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Evaluation of the Cytotoxic Effect and PRRS Antiviral Activity of Glycyrrhizinic Acid in Aqueous Solution and with the Presence of Solid Lipid Nanoparticles

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Authors' contributions

This work was carried out in collaboration between all authors. Author MZUM designed the study, performed the experiments, wrote the protocol and wrote the first draft of the manuscript. Author SEME reviewed the experimental design and protocol, provided material, equipment, and laboratory. Author AJGL wrote the protocol and helped during experimental work. Author HRM reviewed the experimental design and protocol, managed the analyses of the study. Author NMM managed the analyses of the study and performed the literature searches. Author ACC provided the material and equipment and reviewed the protocol. Author DQG reviewed the experimental design, protocol, and first draft of the manuscript, provided material, equipment and laboratory. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To study the effect of glycyrrhizinic acid (GA) solutions on uninfected and PRRS (Porcine Reproductive and Respiratory Syndrome) virus-infected cells in culture. An attempt of testing loaded nanoparticles with GA on cells was also explored.

Study Design: Different concentrations of GA in solution were tested on MARC cells as well as on virus-infected cells and observed during 144 h. Solid lipid nanoparticles with GA were tested on cell culture previously infected. In the end, cytotoxicity (CC_{50}), inhibition of the cytopathic effect (EC_{50}), virus titer and selectivity index were determined. Trypan blue staining (TB) and MTT assay were also performed and statistically analysed.

Place and Duration of Study: Laboratorio de Posgrado e Investigación en Tecnología Farmacéutica y Laboratorio de Microbiología y Virología de las Enfermedades Respiratorias del Cerdo, both at Facultad de Estudios Superiores Cuautitlán, UNAM; between March 2015 and August 2017.

Methodology: For CC_{50} determination solutions of GA were added and TB and MTT assay was performed. EC_{50} was evaluated on virus-infected cells that were treated with GA solutions. Viability and selectivity index were calculated. Virus titer was calculated by the Reed & Muench method. Nanoparticles containing GA (0.54 mg/ml) were obtained by the microemulsion method and tested on cells previously infected with the virus. TB staining and MTT assay were performed at the end of the study.

Results: Cell viability was verified by MTT assay and TB dye exclusion test. Only the 0.7, 0.8 and 0.9 mg/ml solutions were statistically different from controls $P < .05$. The CC_{50} of GA was above 4.2 mg/ml. The EC_{50} was calculated at 0.48 mg/ml. The viral titer decreased two logarithms compared to the control. The selectivity index was 8.7. Viability decreased significantly $P < .05$ compared to controls only at 0.9 mg/ml. An SLN assay on PRRS-infected cells showed that cell viability was comparable to that exerted by the virus-infected control cells $P > .05$. So cells treated with SLN interfere with MTT assay.

Conclusion: GA showed a reduction of PRRS *in vitro* replication. SLN interferes with the MTT assay and it is necessary to perform more assays to conclude on the antiviral activity of GA when administered in nanoparticles.

Keywords: Glycyrrhizinic acid; solid lipid nanoparticles; antiviral activity; cell viability; cytotoxicity; MTT assay.

1. INTRODUCTION

Glycyrrhizinic acid (GA) is the most important saponin of licorice root (*Glycyrrhiza glabra*). It has been used for years because of its anti-inflammatory, anti-ulcer, antitumor, and antiviral properties [1,2,3,4]. Its antiviral activity has proven to be effective against several viruses [5,6,7,8,9,10,11,12,13,14]. GA is a water-soluble molecule comprised of a hydrophilic part (two moieties of glucuronic acid) and a hydrophobic fragment (a moiety of glycyrrhetic acid) that is classified as a GRAS (*Generally Recognized as Safe*) substance by the FDA [15].

In the late 1990s, the etiological agent that causes PRRS was identified as an enveloped, positive-sense RNA, single-stranded virus [16,17,18] belonging to the family *Arteriviridae*. This disease causes significant economic losses worldwide due to clinical presentations manifested in severe reproductive failure in sows and respiratory symptoms in pigs of all ages [19,20]. Due to the lack of an effective treatment, it is now considered the most prevalent porcine disease in the world [21].

On the other hand, since the 90s, solid lipid nanoparticles (SLN) have been used as carriers of a wide variety of different drugs due to their advantages, such as high drug payload, low toxicity, increased bioavailability, capability of including lipophilic and hydrophilic drugs, and drug targeting [22]. SLN are colloidal carriers composed of a lipid matrix stabilised by a surfactant.

