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Potential application of silver nanoparticles to control the infectivity of Rift Valley fever virus in vitro and in vivo

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Abstract

In this work we have tested the potential antiviral activity of silver nanoparticles formulated as Argovit™ against Rift Valley fever virus (RVFV). The antiviral activity of Argovit was tested on Vero cell cultures and in type-I interferon receptor deficient mice (IFNAR $^{-/-}$ mice) by two different approaches: (i) different dilutions of Argovit were added to previously infected cells or administrated to animals infected with a lethal dose of virus; (ii) virus was pre-incubated with different dilutions of Argovit before inoculation in mice or cells. Though the ability of silver nanoparticles to control an ongoing RVFV infection in the conditions tested was limited, the incubation of virus with Argovit before the infection led to a reduction of the infectivity titers both *in vitro* and *in vivo*. These results reveal the potential application of silver nanoparticles to control the infectivity of RVFV, which is an important zoonotic pathogen.

Key words: Silver nanoparticles; Antivirals; Rift Valley fever virus; *In vivo* infection

Rift Valley fever virus (RVFV) is a mosquito-borne pathogen causing an important disease in ruminants often transmitted to humans after epizootic outbreaks. In humans, RVFV manifests as an influenza-like disease, but occasionally leads to more serious complications with high morbidity and mortality. For many decades the disease has been confined to Sub-Saharan Africa but over the last years a spectacular increase in the number of outbreaks, including a more northward geographic spread, with cases reported in Egypt, the Arabian Peninsula and some Indian Ocean islands (Madagascar, Comoros, and Mayotte) confirms the potential of this disease to emerge virtually worldwide.[1,2]

Currently there is no available treatment or licensed Rift Valley fever vaccine for human use; therefore to help combat the spread of the disease prophylactic and/or therapeutic measures need to be developed. The development of effective control strategies, including new antivirals, is a quite active field of research. Experimental antiviral drugs already known to control other viral infections, such as ribavirin and favipiravir, have proven to be efficient also in controlling RVFV infection in animal models.[3,4] Also type-I Interferon (IFN-α/β)-related treatments such as delivery of IFN-α/β itself, or molecules known to be strong IFN-α/β inducers, have shown antiviral potential.[5-7] However these antiviral approaches focused for human treatment are currently restricted to the experimental field.

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Silver has been known for long time to have potent anti-microbial effects, and different formulations of silver nanoparticles have already been reported to display antiviral activity against several viruses belonging to different families,[8] and the number and type of viruses described that are sensitive to this treatment keep growing.[9,10] However, the mechanisms underlying this activity are not yet understood. Compared to the aforesaid antiviral approaches, the use of metal nanoparticles poses many advantages, for instance the non-emergence of resistant variants, as well as its safety and low price. Furthermore, anti-inflammatory and immunomodulatory effects of silver nanoparticles have been also described.[11] Argovit ™ is a commercial formulation of silver nanoparticles already shown to possess a broad spectrum of antimicrobial activity as well as apparent anti-inflammatory effect.[12-14] In this work we have tested the antiviral potential of silver nanoparticles formulated as Argovit™ against RVFV infection, both in cell culture and in a mouse infection model. Our results reveal the potential application of silver nanoparticles to control the infection of this important zoonotic pathogen.

Methods

Silver nanoparticles preparations

Commercial formulation of silver nanoparticles Argovit™ was provided by Vector-Vita Ltd (Russia). Argovit (12 mg/mL metallic silver) consists of spherical silver nanoparticles of 35 ± 15 nm functionalized with poly(vinylpyrrolidone) (PVP, 10-30 kD).[13,15-17]

Viruses and cells

All studies were performed using the MP12 attenuated strain of RVFV on Vero cell monolayers following procedures already described.[18]

Cell viability assays

Vero cells were seeded in M96-multiwell plates, and 24 h later, when 80% confluence was reached, Argovit was added to the medium at serial two-fold dilutions, in quadruplicate. Vero cell viability was tested in two different conditions mimicking the procedures of further viral infection assays. In long exposure experiments, Argovit (from dilution 1/625) was kept in the culture medium all along the time analyzed; in short exposure experiments, designed to mimic virus adsorption, cells were exposed to Argovit-containing medium (from dilution 1/500) for only one hour, then this medium was removed and replaced with fresh one. In both cases, cells were incubated at 37 °C and daily checked at the microscope. Viability of Vero cells after the different treatments at the times indicated was checked by the MTS *Cell Proliferation Assay* (Promega) following manufacturer's instructions.

