. Table 1, which was modified from Juarez-

132   Moreno *et al.* (2017), summarizes the physicochemical characterization of these formulations.

133

White spot syndrome viral stock

135   The WSSV strain (Guasave isolate, 2005) used in this study was kindly provided by the

136   Biochemistry and Molecular Biology Laboratory from CIIDIR, Sinaloa, Mexico. The viral

137   inoculum was prepared according to the methodology described by Alvarez-Ruiz *et al.* (2013).

138   Muscular tissues from moribund shrimp were collected, weighed and homogenized in 10

139   volumes (W/V) of phosphate-buffered saline ($1\times$ PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM

140   Na_2HPO_4, and 2 mM KH_2PO_4) to pH 7.4. The homogenized tissue was centrifuged at 13,000 x *g*

141   (or g-force) for 20 min at 4°C, after which the supernatant was transferred to another microtube

142   (1.6 mL) and centrifuged again at 13,000 x *g* for 20 min at 4°C. The supernatant was passed

143   through a 0.45 μm pore-sized filter and stored at -70°C.

144

Preparation of inoculum from WSSV-infected tissue

146   The experimental challenge with WSSV was conducted by the oral route using infected tissue.

147   The infected tissue was prepared by intramuscularly injecting a batch of shrimp (n=10) of 10 ± 2 g

148   with of 32 SID_{50} in 100 μL of PBS (assuming that 100% of organisms would be infected at 54 h

149   post-infection). The shrimp with observable clinical signs of WSSV infection were individually

150   separated and stored at -70°C until all of the shrimp became infected. Subsequently, after

151   removing the cephalothorax, pleopods and exoskeleton the muscle tissue of the dead WSSV-

152   infected shrimp was weighed and homogenized in 120 mL of marine water for every 24 g of

153   tissue. The homogenized material was used as the inoculum used for the bioassay 4.

154

Shrimps

156   Batches of *L. vannamei* (4 ± 0.5 g) juveniles were obtained from a local farm in Guasave, Sinaloa,

157   Mexico. The shrimps were transported to CIIDIR-IPN and placed in plastic tanks (1000-L

158   capacity) with 500 L of filtered seawater at 30 PSU (Practical Salinity Units) with constant

159   aeration. The shrimps were fed *ad libitum* twice daily with Camaronina® commercial feed

160   (Purina-Cargill LTD, Minnesota, USA) with 35% protein. Subsequently, the shrimps were

161   verified to be free of WSSV, IHHNV and AHPND by endpoint PCR.

162

Experimental conditions

164   All experiments were performed in glass tanks (40-L capacity) containing 25 L of seawater (30

165   PSU) at $27 \pm 1°C$ and continuous aeration with the shrimps acclimatized under these conditions

166   for 24 h before starting the experiments. The experimental shrimp for bioassay 1 and 2 were fed

167   Camaronina® at 4% of their body weight, while in bioassays 3, 4 and 5, the shrimp were fed

168   feed supplemented with AgNPs. The uneaten feed and faecal material were removed by

169   siphoning every 2 days with water exchange (50% volume) during all experimental assays.

170

In vivo viral titration and determination of minimal infectious dose by the intramuscular route

173   A batch of shrimp was used to titre the fresh inoculum using the intramuscular injection method

174   described previously by Alvarez-Ruiz *et al.* (2013) and Apun *et al.* (2017). The inoculum was

175   titrated by *in vivo* injecting 100 μL of serial dilutions from the original extract (10^{-3}, 10^{-4}, 10^{-5}

176   and 10^{-6}) between the 3rd and 4th abdominal segments of shrimp (n=5 shrimp per dilution). The

177   experimental assay was finished at 96 h post-infection (hpi). The viral titre was calculated using

178   the method of Reed & Muench (1938), for the concentration of a test substance that produces an

179   effect of interest in half of the test units (50% tissue culture infectious dose of a virus), which

180   was described as SID_{50}/mL.

181

Preparation of Argovit-4 supplemented feed

183   Feed supplemented with AgNPs at different concentrations was made according to the modified

184   method of Gutiérrez-Dagnino *et al.* (2015). Camaronina® 35% protein pellets (commercial feed

185   for shrimp) were pulverized in a coffee grinder. Three homogeneous pastes supplemented with

186   Argovit-4 AgNPs (10, 100 and 1000 μg/g feed), sodium alginate (20 μg/g feed; Sigma-Aldrich®,

187   St. Louis, MO, USA), and distilled water (0.5 mL/g feed) were generated. In the control feed,

188   AgNPs were replaced by distilled water. Subsequently, the pastes were manually pelletized with

189 a 50-mL disposable syringe (Millipore®, Merck, Darmstadt, Germany) and dried for 72 h at 4°C

190 in darkness.

191

BIOASSAYS

Bioassay 1: Determination of the minimum WSSV infectious dose by intramuscular route

195 Bioassay 1 was designed to determine the effective dose of WSSV that is capable of infecting

196 100% of the shrimp (as determined by conspicuous clinical signs, such as a lack of appetite,

197 erratic swimming and conspicuous atypical coloration). Three groups of shrimp (30 organisms

198 per group, n=10 shrimp in three replicates) were intramuscularly inoculated between the 3rd and

199 4th abdominal segment with different WSSV infectious doses (7, 10 and 100 SID_{50} in l00 μL of

200 PBS). The control group was a mock inoculation with 100 μL of PBS. The mortality of the

201 shrimp in every group was recorded and the bioassay finished was when all of the infected

202 shrimp died.

203

Bioassay 2: Inhibition of pathogenicity by the application of a WSSV-Argovit-4 mixture

206 Several viral inoculums were calibrated at 7 SID_{50} in 100 μL volumes and exposed at 40, 100,

207 200 and 1000 ng of Argovit-4. The viral inoculums and Argovit-4 (WSSV + AgNPs) mixtures

208 were incubated for one hour on ice and then intramuscularly injected into 30 shrimp for every

209 group (n=10 organisms per replicate) for each treatment. The positive control group was

210 inoculated with WSSV (without AgNPs), while the control group was mock-treated with 100 μL

211 of PBS. The experiment lasted up to 96 hpi, and every six hours the presence of dead organisms,

212 clinical signs and the behaviour of the surviving shrimp were recorded.

213

Bioassay 3: Toxic effect of Argovit-4 supplemented in feed

215 The toxic effect of Argovit-4 supplemented in feed at different concentrations was tested first.

216 The experiment consisted of four treatments with three replicates each (n=10 shrimp per

217 replicate) that were organized as follows: (1) shrimps receiving feed without AgNPs (control

218 group), (2) shrimps receiving AgNPs at 10 μg/g feed, (3) shrimps receiving AgNPs at 100 μg/g

219 feed, (4) shrimps receiving AgNPs at 1000 μg/g feed. The shrimps were fed twice daily at 4% of

220 their body weight. The signs of toxicity (lethargic movement, empty gut or atypical colour

221 change) and cumulative mortality were recorded daily. Previously a toxicity bioassay was carried

222 out by intramuscular route in the shrimp for each of 5 different designs of Argovit, which did not

223 occurred mortality and Argovit-4 was selected (data not shown).