According to literature, some efforts have been made to encapsulate antiviral agents in SLN, for example, lopinavir, saquinavir, ganciclovir, and resveratrol [23,24,25,26]; but none of them for an application intended exclusively for animal disease.

Taking into consideration the demonstrated antiviral activity of GA against various viruses, the aim of the present study was to evaluate the effect of GA aqueous solutions on uninfected and PRRS virus-infected cell cultures in an attempt to discover new therapeutic alternatives for use against this virus. Cytotoxicity and antiviral activity were evaluated using the TB dye exclusion test, MTT and virus titer reduction. Also, the cell viability of virus-infected cells after treatment with a SLN dispersion containing GA was evaluated as an early stage in the development of a pharmaceutical product that can exploit the important advantages of these nanocarriers.

2. MATERIALS AND METHODS

2.1 Glycyrrhizinic Acid Solutions

Monoammonium glycyrrhizinate (the ammonium salt of the glycyrrhizinic acid, Sigma Aldrich, St. Louis, MO, USA) solutions were prepared by dissolving the drug in RPMI giving concentrations of 1, 10 and 30 mg/ml. Concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 mg/ml were then prepared from a stock solution of 10 mg/ml diluted to the adequate volume of RPMI. All solutions were sterilised with a 0.22 μm membrane filter.

2.2 Cells and Virus

MARC 145 cells were maintained in RPMI medium (Gibco®, California, USA) and supplemented with 10% fetal bovine serum (FBS) (Gibco®, California, USA) and a 1% penicillin and streptomycin solution (In vitro®, DF, México) in an incubator at 37°C and 5% CO_2. The VR2332 PRRS strain (donated by Avimex, S.A. de C.V., Mexico) was propagated in MARC cells using the same medium. Virus titers were obtained by the Reed and Muench method and expressed as 50% tissue culture infective dose per ml ($TCID_{50}$/ml).

2.3 Cytotoxicity Assay

Cells (3.5×10^3 cells/well) were placed in a 96-well culture plate with the supplemented medium. Once confluence was observed, the supernatants were removed and 100 µl of the GA solutions (1-30 mg/ml in the first stage of the assay, and 0.1-0.9 mg/ml in the second) were added, followed by 100 µl of the supplemented medium (2% FBS). Plates were kept at 37°C and 5% CO_2 for 7 days. Cells were used as the control. Cell morphology was analysed daily during one week using inverted microscopy to observe morphological alterations (*i.e.*, loss of confluence, cell-rounding or shrinking, and the presence of vacuoles). At that time, cytotoxicity was evaluated by the TB dye exclusion test and MTT assay. The percentage of viability was calculated as (AT/AC)*100, where AT and AC are the absorbances of treated and control cells, respectively. The cytotoxic concentration of GA that reduced cell viability to 50% (CC_{50}) was estimated from concentration-effect curves after linear regression analysis.

2.4 Antiviral Activity Assay

The antiviral activity of GA was assessed by inhibiting the cytopathic effect on infected cells. Briefly, cells were incubated in a 96-well culture plate. When confluence was reached, the medium was discharged and 100 µl of the virus at a concentration of 1×10 $TCID_{50}$/ml in RPMI were added to each well. The plate was then incubated at 37°C for 1 h. Afterwards, the cells were treated with 100 µl of the GA solutions at concentrations of 0.1-0.9 mg/ml with 100 µl of RPMI supplemented with 2% FBS, and maintained for 144 h at 37°C and 5% CO_2. Controls were run on the same plate with 8 wells per control. Drug-treated cells (0.9 mg/ml GA solution) were used as the negative control and virus-treated cells as the positive control. A second negative control consisted of cells held in the maintenance medium. The TB dye exclusion test and MTT assay were performed. Antiviral activity was calculated as follows: ((mean absorbance of eight GA treated cells - mean absorbance of eight virus controls)/(mean absorbance of cell controls - mean absorbance of eight virus controls)) × 100.

The effective concentration of GA required to inhibit the cytopathic effect to 50% of the control value (EC_{50}) was calculated by a regression analysis of the concentration-effect curve for the MTT assay. The selectivity index was calculated as the ratio between CC_{50} and EC_{50}.

Another mode of the antiviral assay was performed as follows. Briefly, confluent cells in a 96-well plate were infected with 100 µl of the virus dilutions at concentrations of 1×10^1 to 1×10^8 $TCID_{50}$/ml. The plate was kept at 37°C for 1 h before adding 100 µl of the GA solutions (0.1-0.9 mg/ml) with 100 µl of supplemented medium, and then maintained for 168 h at 37°C and 5% CO_2. The controls specified above were used.