In vitro infection experiments

For "long-exposure" infections, semi-confluent Vero cells on MW24 plates were infected with RVFV-MP12 at a multiplicity of infection (moi) of 0.5 plaque-forming units (pfu) per cell, and after 1 h of adsorption at 37 °C the inoculum was removed and replaced with fresh medium containing Argovit at dilution 1/5000 and 1/10000. Controls corresponding to 100% infectivity (infection in the absence of Argovit) as well as 100% viability (non-infected cells in medium containing Argovit) were also included. Assays were performed in triplicate. Development of cytopathic effect (cpe) as well as normal cell growth was checked daily at the microscope. At the indicated times post-infection (24, 48 and 72 h) supernatants were collected and further titrated in a plaque assay on Vero cells as described.[18] For "short-exposure" experiments, a small fixed amount of virus (about 30-300 pfu) was incubated with rotation for 1 h at 4 °C with serial two-fold dilutions of Argovit beginning from 1/1000. Identical input of virus was pre-incubated in the same conditions with medium alone to determine 100% of infectivity. After incubation each mix of virus and Argovit was inoculated by triplicate onto Vero cells grown in MW6 plates. After 1 h of adsorption the inoculum was removed, the cells were washed and semi-solid medium with agar was added. Plates were incubated until infection plaques were clearly developed, then fixed and crystal violet stained.

Animals and ethics statement

8 to 10 week-old transgenic 129Sv/Ev IFNAR$^{-/-}$ mice (B&K Universal) were used in the experiments described here. During the course of the experiment all mice were housed in a BSL-3 containment area with food and water supplied *ad libitum*. All experimental procedures were approved and supervised by the Biosafety and Bioethics Committees from Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (National Research Institute for Agricultural and Food Technology, INIA), following regulatory guidelines from the European Community Council Directive 86/609/EEC.

RVFV infection of mice

Groups of 5 IFNAR$^{-/-}$ mice were inoculated by the intraperitoneal (i.p.) route with 10^4 pfu of RVFV-MP12 strain, either alone or preincubated with serial dilutions of Argovit. As described in the text, at the indicated times after infection, Argovit was administrated at the dose, route and schedule indicated in the corresponding experiment. Clinical signs including ruffled fur, hunched posture, reduced activity or conjunctivitis were monitored for days 10-15 post infection and scored from 1 to 4 respectively depending on their severity to determine the clinical score. Moribund animals were euthanized.

To monitor viremia, blood samples were taken at 24, 48 and 72 h after RVFV infection, prior to Argovit administration, and tested for virus isolation on cell culture as described.[18] Viral load is expressed as log10 of the infective dose 50 ($TCID_{50}$) per microliter of blood. In some cases, serum samples collected at later times post-infection were analyzed for the presence of neutralizing antibodies as described.[18]

Results

Analysis of the antiviral activity of Argovit against RVFV on cultured cells

Effect of Argovit on Vero cells viability

In order to determine the efficacy of Argovit against RVFV-MP12 *in vitro*, we first analyzed its cytotoxicity on