224

225 **Bioassay 4: Argovit-4 supplemented in feed against WSSV**

226 For bioassay 4, the experimental design was performed in triplicate (n=10 shrimps by replicate).

227 The shrimp were fed Argovit-4-supplemented feed at 0 and 12 h for the first and second

228 feedings, respectively. In both cases, the feed weight corresponded to 4% of the shrimp body

229 weight (4 g/organism). Subsequently, at 24 h, the shrimps were fed the WSSV inoculum (200 mg

230 infected tissue per shrimp). Every shrimp received two daily rations of food corresponding to 4%

231 of their body weight, and at 12 h post-treatment with AgNP in feed were challenged with 200 mg

232 of infected tissue per shrimp for each replicate. The cumulative mortality was recorded twice a

233 day until 192 h.

234

235 **Bioassay 5: Immunostimulatory activity of Argovit-4 towards immune-related**

236 **genes**

237 To determine the immunostimulatory activity of Argovit-4 on shrimp *L. vannamei* juveniles, a

238 bioassay was established in two glass tanks (40 L) with 25 L of seawater and continuous

239 aeration. Two groups (n= 12 shrimp per tank) were treated with either feed supplemented with

240 Argovit-4 (1000 μg/g) or control feed (feed without Argovit-4) at 4% of their body weight twice

241 daily.

242

243 **Haemolymph sampling**

244 Shrimp haemolymph samples from each tank were collected in pools of three to obtain ~400

245 μL/shrimp every 0, 6, 12 and 48 h post-treatment. A 1 mL syringe (27 G×13 mm needle) was

246 used for rinsing with 5% potassium oxalate in isotonic saline (850 mOsm/kg) to obtain

247 haemolymph from the ventral sinus (base of the first abdominal segment (Apun-Molina *et al.*,

248 2017; Guemez-Sorhouet *et al.*, 2019). The pools of haemolymph samples were centrifuged at

249 800 x *g* for 10 min at 4°C, after which the plasma was removed and the pellet was washed with

250 250 μL of cold PBS centrifugation as described above. The supernatant was removed, and the

251 haemocytes were suspended in microtubes containing 200 µL of precooled TRIzol Reagent (TRI

252 Reagent®, Molecular Research Center, Ohio, USA). The haemocyte samples were stored at -

253 70°C until use.

254

Isolation of RNA and cDNA synthesis

256 Total RNA from the haemocyte and intestinal samples was extracted with TRIzol reagent

257 according to the manufacturer's protocol. The RNA was quantified at 260 nm using a NanoDrop

258 instrument (NanoDrop Technologies, Wilmington, DE) and then treated with DNase 1 (1 U/µL,

259 Sigma-Aldrich®, St. Louis, MO, USA) to remove residual genomic DNA. cDNA synthesis was

260 performed using the Improm II® Reverse Transcription System (Promega, Wisconsin, USA)

261 with 500 ng of total RNA and random primers from the kit. The obtained cDNA was suspended

262 in 80 µL of ultrapure water and stored at -70°C until analysis.

263

Quantitative real-time PCR analysis

265 The transcriptional responses of genes encoding immune-related proteins (PEN4, Crustin,

266 proPO, PAP and Rab6) from *L. vannamei* haemocytes were determined by quantitative real-time

267 PCR (RT-qPCR) using a CFX96 system and CFX Manager version 3.0 (Bio-Rad Laboratories,

268 Hercules, CA, USA). The expression of target genes was normalized to that of the *β-actin* gene

269 as an endogenous control. Table 2 shows the sequences of specific primers used in this study. All

270 amplifications were performed (in duplicate) in 15 µL volume reactions and contained 7.5 µL of

271 PCR master-mix 2× (3.0 µL of 5× Colorless GoTaq Flexi Buffer [Promega, Wisconsin, USA],

272 1.5 µL of 25 mM $MgCl_2$ [Promega], 0.3 µL of 10 mM dNTPs [Bioline, Taunton, MA, USA],

273 0.75 µL of 20× EvaGreen® [Biotium, Fremont, CA, USA], 0.1 µL of 5 U/µL GoTaq® Flexi

274 DNA Polymerase [Promega, Wisconsin, USA], and 1.85 µL of ultrapure water). Each master-

275 mix aliquot was mixed with 0.35 µL of each primer (10 µM, Sigma-Aldrich®), 1.8 µL of

276 ultrapure water and 5 µL of cDNA as template. The thermocycling program was as follows: an

277 initial cycle of 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 58°C for 20 s, 72°C for

278 30 s and 79°C for 5 s (to acquire the fluorescence signal). After each reaction, a dissociation

279 curve from 65 to 90°C was recorded at 0.5°C increments and examined for unique and specific

280 products. The Pfaffl method was used to analyse the relative quantification data from the real-

281 time PCR experiments (Pfaffl, 2003).

282

Statistical analysis

284 The percentage data of cumulative mortality (**Bioassays 2, 3 and 4**) were initially arcsine-
285 transformed to perform the statistical analysis. Normality and homogeneity of variances
286 assumptions were analysed by Kolmogorov-Smirnov and Levene's tests. Thereafter, Kruskal-
287 Wallis ANOVA test (non-parametric ANOVA) followed by a multiple comparison test was used
288 to determine significant differences between the experimental groups ($p<0.05$).
289 To determine the effect of Argovit-4 on the transcriptional response of five immune-relevant
290 genes (**Bioassay 5**), the data were Log10-transformed to confirm its normality before statistical
291 analysis. The data were analysed by Student's t-test to determine significant differences
292 ($p<0.05$). All bioassays were performed in triplicate and the results of cumulative mortality were
293 expressed as mean ± standard deviation. While, the immune-related gene expression results are
294 reported as the mean values ± standard error. Statistical analyses were performed using
295 STATISTICA version 8.0 (StatSoft, Tulsa, OK).

296

Results

298

A 7 SID_{50} inoculum is the minimum effective dose to generate 100% shrimp mortality

301 In the challenge bioassay, the three assayed doses of WSSV resulted in 100% shrimp mortality at
302 different time intervals. The shrimp group infected with 100 SID_{50} showed mortality after 30 hpi
303 with values above 3%, reaching 100% of mortality at 54 hpi. In contrast, the shrimp group
304 infected with 10 SID_{50} exhibited mortality after 36 hpi with values above 17%, reaching 100% at
305 60 hpi. Finally, in the shrimp group infected with 7 SID_{50}, mortality began after 36 hpi with
306 values above 13%, reaching 100% at 72 hpi. The control group (PBS) did not show mortality
307 during the bioassay (Fig. 1). The results revealed that 7 SID_{50} as the minimal dose capable
308 causing 100% of shrimp to be infected with conspicuous clinical signs (lack of appetite, erratic
309 swimming and atypical coloration).