Virus titers were calculated daily using the Reed and Muench method. At the end of testing, the TB dye exclusion test and MTT assay were performed.

2.5 Solid Lipid Nanoparticles Preparation

Nanoparticles were prepared by the microemulsion cooling method [27]. Briefly, SLN were prepared with a mixture of mono-, di-, and triglycerides (Gelucire® 44/14), a non-ionic surfactant (Pluronic® F127) and GA, maintaining the following ratios: 10, 1.5 and 3% w/w, respectively. Also, 85.5 g of water was heated to, and maintained at, 90°C during the process. GA was dissolved using a mechanical stirrer (Caframo® RZR-1; Ontario, Canada; propeller: PR 31; Heidolph, Schwabach, Germany) at 800 rpm for 2 min. Next, the polymeric surfactant was added while maintaining the system at the same stirring rate until total dispersion was reached. The pre-defined amount of melted lipid was then added at 90°C and 1600 rpm for 5 min to form a clear liquid O/W microemulsion. To obtain the SLN, this microemulsion was cooled rapidly in a water bath at 5°C and a stirring rate of 1200 rpm for 3 min. Nanoparticle separation was performed by ultracentrifugation at 50,000 rpm for 1 h (Beckman® Optimal LE-80K, CA, USA).

2.5.1 Particle size

The dynamic light-scattering technique (Coulter N4, CA, USA) was performed in triplicate. Measurements were made at a 90°C fixed-angle for 180 s at 25°C. The laser light wavelength (He/Ne, 10 mW) was set at 678 nm. To analyze the scattering intensity data, a digital correlator was used with the unimodal analysis mode.

2.5.2 Z-potential

The electrophoretic mobility of the dispersions was measured and transformed into Z-potential by applying the Smoluchowski approximation (Malvern Instruments NS ZEN 3600, Worcestershire, UK) at 25°C in a capillary cell. All samples were analysed in triplicate.

2.5.3 Scanning electron microscopy

After removing the excess stabiliser, or free drug, from the sample, a few drops of the dispersed nanoparticles were placed on a slab and dried at room temperature. The dried samples were then coated with gold (~20 nm thickness) using a JFC-1100 Sputter Coater (JEOL, Tokyo, Japan) and observed with a microscope of light vacuum LV-SEM JSM 5600 (resolution 5 nm), at a voltage of 20 kV and a chamber pressure of 12-20 Pa.

2.5.4 Effect of SLN with and without GA on PRRS-infected cells

Cells (3.5×10^3 cells/well) were seeded in a 96-well culture plate and allowed to grow for 24 h. Once confluent, the medium was removed and the cells were infected with 100 µl of the virus dilutions at concentrations of 1×10^1 to 1×10^8 $TCID_{50}$/ml. The plate was kept at 37°C for 1 h and then 100 µl of supplemented medium was applied. Next, 100 µl of the SLN dispersion with GA was added to each well. The SLN dispersion was previously sterilised and then diluted in RPMI to a concentration of 0.5 mg/ml of the drug. The cells, virus, GA and SLN without GA controls were evaluated simultaneously. Plates were incubated and observed for 72 h, and at that point cell viability was determined by the TB dye exclusion test and MTT assay. Cell viability of the SLN-treated wells was evaluated as the percentage of the mean absorbance value that resulted from eight mock-treated cell controls, which was set at 100%.

2.6 Trypan Blue Dye Exclusion Test

Cell monolayers were incubated as described above. The medium was discharged from the plate and 20 µl of TB mix (0.5% w/v in PBS) were added to each well. The plate was then incubated for 5 min at room temperature, and the dye was discharged for observation and direct cell-counting in a Neubauer chamber.

2.7 MTT Assay

Cells were washed with RPMI followed by PBS. Immediately, 50 µl of MTT reagent were added per well. Incubation was carried out at 37°C for 3 h. Culture supernatants were removed and 100 µl of DMSO was added to dissolve the formazan precipitate by shaking the plate gently for 5 min. Finally, the absorbance of the wells was determined using a plate reader at a wavelength of 540 nm and a wavelength reference of 630 nm.

2.8 Statistical Analysis

Data were analysed by one-way ANOVA and a Tukey multiple-range test for comparison of means at a confidence level of 0.05. ~~A~~ $P < .05$ was considered significant. Results are

expressed as mean ± standard error of the mean values of at least three experiments.