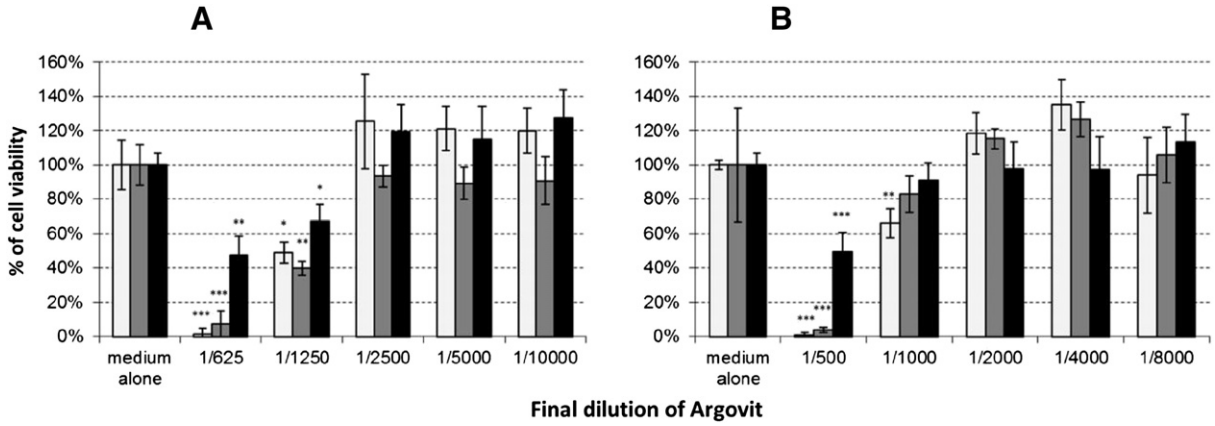

Figure 1. Effect of Argovit on viability of Vero cultured cells. Serial two-fold dilutions of Argovit were added in quadruplicate to the medium of Vero cells seeded in M96-multiwell plates. Viability at 24 (white bars), 48 (grey bars) and 72 h (black bars) was checked by the MTS *Cell Proliferation Assay* by Promega and calculated as described in Methods. (**A**) Long exposure: Argovit was kept in the culture medium all along the time analyzed. (**B**) Short exposure: cells were exposed to Argovit-containing medium for only one hour; then the medium was removed and replaced with fresh one. Those values rendering statistically significant differences when compared to the corresponding 100% of cell viability (by unpaired *t* test) are indicated. (*) $P < 0.05$; (**) $P < 0.005$; (***) $P < 0.0001$.

cultured Vero cells. Cells were seeded in M96-multiwell plates and Argovit was added to the medium at serial two-fold dilutions, in quadruplicate. For a more accurate reproduction of the specific conditions for the viral assays to be performed later, Vero cell viability was tested in two different conditions: after (i) long or (ii) short exposure to Argovit, as detailed in Methods, beginning from dilutions 1/625 or 1/500 respectively. These slight differences in the initial dilution were due to the particular procedure associated to the corresponding viral assay. Results are shown in Figure 1. Panel *A* shows cell viability after long exposure to Argovit, while panel *B* shows the results when cells were exposed to Argovit for only one hour, then further cultured in fresh Argovit-free medium. When cells were growing in the presence of Argovit over 24 h, high viability values were reached only for Argovit dilutions over 1/2500 (Figure 1, *A*). As expected, short-time exposition to Argovit was less toxic than longer exposition (Figure 1, *B*). In this case, high viability values were reached for a 1/1000 dilution, i.e., about 2.5-fold higher than long exposure. Since Vero cells must keep their viability in order to support viral infection, the minimal dilution of Argovit selected for further infection assays should be higher than 1/2500 for long exposure experiments and 1/1000 for short exposition experiments. These values correspond to 4.8 and 12 μg/ml of metallic silver, respectively.

Addition of Argovit to infected cells

Once the toxicity of Argovit for Vero cells had been determined, we analyzed its effect on virus infection. First, we wanted to determine whether the addition of Argovit to already infected cells was able to reduce to some extent the final viral production. For this purpose Argovit dilutions 1/5000 and 1/10000 selected on the basis of the previous cytotoxicity results (Figure 1, *A*), were added to Vero cells infected with RVFV so that infection proceeded in the presence of silver nanoparticles. At 24, 48 and 72 h post-infection (pi) supernatants were collected and titrated in order to determine virus production at the different conditions assayed. Virus titers obtained in infections occurring in the presence of Argovit were compared to those reached in medium alone for the corresponding time pi analyzed. Results are shown in Figure 2. Although addition and maintaining of Argovit to infected cell cultures did not totally abolish viral production, infection was controlled to some extent since a reduction of about 50% in the final virus production was observed in all conditions tested when compared with infection occurring in the absence of silver nanoparticles. Although this reduction in viral yield was repeatedly observed, differences were not statistically significant.

Incubation of RVFV with Argovit before infection

On the other hand, we also analyzed whether preincubation of virus with silver nanoparticles could have some effect on the infectivity of RVFV, as reported for other viruses.[19,20] A small fixed amount of virus (about 30-300 pfu) was preincubated with Argovit dilutions from 1/1000 to 1/8000 (12 to 1.5 μg/ml of metallic silver), in the range previously established for these assay conditions (short-time exposition, Figure 1, *B*), and the effect on infectivity analyzed by a plaque reduction assay. Results show that Argovit reduces RVFV-MP12 infectivity in a dose dependent manner (Figure 3). At 1/8000 dilution, infectivity was reduced in only 10% whereas over an 80% reduction was achieved at 1/2000 dilution. When the virus was preincubated at the maximum concentration allowed to be tested below the cytotoxicity threshold (Figure 1, *B*), i.e., Argovit diluted 1/1000 (12 μg/ml of metallic silver), a reduction in infectivity of 98% was reached. These results show that RVFV infectivity is drastically affected upon interaction with silver nanoparticles.