310

Argovit-4/WSSV decreased the mortality of shrimp

312   The shrimp group treated with 1000 ng/SID$_{50}$ showed the lowest mortality (50%) at 60 hpi,

313   whereas the shrimp groups treated with 40, 100 and 200 ng/SID$_{50}$ of Argovit-4 showed

314   mortalities of 90, 83 and 70% at 60 hpi, respectively. The positive control showed 100%

315   mortality at 72 hpi compared with 0% in the control group. Statistical analysis revealed that the

316   susceptibility of the shrimp to WSSV mixed with Argovit-4 significantly decreased (Kruskal-

317   Wallis test; p=0.0169) in a dose-dependent manner across the groups (Fig. 2).

318

319   **Argovit-4 supplemented in feed is non-toxic**

320   None of the assayed Argovit-4 doses supplemented in feed caused significant shrimp mortality

321   (Kruskal-Wallis test; p=0.0000) and not affect the health or survival of shrimp, given that they

322   did not show atypical behaviour (evaluated by observations for empty guts, lethargy, and erratic

323   movement) by toxicity effects during the course of the bioassay at 192 h post-treatment.

324

325   **Argovit-4 supplemented in feed has an antiviral effect against WSSV**

326   The shrimp group exposed to infected WSSV tissue (without Argovit-4) showed a mortality of

327   43% at 84 hpi. The cumulative mortality in shrimp fed the 1000 µg/g doses was 3% after

328   infection with WSSV at 192 h, followed by 10 and 16% for the 100 and 10 µg/g doses,

329   respectively. In contrast, the uninfected control group did not show mortality. Interestingly,

330   statistical analysis (Kruskal-Wallis test; p=0.0001) did not reveal a dose-dependent effect (Fig.

331   3). However, the results revealed an obvious antiviral effect with both doses assayed.

332

333   **Argovit-4 does not induce an early immunostimulatory effect for immune-related**

334   **genes**

335   The expression analysis of genes encoding Crustin, PEN4, PAP and Rab6 showed no significant

336   (p<0.05) changes in the haemocytes of shrimp treated with Argovit-4 supplemented in feed with

337   respect to the control (Fig. 4A, 4B; 5A, 5B). Interestingly, only the gene encoding proPO was

338   downregulated at 48 h (t-student test; p= 0.024), which was possibly an indirect effect of low

339   levels of AgNPs in the haemolymph (Fig. 6).

340

341   **Discussion**

342   Several studies have evaluated the effectiveness of AgNPs in aquatic diseases control because to

343   their antimicrobial potential against bacteria, virus and parasites (Barakat, El-Sayed & Gohar,

344   2016; Morales-Covarrubias *et al.*, 2016; Acedo-Valdez *et al.*, 2017; Pimentel-Acosta *et al.*,

345   2019). However, this is the first work that examine the efficacy of Argovit-4 (AgNPs-based

346   formulation) as a potential antiviral additive in feed to protect the health of shrimp *L. vannamei*

347   against WSSV.

348   In order to evaluate the protective effect of AgNPs on shrimp against WSSV, firstly, we

349   determinate that 7 SID_{50} was the minimum effective dose to generate 100% shrimp mortality

350   according to WSSV inoculum used (**bioassay 1**) and then, we preincubated infectious WSSV

351   inoculums (calibrated to 7 SID_{50}) with Argovit-4 at different concentrations, which were

352   subsequently intramuscularly injected in shrimp (**bioassay 2**). Our results indicated that WSSV

353   infectivity is drastically affected due to a delay in the onset of clinical signs and mortality in a

354   manner dose-dependent ($p<0.05$) (Figure 2), compared with that observed in the control (WSSV

355   without Argovit-4). Some authors have observed that silver nanoparticles interaction with

356   envelope glycoproteins of virus can inhibit post-entry stages during replication cycle (Rogers *et*

357   *al.*, 2008; Lara *et al.*, 2010; Borrego *et al.*, 2016). Elechiguerra *et al.* (2005) suggested that

358   AgNPs undergo specific interaction with HIV-1 due to their affinity to disulfide bonded regions

359   (thiol groups provided in viral envelope protein domains). While, Lara *et al.* (2010) postulated

360   that silver nanoparticles not only bind to the viral protein, but can also structurally modify it

361   through the denaturing of disulfide-bound domain. Hence, we hypothesize that AgNPs could

362   interact with WSSV envelope proteins (e.g. VP28; Tang et al., 2007) and block the specific

363   binding to membrane proteins of shrimp host cells. This can be seen in our results of the ability

364   of AgNPs to decrease residual infectivity of viral particles after 60 minutes of incubation, as

365   described by Lara *et al.* (2010). Nevertheless, further research is needed to elucidate the antiviral

366   mechanism of silver nanoparticles against WSSV.

367   Recently, Juarez-Moreno *et al.* (2017) reported that prophylactic doses 50 ng/g Argovit®

368   (AgNPs-based formulation) administered by intramuscular route in infected shrimp with WSSV

369   evinced a rate survival of 80% without toxic effects after 96 h post infection. While, Ochoa-

370   Meza *et al.* (2019) showed evidence that a single therapeutic dose 1.2 µg/g AgNPs can delay the

371   onset of clinical signs and enhance the response of shrimp immune system without toxic effects

372   in healthy shrimps. However, this results are not comparable with Juarez-Moreno *et al.* (2017)

373   and Ochoa-Meza *et al.* (2019) in effectiveness, due to the different: (1) AgNP preparations used,

374 (2) weights of the shrimp and, (3) routes of exposure; considering that intramuscular

375 administration is impractical in aquaculture due to the enormous quantity of organisms that are

376 need to be treated (Kumar & Roy, 2017).

377 The administration by feeding represents the most practical prophylactic or therapeutic method

378 for *in situ* shrimp culture (Alvarez-Sanchez *et al.*, 2017). Therefore, we prepared feed

379 supplemented with Argovit-4 at 10, 100 and 1000 µg/g doses and determined their effects on

380 shrimp health or mortality (**bioassay 3**). Our results showed no obvious toxicity effects on the

381 behavioural activity of shrimp in the form of erratic swimming, changes in feeding rate, atypical

382 body colour body, and increased mortality (1 shrimp death) in all treatments by the oral route.