3. RESULTS

According to microscope observation, the non-toxic concentrations of GA were below 0.9 mg/ml; but upon exceeding this concentration a marked loss of confluence together with the presence of cell-rounding and vacuoles were seen to follow an apparent concentration-dependent response. Cell viability was verified by MTT assay and the TB dye exclusion test at the end of the assay. Only the 0.7, 0.8 and 0.9 mg/ml solutions were statistically different from controls, Tukey $P < .05$, but a relatively high viability was observed in all treatments (>80%; Figs. 1a, 1b) and there was no clear evidence of a concentration-dependent response. The CC_{50} value estimated from the linear analysis of the concentration-response curve was 4.2 mg/ml. TB staining (Fig. 2) showed that viable cells decreased significantly in a concentration-dependent manner (70-75% for the concentrations tested *vs.* controls). In concentrations above 10 mg/ml, gelation of the solution occurred, so those solutions were not applied to the cells.

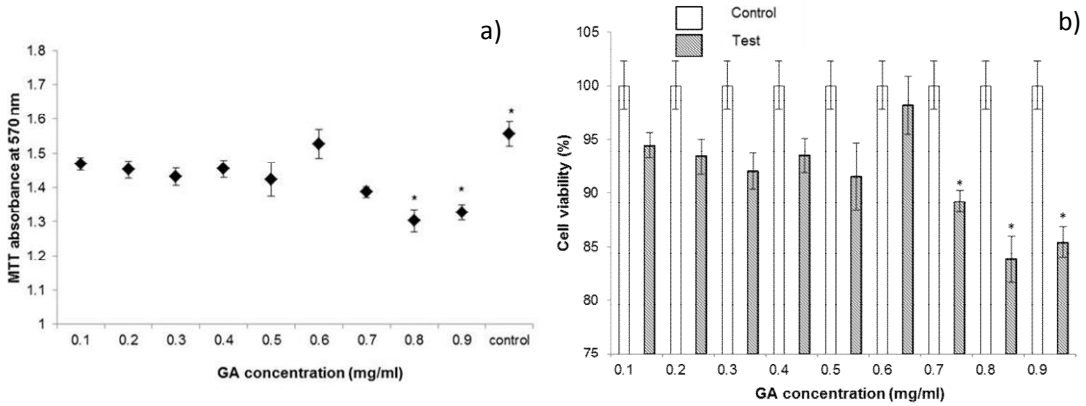

Fig. 1. MTT assay, absorbance as a function of GA concentration (a). Cell viability obtained by MTT assay expressed as the percentage of control (b). Cytotoxicity was evaluated after 144 h exposure of MARC 145 cells to GA aqueous solutions. n= 5; *$P < .05$ *vs.* controls (Tukey test)

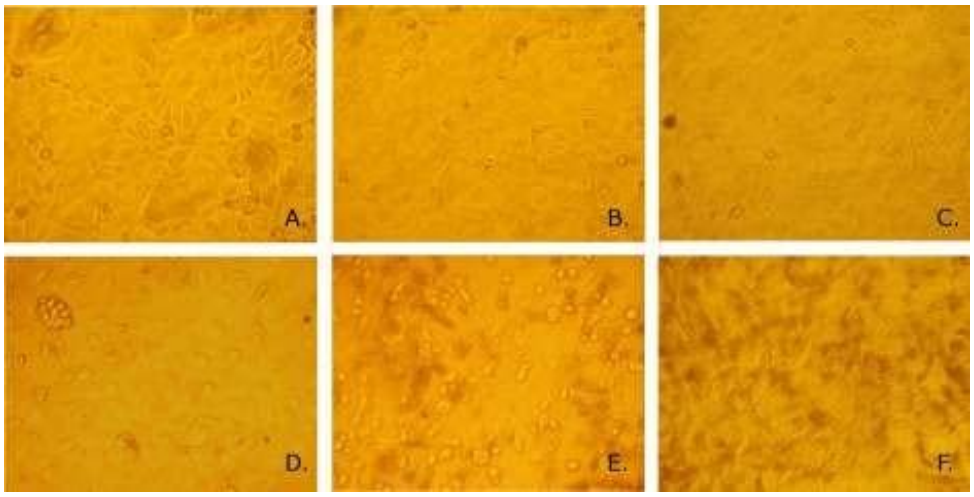

Fig. 2. Trypan blue dye exclusion test for the cytotoxic assay. 48 h (before staining as a representative comparison): A. cell control; B. cells treated with GA at 0.5 mg/ml; C. cells treated with GA at 0.9 mg/ml. 144 h (after staining): D. cell control; E. cells treated with GA at 0.5 mg/ml; F. cells treated with GA at 0.9 mg/ml