In summary, our results from cell culture experiments show that the addition and maintenance of Argovit silver nanoparticles into the medium of cells infected with RVFV in the range of 1.2-2.4 μg/ml (Argovit dilutions 1/10000 and 1/5000) was able to control only partially viral production, with a reduction of about 50% of the total viral yield. On the other hand, preincubation of virus with a 1/1000 Argovit dilution, corresponding to 12 μg/ml, was much more effective in impairing

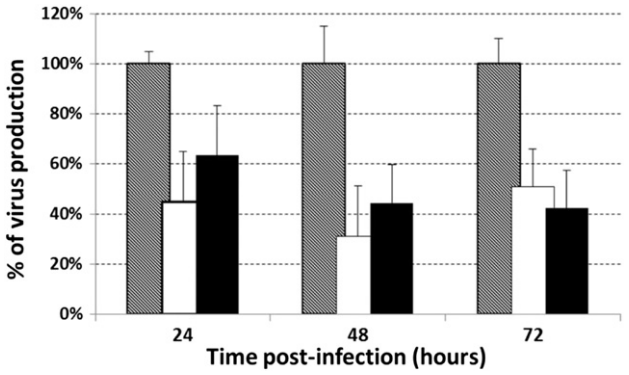

Figure 2. Production of RVFV-MP12 in the presence of Argovit. Semi-confluent Vero cells were infected with RVFV-MP12 at a moi of 0.5 pfu/cell. After adsorption inoculum was removed and replaced with fresh medium alone (grey bars) or containing Argovit at 1/5000 (white bars) or 1/10000 (black bars) dilutions. At 24, 48 and 72 hrs post-infection supernatants were collected and further titrated in a plaque assay. Titers obtained in the different conditions were compared to those in the absence of Argovit, considered as 100%.

viral infectivity, being able to abolish almost totally viral infection (a reduction of 98% infectivity was observed). The results obtained *in vitro* encouraged us to test the antiviral effect of Argovit in *in vivo* experiments. Previous observations[17] (Almanza-Reyes, unpublished) suggested that different laboratory rodent species were well tolerant to silver nanoparticles formulated as Argovit allowing the assay of higher doses as those tested *in vitro*.

Evaluation of the efficacy of Argovit against RVFV in vivo

Administration of Argovit to RVFV-infected mice

To test the efficacy of Argovit against RVFV infection *in vivo*, animal experiments were performed using IFNAR$^{-/-}$ deficient mice, a mouse strain extensively used as animal model for RVFV infection studies. These mice lack a functional IFN-α/β system, thus becoming highly susceptible to infection with attenuated strains of RVFV such as MP12.[21] When infected with RVFV MP12, IFNAR$^{-/-}$ mice develop clinical signs of disease within 2 to 4 days, a peak of viremia is detected at 48 h pi and animals die within 3-5 days after challenge.[18,22]

In a preliminary assay IFNAR$^{-/-}$ mice infected with RVFV-MP12 received 24 h after infection one single i.p. dose of 200 μl of Argovit diluted 1/20, corresponding to 6 mg/kg of metallic silver. Selection of doses to be tested *in vivo* was based on those tolerated for other rodent lab species including Balb/c mice, in the range of repeated daily doses of 2.5-10 mg/kg (Almanza-Reyes, unpublished).[17,23] This dosage corresponded to a higher amount of nanoparticles than the maximum assayed on cultured cells as described in the previous sections. Other authors have reported this same phenomenon, pointing out that *in vitro* experiments do not represent a replica of conditions expected for *in vivo* exposure.[24] However this dosage had no effects on the progression of viral infection, since all the infected animals, whether receiving or not Argovit, showed the same kinetics of disease development and death (not shown). Since mock-infected animals receiving the same Argovit treatment as a toxicity control showed no signs of sickness and remained