383 The lack of mortality and clinical signs can be explained by the structural design of Argovit®

384 nanoparticles having been approved by sanitary international organizations for their use in

385 veterinary and medical applications (Vazquez-Muñoz *et al.*, 2014; Bogdanchikova *et al.,* 2016;

386 Borrego *et al.*, 2016).

387 Despite the potential of AgNPs as antimicrobial agent, in aquaculture industry, concerns remain

388 regarding the potential negative impact to the marine and freshwater environment. However, to

389 date, there have not been clear the *in vivo* toxicity of silver nanoparticles in marine environment,

390 as the effects depend on AgNP properties (particle size, surface area, shape), concentration,

391 AgNP colloidal stability, aggregation grade, sedimentation, and on the amount and species of

392 products yielded from chemical interactions between AgNPs and other variables (Fabrega *et al.,*

393 2011; Sharma *et al.*, 2019; Pimentel-Acosta *et al.*, 2019),

394 It is important to clarify that Argovit-4, given its recent design, still does not present toxicity

395 reports with which this AgNP should be better studied in terms of its safety if it is intended to

396 apply to animals for human consumption and in turn study bioaccumulation in the same shrimp

397 and in the ecotoxicological environment. We have presented in this research some results of

398 AgNP exposure considering only the acute toxic response, that is, in the short term. This is

399 because the antiviral activities observed are a consequence of the application of a single dose.

400 The main reason why a chronic toxicological study was not performed is precisely because of the

401 observed antiviral effect. Fabrega *et al.* (2011), points out that invertebrates such as water flea

402 *Daphnia pulex*, acute and chronic toxicity studies do not generate bioaccumulation, although

403 other authors point out that in the event that AgNPs accumulate in the gills, they do not have

404 harmful side effects (Xiang *et al.,* 2019), however, this could dependent of administered doses

405 (Monfared *et al.,* 2015). It is important to mention that more experiments should be carried out to

406 consider the possibility of applying this nanoparticular design in the field. For example,

407 bioassays that generate enough information to evaluate the bioaccumulation of flora and fauna

408 accompanying the bodies of water where the shrimp are raised.

409 To determine the protective properties of Argovit-4 supplemented in feed against WSSV, the

410 results of **bioassay 4** indicate that the 1000 µg/g treatment provided the best protective effect in

411 shrimp against the inoculum of WSSV by the oral route than by injection (Fig. 3). This result

412 was probably due to the high bioavailability of the silver nanoparticles in the digestive tract,

413 which allowed the AgNPs-virus interactions to improve and promote entry by clathrin-mediated

414 receptor routes (Coutiño, Ávila-Lagunes & Arroyo-Helguera, 2017). This is the first study that

415 describes the use of AgNPs as an antiviral additive in feed against WSSV. In recent years, the

416 use of metallic nanoparticles in aquaculture has been increased, for example in fish. For *Clarias*

417 *gariepinus*, selenium nanoparticles (SeNPs) at a 680 µg/g dose was observed to improve feeds

418 by increasing the proportion of fish food nutrients that pass across the gut tissue (Chris, Sing &

419 Agarwal, 2018). For *Clarias batrachus*, it was also shown that iron nanoparticles (FeNPs) at a

420 400 µg/g dose is sufficient to ensure the growth and health of fish (Akter *et al.*, 2018).

421 Previous studies have demonstrated that because metallic nanoparticles are foreign elements to

422 organisms, they can be recognized and internalized by immune system cells through different

423 routes according to their size and shape (Jiang *et al*., 2008*;* Coutiño, Ávila-Lagunes & Arroyo-

424 Helguera, 2017; Dacoba *et al*., 2017; Boraschi *et al*., 2017). To test this possibility, and to

425 determine the immunostimulatory effect in the early stages by assessing the expression of

426 immune-relevant genes, **bioassay 5** was performed in which shrimp were fed Argovit-4 at a

427 1000 µg/g dose due to it being the therapeutic dose that best-protected shrimp against WSSV.

428 Five genes encoding immune-related proteins were examined at different time intervals in

429 shrimp haemocytes (PAP, Rab6, Crustin, PEN4, and proPO) by RT-qPCR analysis. PAP

430 (Phagocytosis-Activating Protein) and Rab6 have been shown to be associated with phagocytosis

431 in shrimp immune response (Deachamag *et al*., 2006; Zhao *et al*., 2011; Ye, Zong & Zhang,

432 2012; Ye, Tang & Zhang, 2012; Soto-Alcalá *et al.,* 2019). Crustin and PEN4 are antimicrobial

433 peptides (AMPs) that participate in the humoural defence against pathogens or foreign particles

434 (Wang *et al*., 2010; Hirono *et al*., 2011), while proPO is an important humoural effector in innate

435 immunity, catalysing the upstream steps of melanization in shrimp and others invertebrates

436   (Gollas-Galván, Hernández-Lopez & Vargas-Albores, 1999; Wang *et* al., 2010; Amparyup,

437   Charoensapsri & Tassanakajon, 2013). The expression of the genes encoding PAP, Rab6,

438   Crustin, and PEN4 in the treated shrimps showed no significant differences in the time intervals

439   proposed with respect to the control (feed without Argovit-4). The expression of the gene

440   encoding phagocytosis activating protein was not activated in samples from the silver

441   nanoparticle-treated shrimp compared to that observed in the control shrimp samples, suggesting

442   that the size of the particles is important, since this parameter facilitates their incorporation and

443   internalization in haemocytes. This observation is reinforced by the same observed behaviour of

444   the Rab6 gene, which participates in the endocytic pathway (Soto-Alcala *et al*., 2019), since its

445   expression was not altered at any time by the silver nanoparticles. On the other hand, peneaidins

446   are a family of antibacterial proteins that have recently been shown to contribute to the arrest of

447   WSSV-type viral particles. However, the expression of the PEN4 gene was not altered with

448   respect to the time recorded from zero to 48 hours, which indicates that the AgNPs do not

449   interact with the effectors of this gene. The same pattern was observed for the expression of the

450   gene encoding Crustin, which has been shown to be activated in the first hours of WSSV

451   infection (Xiao *et al*., 2018; Sun, Wang & Zhu, 2017). Taken together, the patterns of the four

452   assayed genes encoding PAP, Rab6, PEN3 and Crustin strongly suggests that the antiviral effect

453   of the nanoparticles occurs by a different pathway than antibacterial peptides or endocytic

454   phagocytosis.

455   The proPO system is one of the most studied in arthropods, where particles generated by cell

456   destruction and attacks by pathogenic organisms are coated by melanin via the oxidation of

457   phenols by the enzyme phenoloxidase, which are subsequently secreted as melanized corpuscles

458   (Amparyup, Charoensapsri & Tassanakajon, 2013). The proPO gene encodes the phenoloxidase

459   precursor enzyme, with *L. vannamei* encoding LvproPO1 and LvproPO2 (Wang *et al*., 2010).

460   Interestingly, proPO has been shown to be active during WSSV infection (Abad-Rosales *et al.,*

461   2019). In this study, proPO gene expression exhibited a significant decrease at 48 h compared

462   with that observed in the control. Several authors have reported that during infection by WSSV,

463   the virions are not covered by melanin due to the participation of at least two viral proteins

464   identified in *P. monodon*. wsv164 interacts directly with PmproPO1 and PmproPO2 (Sangsuriya

465   *et al*., 2018), probably for an alternative route of infection. Similarly, the WSSV453 protein

466   interacts with the proPO activating enzyme 2 (PmPPAE2), which could explain the observed

467   decrease in the expression of the proPO gene due to the presence of AgNPs, taking into account

468   that no challenge against WSSV was performed in this experiment (Sutthangkul *et al.*, 2015;

469   Sutthangkul *et al.*, 2017). Taken together, these results suggest that Argovits-4 does not induce

470   the expression in of the assayed early stage immune-related genes, suggesting that the antiviral

471   activity or immunostimulation by AgNP involves a different route to the endocytic pathway. A

472   previous study by our group (Ochoa-Meza *et al.*, 2019) reported that the LGBP and Cu,ZnSOD

473   genes are negatively deregulated by the presence of AgNPs (Argovit®), which suggested that

474   there must be a path for the internalization of the nanoparticles and the activation of the immune

475   system of haemocytes. However, such a path does not require the participation of any of the five

476   genes analysed in this study.

477

Conclusions

479   In this study, we explored whether a new AgNP formulation (Argovit-4), which replaced

480   Argovit®, has similar properties to protect and immunostimulant shrimp, allowing them to avoid

481   infection by the white spot virus without causing toxic effects. In addition, we assessed if the

482   expression of some genes immune system-related suggested a common means of activation.

483   1. Decreased or high concentrations of Argovit-4 nanoparticles do not generate mortality by

484   causing toxicity towards shrimp.

485   2. The mixture of WSSV-Argovit-4 was capable of decreasing the mortality of shrimp by at

486   least 50% by intramuscular administration.

487   3. Argovit-4 included in feed could be used to prevent infection by WSSV and can be

488   considered a safe candidate to safeguard aquaculture systems in the future.

489   4. The zero expression of the PAP, Rab6, PEN4 and Crustin genes, suggest that the antiviral

490   activity of Argovit-4 does not include any of the mechanisms by which such genes

491   perform their function in the immune system of *L. vannamei*.

492   5. The late down regulation of proPO gene expression, suggests that the antiviral activity of

493   Argovit-4, could to modify a different and unknown route to inhibit a WSSV infection.

494

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506

Competing interest
508 The authors declare that there are no competing interests

509

Author contributions
511 Romo Quiñonez CR. PhD student. Performed all of the experiments.

512 Alvarez Sanchez AR. Participated in the experimental design and the discussion of the results.

513 Alvarez Ruiz P. Participated in the design and execution of the experiments and advised on the

514 analysis of molecular techniques. Co-director of the PhD student.

515 Chavez Sanchez MC. Leader of the global project and participated in the discussion of results

516 and the draft of the paper.

517 Bogdanchikova N. (nina@cnyn.unam.mx) Provided the AgNPs and participated in the writing of

518 the paper, providing information regarding the chemical properties of Argovit-4.

519 Pestryakov A. Designed the new AgNPs (Argovit-4) and provided important data for its

520 management.

521 Mejia Ruiz CH. Project leader in the management of AgNPs for their application in shrimp.

522 Participated in the experimental design, analysed results and the final manuscript. Principally

523 responsible for the paper and co-director of the PhD student.

524

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735

Figure 1

WSSV-Inoculum title

Cumulative mortality of shrimp *L. vannamei* injected with three different WSSV doses by intramuscular route. Shrimps infected with 100, 10 and 7 SID. (f) Control negative shrimp treated with PBS. The results are presented as the mean ± SD.