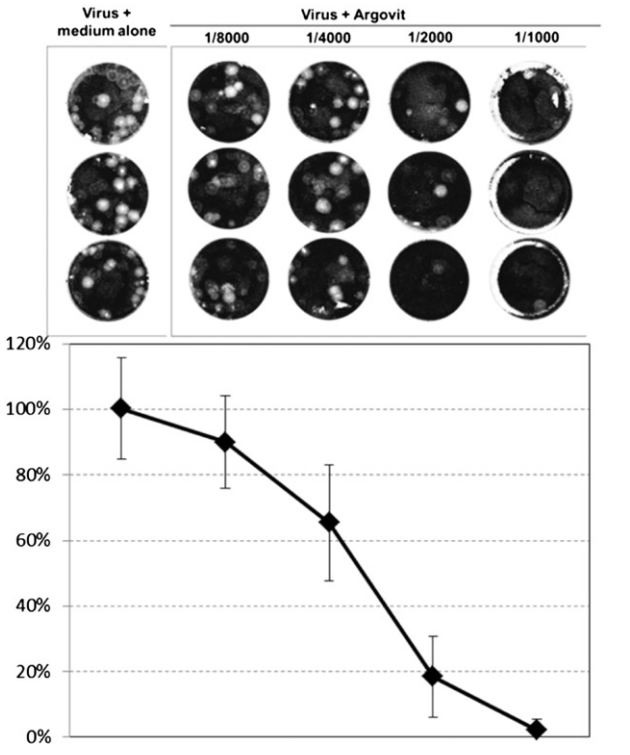

Figure 3. Reduction in the infectivity of RVFV MP12 by preincubation with Argovit. A fixed amount of virus was incubated with serial dilutions of Argovit or with medium alone, and then plaqued onto Vero cell monolayers. Cells were fixed and stained at 5 dpi and plaques counted to calculate the percentage of infectivity reduction reached at the different conditions assayed.

healthy (not shown), we decided to perform a new infection assay testing higher and repeated doses of Argovit delivered by different routes.

In a further set of experiments mice were i.p. infected with the same lethal dose of RVFV-MP12 and 20 h after infection, they were Argovit-treated every 24 h, with 200 μl of Argovit solutions undiluted or diluted 1/2, that is, repeated doses 20- and 10-fold higher than the single one assayed before. In this experiment animals were split into two groups depending on the route of delivery, either oral or intraperitoneal. The corresponding control groups of viral infection and Argovit alone (using only the higher concentration) were included. The animals were monitored daily and the development of disease and survival rate was evaluated.

Delivery of Argovit by the i.p. route in repeated doses resulted to be lethal for IFNAR$^{-/-}$ mice. Mock-infected mice receiving only Argovit at doses of 120 mg/kg were all found dead 24 h after its administration, dying at the same time or even before than control infected animals. In those receiving a dose corresponding to 60 mg/kg no effects in disease development and death were noticed when compared to the control infected group (not shown), thus delivery by the i.p. route was discarded.

On the contrary, the administration of the same doses of Argovit by the oral route had no noticeable effects on mice health and behavior, and after receiving a dose of 200 μl of Argovit undiluted every 24 h for four days, 100% of the animals in the control group were alive and healthy. In infected animals the first

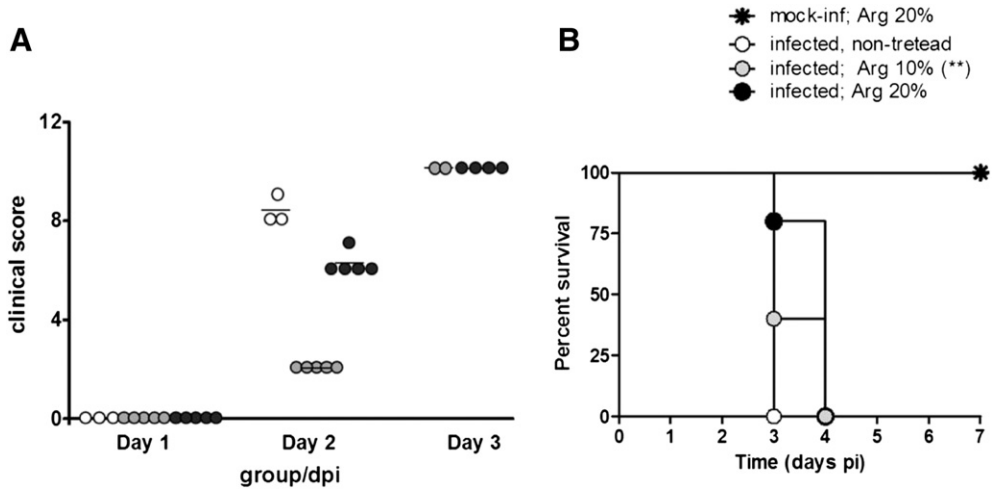

Figure 4. Effect of oral administration of Argovit to RVFV-infected mice. Evolution of clinical signs (**A**) and survival rate (**B**) in IFNAR$^{-/-}$ mice infected with RVFV-MP12 receiving Argovit by oral gavage. Animals received the first doses of Argovit 20 h post infection and then daily every 24 h. Clinical score of infected animals (**A**) was determined by quantifying the severity of clinical signs as described in Methods. Asterisks (**) denote statistical significance differences between the corresponding Argovit-treated and control groups ($P < 0.01$).

signs of disease were observed at day 2 post challenge in all the groups although, within the Argovit-treated groups, disease severity was lower (Figure 4, *A*). These differences in clinical score were statistically significant when subjected to ANOVA non-parametric test ($P = 0.0031$). At day 3 post challenge all the RVFV infected mice had died while in the groups receiving Argovit some animals remained alive, although their clinical score was indicative of severe disease. The survival rates at this time were 80% (4/5) and 40% (2/5) in those receiving Argovit undiluted or diluted 1/2 respectively. At day 4 all the animals had succumbed to the infection (Figure 4).

Blood samples collected at different times post infection, prior to administration of Argovit, were analyzed for viremia. All samples collected 20 h pi when Argovit was first administrated were negative regardless of the group analyzed. In samples collected at day 2 pi, when the peak of viremia is expected, Argovit-treated animals showed a slight decrease in viral loads, with average values of 2.7 logs in the group that was treated with undiluted Argovit. In the infected control group the average value was 3.1. However, this difference was not statistically significant. Samples from the group receiving Argovit diluted 1/2 were not collected.

Thus, these results from lethally infected mice suggest that the pattern of daily administration of Argovit by oral gavage, beginning one day after infection, could slightly reduce the viral load in infected animals, although this reduction was not enough to prevent the onset of acute disease and death. However, since no signs of toxicity were recorded in the Argovit-treated group, some room is left for higher dosage treatments.

Infection of mice with Argovit-pretreated RVFV

Even though the above mentioned results suggest that the ability of Argovit silver nanoparticles to control *in vivo* a current RVFV infection is limited in the conditions tested, our previous data in cultured cells had shown that incubation of virus with Argovit before the infection led to a clear reduction of infectivity (Figure 3), thus opening the possibility of using Argovit for viral inactivation and, eventually, for the production of inactivated RVFV vaccines, provided that Argovit treatment is not affecting antigenicity. Therefore another *in vivo* assay was designed to test whether preincubation of RVFV with Argovit at higher concentrations than those allowed for testing on Vero cells could totally abolish viral infectivity in mice. In this case a lethal dose of RVFV-MP12 was mixed with Argovit dilutions corresponding to the highest concentration of Argovit tested in cell culture, and 10-fold higher doses; i.e., final dilutions of 1/1000, 1/100 and 1/10 and incubated with rotation for 60 min at 4 °C. Mice were i.p. inoculated with the corresponding mixtures of virus and silver nanoparticles and then monitored daily to evaluate development of disease and survival rates. The highest concentration of Argovit tested in this assay corresponded to a single i.p. dose of 6 mg/kg of silver. The corresponding control groups for viral infection and toxicity of silver nanoparticles were also included.

As shown in Figure 5, in the groups of animals receiving virus previously incubated with 1/1000 and 1/100 Argovit dilutions no survivors were found, although time to death in these groups was recorded one day later than in the control group (Figure 5). However, in the group of mice that received virus preincubated with 1/10 Argovit dilution, a 60% of survival rate was recorded by day 10 post challenge. The differences observed reached statistical significance as determined by the log-rank (Mantel-Cox) test ($P = 0.0171$). Furthermore, in this group the first decease was recorded at day 5 post infection, 2 days later than in animals inoculated with non-treated virus. Blood samples from the surviving animals were negative for viremia at all the time points analyzed (not shown). All the animals in the control group that received Argovit alone were alive and healthy at the end of the experiment.