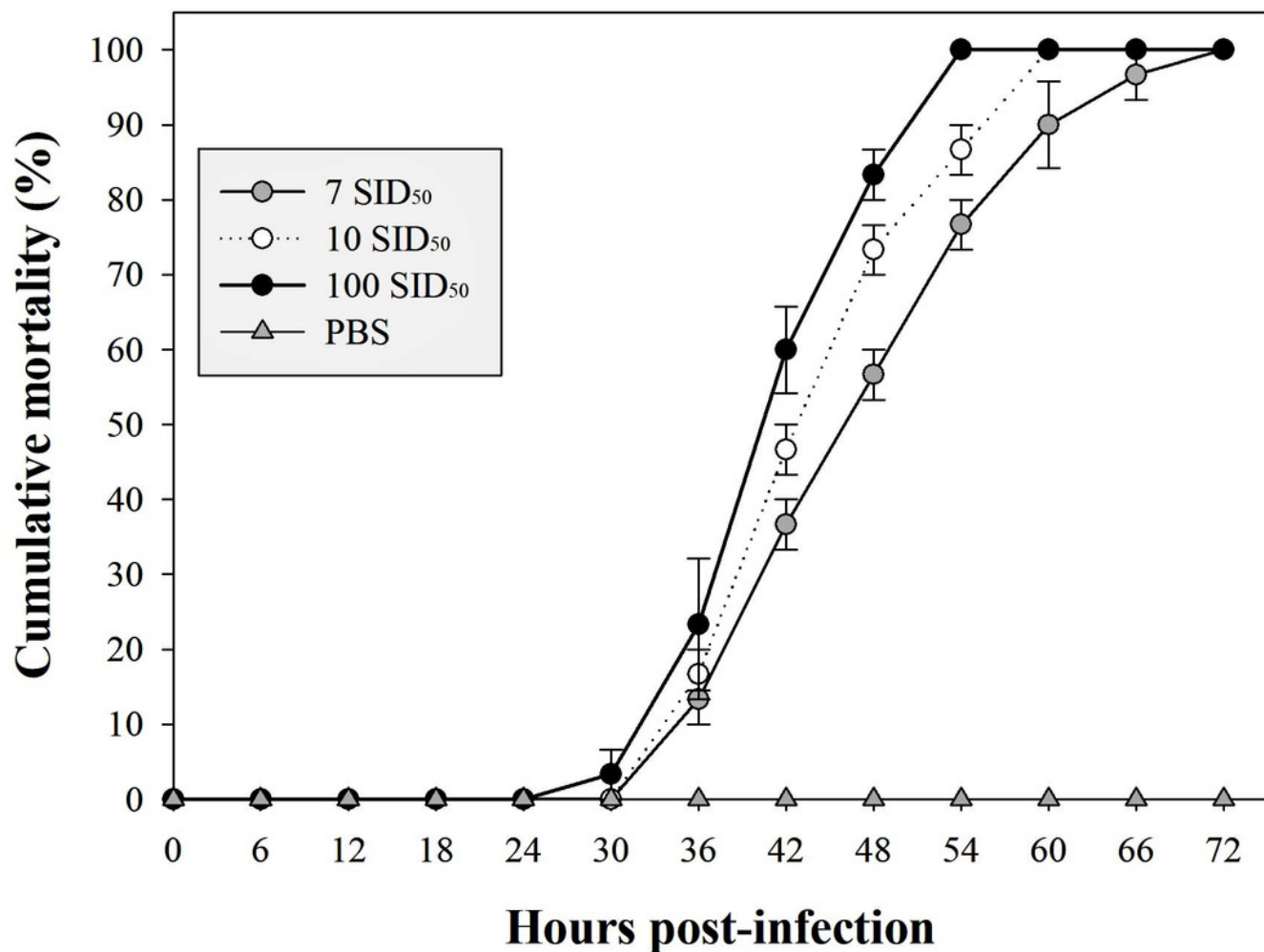



Figure 2

Effect of mixture Argovit-4/WSSV of different doses injected by intramuscular route

Cumulative mortality of *L. vannamei* juveniles: 40, 100, 200, 1000 ng/(7)SID. (Control −) shrimp treated with PBS and were not infected; (Control +) shrimp treated with PBS and infected with WSSV inoculums. Statistical differences are represented by different letters (p<0.05). Points are presented as the mean ± SD.

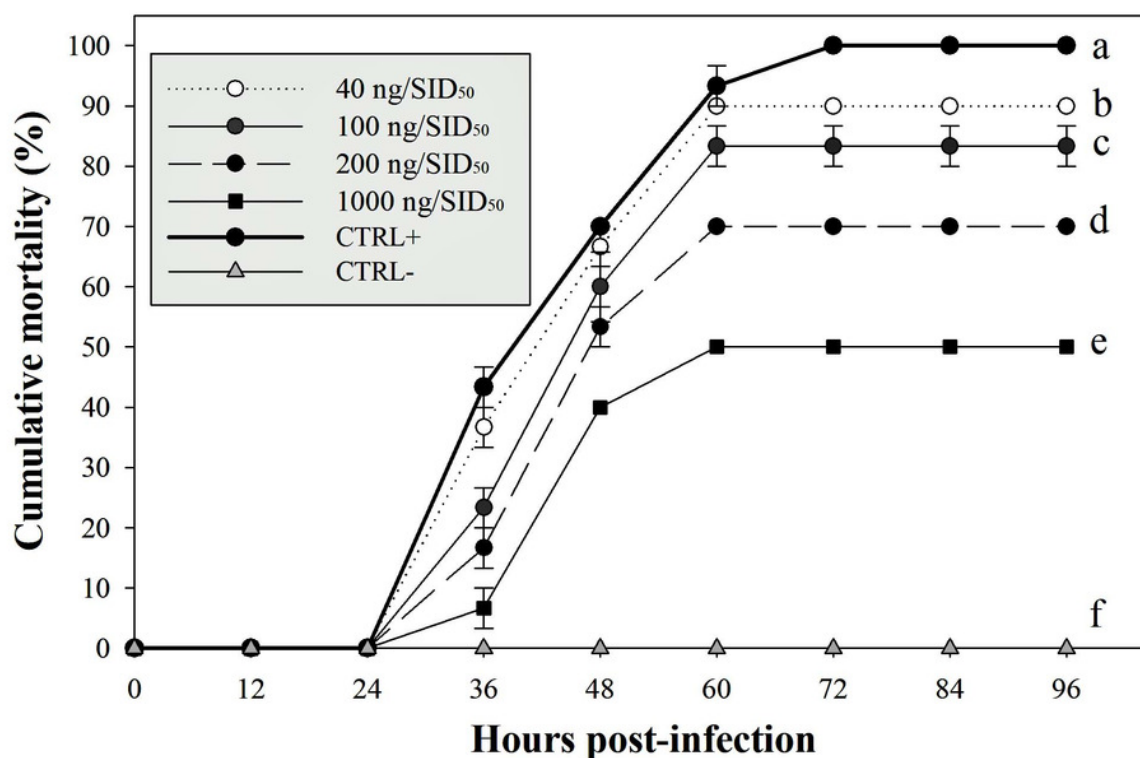



Figure 3

Effect of Argovit-4 supplemented in feed at different doses on cumulative mortality of *L. vannamei* juveniles

Shrimp nourished with Argovit-4 supplemented feed with concentration of silver: 10, 100, 1000 µg/g. (Control +) shrimp fed only with WSSV inoculum. (Control -) shrimp fed with feed lacking Argovit-4 additive. Procedure sequence: at 0 h the feeding with Argovit-4 supplemented feed was executed for the first time; at 12 h feeding with Argovit-4 supplemented feed was performed for the second time and at 24 h feeding with WSSV inoculum (200 mg infected tissue per shrimp) was performed. After that, every 12 h the shrimps were fed with commercial feed. Statistical differences are represented by different letters (p<0.05). The results are presented as the mean ± SD.