These results suggest that, as previously observed in the *in vitro* assays, pre-incubation with Argovit reduced the viral infectivity in a dose dependent manner. However even at the highest silver

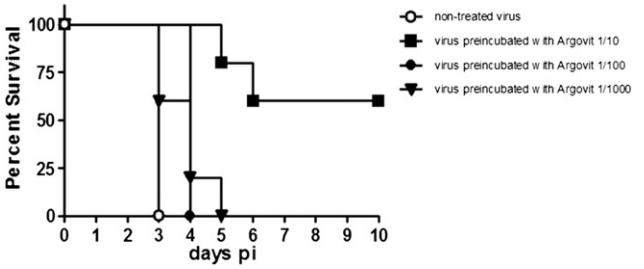

Figure 5. Infection of IFNAR$^{-/-}$mice with RVFV pre-incubated with Argovit. Survival rates in mice infected with a lethal dose of virus pre-incubated with different dilutions of Argovit (triangle, 1/1000; circle, 1/100; square, 1/10). Non treated virus: open circles.

nanoparticles concentration tested, a small fraction of infective virus remained still able to initiate a productive infection to finally cause the death of some animals, although later upon challenge.

To verify whether inoculation of Argovit-pretreated virus was able to induce an immune response and subsequent protection of the surviving animals, blood samples were taken 12 days after infection and analyzed for the presence of neutralizing antibodies. In addition, at day 17 post challenge mice were further subjected to a second viral infection. Neutralizing antibodies were not detected in any sample and all the animals died as the control group (not shown), suggesting that pretreatment of virus with Argovit inactivates the virus reducing its infectivity and antigenicity so that inoculated mice did not become properly immunized.

Discussion

Rift Valley fever virus (RVFV) is a mosquito-borne pathogen causing an important disease in ruminants often transmitted to humans after epizootic outbreaks. It is also important to mention that RVFV is considered as potential biological weapon.[25,26] Since there is no available treatment or licensed Rift Valley fever vaccine(s) for human use, the development of new approaches able to inhibit viral replication and transmission for an efficient control of the disease is a must. Different formulations of silver nanoparticles have already been reported to display antiviral activity against several viruses belonging to different families.[8,27] In this work we have tested the potential antiviral activity against RVFV of silver nanoparticles formulated as Argovit, a commercial preparation consisting of spheroid silver nanoparticles 35 nm-average-sized. Argovit has already shown to possess a broad spectrum of antimicrobial activity and does not show any cyto- or genotoxic effect in human lymphocytes.[12,13] (Juarez-Moreno, manuscript in preparation). Therefore it has been approved by international instances as food supplement, cosmetic, and medical devices for veterinary and human use.[14,28-31] The effect of Argovit on RVFV infectivity was estimated by comparing virus yields in cell cultures testing different Argovit concentrations; also we tested its antiviral potential in an animal model comparing clinical disease and mortality rates between groups of lethally infected mice receiving different treatments. Many works have already described the effectivity of silver nanoparticles to inhibit various viruses *in vitro*, but to our knowledge, there is only one previous report describing the capacity of silver nanoparticles to control influenza infection in animal models.[20] Thus *in vivo* application of the antiviral properties of silver nanoparticles in more complex systems remains an unexplored field.

As a previous step for the *in vitro* analysis of the efficacy of Argovit against RVFV, we determined its cytotoxicity on cultured Vero cells. We found that the highest doses allowed to keep high cell viability corresponded to 4.8 and 12 μg/ml of Argovit metallic silver, depending on how long cells were exposed to silver nanoparticles. In previous works assaying silver nanoparticles formulations on Vero cells, toxic concentrations were reported in the range of 30 to 300 μg/ml of their corresponding formulations.[10,19,32-34] Although the cytotoxicity of our formulation seems to be higher, a direct comparison between those and our results is not easy, since the nature of the preparations assayed is not the same. Our results referred to silver content in Argovit while other investigations often refer their results to the weight of nanoparticles as a whole, i.e. capping agent and metallic silver. The concentrations of Argovit (metallic silver and PVP) that correspond to 4.8 and 12 μg/ml of metallic silver are 80 and 200 μg/ml respectively, hence in the range of previous reports. This information is critical since silver, in either its metallic or ionic form, is actually responsible for impairing viral activity. The formulations differ in nanoparticles size and shape, synthesis procedure, content of metallic silver, surface functionalization and interactions with the capping agents, and all these features affect the cytotoxicity. Also the surface chemistry plays a major role into the interaction of nanoparticles with biological systems.[35-37] Silver nanoparticles in Argovit are coated by PVP exhibiting a negative zeta potential (−15 mV), which confers them high stability in solution[32,38] for up to 2 years at 4 °C. Furthermore, it has been reported that silver nanoparticles functionalized with PVP showed better performance in antiviral assays than those non capped or capped with other agents,[9,24,33,39,40] because the presence of a biocompatible polymer such as PVP allows the use of non-cytotoxic doses of Ag nanoparticles for the host cells but that effectively exert an antiviral activity.

In cells previously infected with RVFV, the presence of Argovit silver nanoparticles in the medium at the dilutions restricted by its cytotoxicity was able to control viral production only partially with a 50% reduction of the total virus yield. In contrast to the limitations imposed by the intrinsic toxicity of silver nanoparticles on cultured cells, laboratory animals offer a more permissive system where higher doses can be assayed, as well as a high number of delivery variations. Furthermore, studies in animal models are necessary to ascertain whether the use of silver nanoparticles may constitute a realistic approach to control viral infections.

There are not many studies on the *in vivo* toxicology profiles of silver nanoparticles but it seems clear that the effects may vary depending on animal species under study, gender and age, but also on a number of factors not only related to the physical properties of the silver nanoparticles administered but especially the route, dose and time of delivery.[36,41] In order to optimize the

antiviral properties of silver nanoparticles, the administration pattern is critical depending on how viral infection proceeds. In the work by Xiang et al daily delivery of 5-20 mg/kg of nanoparticles by intranasal administration led to a significant enhanced survival of mice infected with the H3N2 influenza virus, a respiratory virus.[20] For RVFV, having mainly hepatic tropism, we tried oral and i.p. delivery. Previous studies on the effect of Argovit administration on the immune system and its body distribution[15,23] suggested that oral gavage could be an appropriate way of delivery, and our results show that although mice were only monitored macroscopically the doses assayed were well tolerated by oral gavage while these doses were lethal when administrated by the i.p. route. Furthermore, we found that daily administration of 120 mg/kg of Argovit silver by oral gavage beginning one day after infection was able to slightly reduce the viral load in lethally infected animals. However this reduction was not enough to prevent their final death. This result resembled our previous finding in cell culture assays, where Argovit silver nanoparticles displayed only a partial control on an ongoing RVFV infection, which could lead to a minor delay in the appearance of signs of disease and death. Since no signs of toxicity due to Argovit administration by oral route were recorded, some room is left for higher dosage treatments. Other delivery patterns, with earlier, higher and/or more frequent doses should be tested in order to impair viral production more drastically with significant delay or prevention of disease signs and death, so that the use of Argovit might be considered as a therapeutical post-exposure antiviral treatment.

In contrast, preincubation of RVFV with Argovit was much more effective in reducing viral infectivity, both *in vitro* and *in vivo*. Incubation with concentrations near the cytotoxicity threshold (12 μg/ml) abolished almost completely viral propagation, leading to a 98% of infectivity reduction. In a similar way, mice inoculated with a lethal dose of virus previously incubated with 1.2 mg/ml of Argovit silver showed a delayed-onset clinical disease and mortality, with a survival rate of 60%.

These results show that interaction of silver nanoparticles with RVFV severely affects its infectivity, maybe by interfering with virus-cell attachment and viral entry as suggested for other viruses such as HIV[42] and vaccinia virus[34] respectively, or by morphological changes in the virion that render it un-infectious, as described for influenza.[20] The latter mechanism may explain the fact that mice surviving in this experiment did not become protectively immunized as a result of the inoculation. Although still to be defined, the physical interaction of RVFV with a certain amount of silver nanoparticles seems to destroy the virus in a way that it is no longer able to productively infect animals to cause disease or death, but at the same time its antigenic structure or immunogenicity is strongly affected so that the virus is no longer recognized properly by the immune system. Thus, regardless the ability of silver nanoparticles to reduce RVFV infectivity, their use for RVFV inactivation would not be recommended.

In summary, our results open the possibility of using silver nanoparticles formulated as Argovit to control the infectivity of RVFV, which represents an important zoonotic pathogen and potential biological weapon. In order to support the use of Argovit as an antiviral agent for post-exposure treatment, further studies must be done to optimize *in vivo* delivery protocols allowing a more effective interaction between nanoparticles and virions able to control an ongoing infection, since the administration routes assayed in this work do not result efficient. In contrast, the incubation of virus with Argovit before the infection leads to a clear reduction of its infectivity both *in vitro* and *in vivo*. Further investigations on the mechanisms by which silver nanoparticles interact with the virus and affect its infectivity will help to design a more effective application of Argovit against viral infections both prophylactically and therapeutically.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2016.01.021.

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