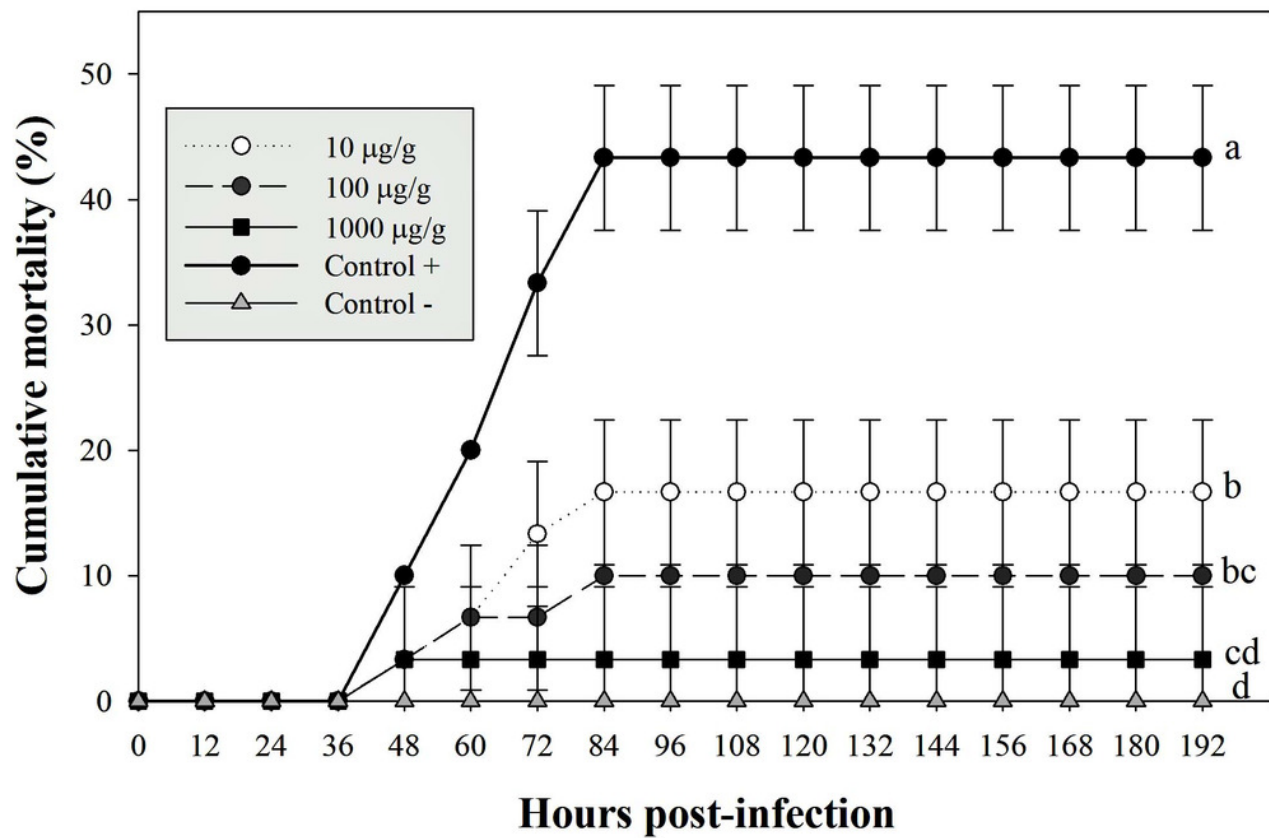



Figure 4

Relative expression of PEN4 and Crustin genes

The graphic show the RT-qPCR of; (A) Relative expression of PEN4 gene with no significant differences between AgNP treatment (black box) and control (white box); (B) Relative expression of Crustin gene, also no significant differences between AgNP treatment (black box) and control sample (white box). The data of the statistical analysis are represented in mean ± standard error.

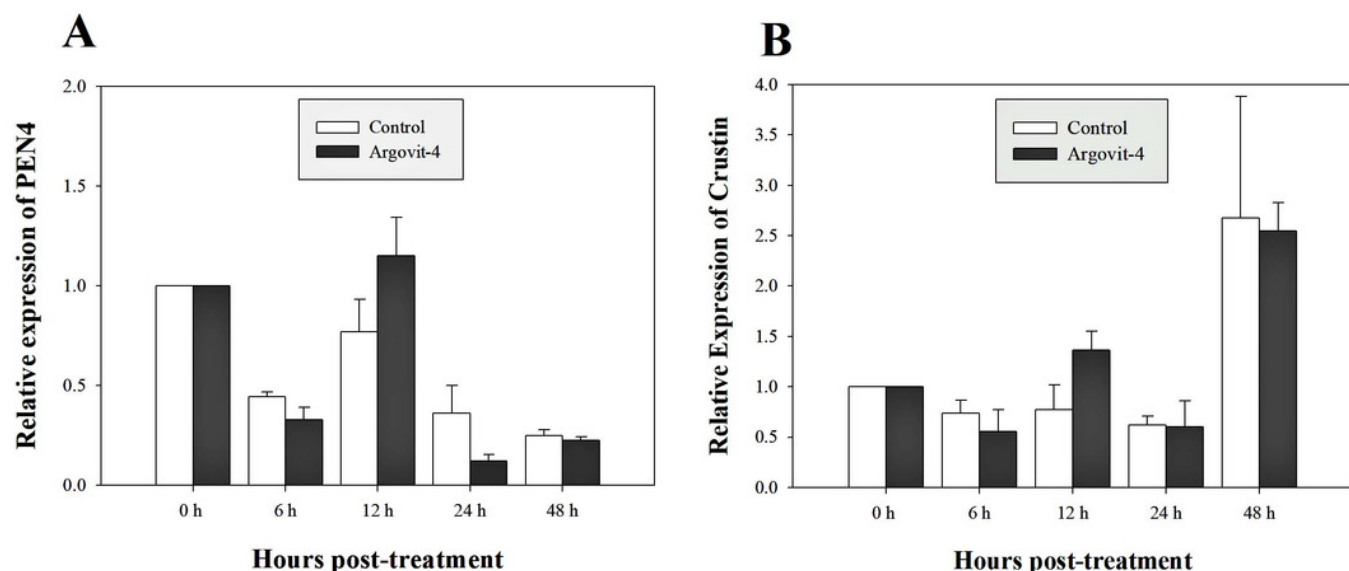



Figure 5

Relative expression of Rab6 and PAP genes.

The graphic show the RT-qPCR of; (A) Relative expression of Rab6 gene with no significant differences between AgNP treatment (black box) and control (white box); (B) Relative expression of PAP gene, also no significant differences between AgNP treatment (black box) and control sample (white box). The data of the statistical analysis are represented in mean ± standard error.

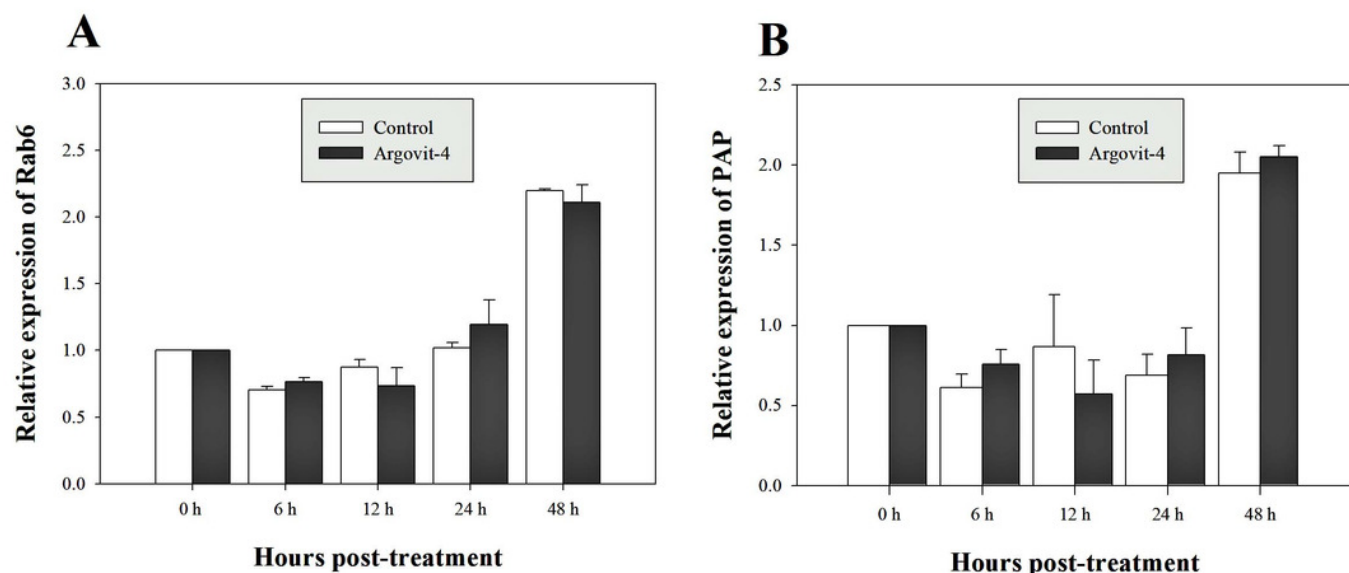
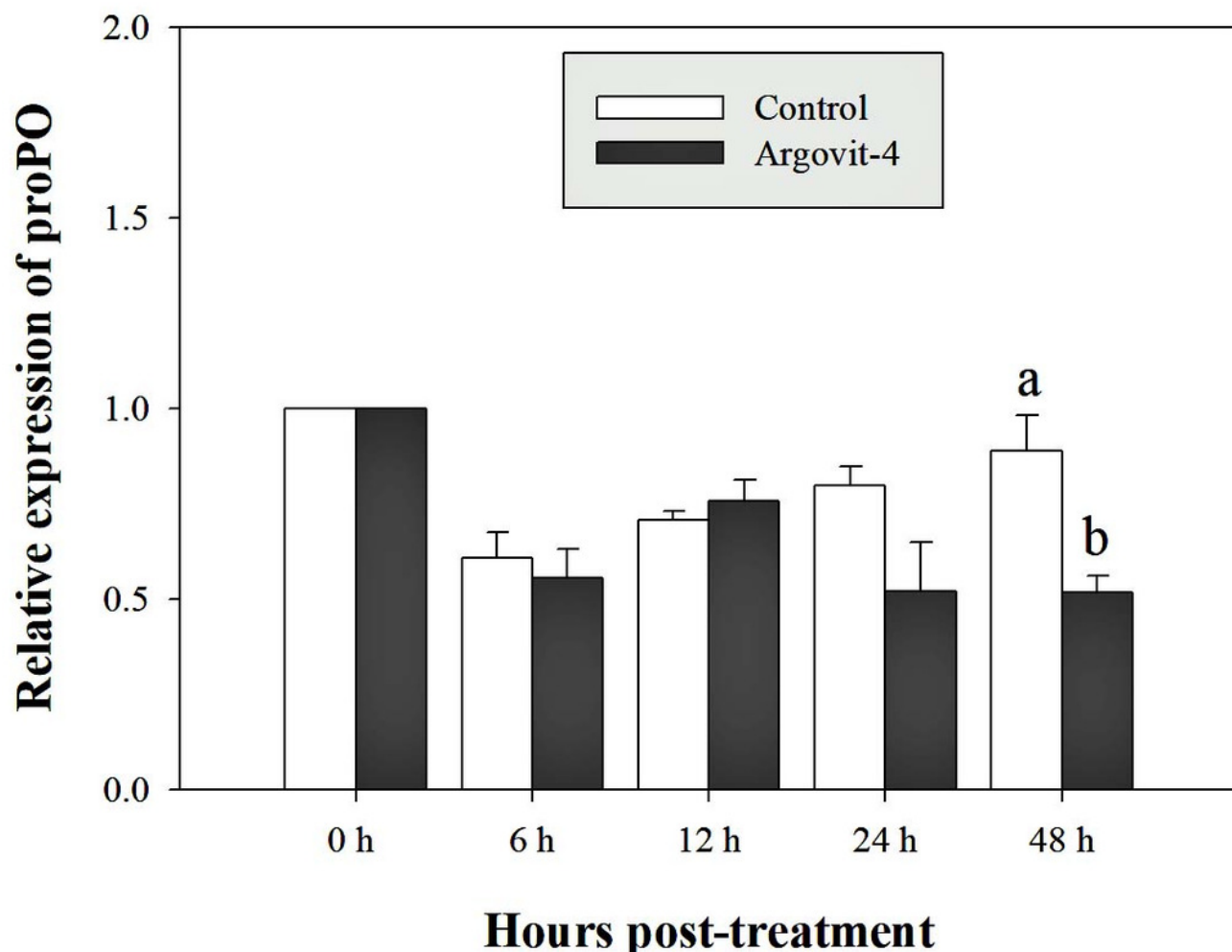



Figure 6

Relative expression of proPO gene

The graphic show the RT-qPCR of the relative expression of proPO gene with no significant differences between AgNP treatment (black box) and control (white box) only at 48 hrs. The data of the statistical analysis are represented in mean ± standard error (Analysis test in text).

Physicochemical Characteristics of silver nanoparticles

Physicochemical Characteristics of silver nanoparticles modified to Argovit-4 from Juarez-Moreno et al., 2017.

Properties	Mean
K- value	K30
PVP content	18.8 (% wt.)
Metallic silver content	1.2 (% wt.)
Silver nanoparticle morphology	spheroidal
Average diameter of metallic silver particles by TEM data (nm)	35±15
Size interval of metallic silver particles by TEM data	1 to 90 (nm)
Hydrodynamic diameter: metallic Ag with PVP	70 (nm)
Zeta potential	-15 (mV)
Surface plasmon resonance	420 (nm)
PVP structure by FTIR	Confirmed

Specific primers used

Specific primers used for PCR amplifications of immune system-related genes.

Genes	Primer sequences (5′-3′)	Amplification Size (bp)	Reference
ProPO	Fw: CTGGGCCCGGGAACTCAAG Rv: GGTGAGCATGAAGAAGAGCTGGA	125	Soto-Alcalá et al., 2018
PEN 4	Fw: GCCCGTTACCCAAACCATC Rv: CCGTATCTGAAGCAGCAAAGTC	106	Wang et al., 2010
PAP	Fw: CGAAGTTCAGGTTGTGCGTG Rv: ACTGATGCACCATTGGCCTT	126	Soto-Alcalá et al., 2018
Rab 6	Fw: GTTCCGCAGCCTTATTCCCT Rv: ATCACTGCCTCGCTCTGTTC	133	Soto-Alcalá et al., 2018
Crustin	Fw: GAGGGTCAAGCCTACTGCTG Rv: ACTTATCGAGGCCAGCACAC	157	Wang et al., 2010
β-Actin	Fw: CCACGAGACCACCTACAAC Rv: AGCGAGGGCAGTGATTTC	142	Wang et al., 2007