The potential antiviral effect of the GA solutions was evaluated on PRRS-infected cells by the cytopathic inhibition assay, MTT assay, and TB dye exclusion test. The MTT assay showed no statistical differences for any of the concentrations tested, ANOVA $P > .05$, but all groups showed statistical differences compared to both controls, Tukey $P < .05$. The percentages of viral inhibition obtained were above 50%, and the EC_{50} was calculated at 0.48 mg/ml after linear regression analysis based on the concentration- effect curve (Fig. 3). GA showed good activity against the PRRS virus (SI 8.7). Direct counting of viable cells revealed that viability decreased significantly, $P < .05$, compared to controls (60% *vs*. controls) only at 0.9 mg/ml.

After infecting the cells for 1 h with PRRS at different virus titers and treating those cells with GA solutions, daily observations were made for 168 h. At that point, the TB dye exclusion test showed that the number of viable cells decreased significantly at concentrations above 0.7 mg/ml. The antiviral activity of GA estimated by the limit-dilution method demonstrated that the virus titer decreased two logarithms compared to the titer of the control titration without GA treatment (1 x $10^{3.7}$ $TCID_{50}$/ml vs 1 x 10^{6} $TCID_{50}$/ml) (Fig. 4).

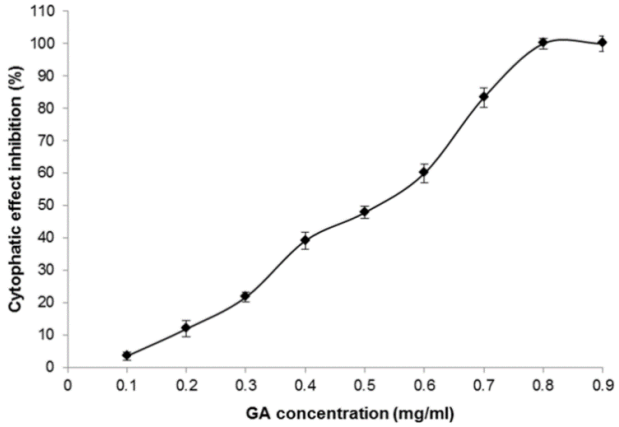

Fig. 3. Concentration-effect curve of GA activity against PRRS. Cells were infected with PRRS and at 1 h post-infection different concentrations of GA solutions were added. Inhibition of cytopathic effect was evaluated daily for 6 days. n= 5

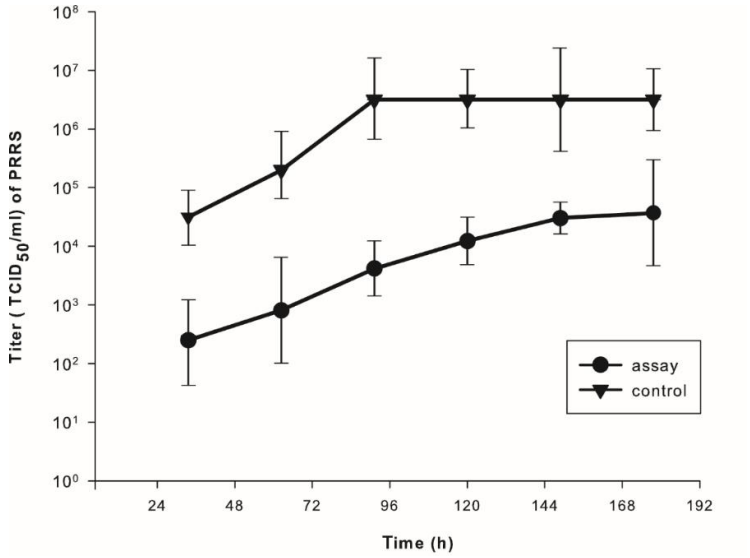

Fig. 4. Virus titer calculated by the Reed and Muench method, where increased concentrations of GA were added after 1 h of incubation of the cells infected with PRRS. n= 3

The SLN was obtained by the microemulsion cooling method. The characterisation of this formulation is presented in Table 1.

An increase in mean diameter was observed during particle size evaluation, though the polydispersity index always maintained its value below 1. However, in some batches, the presence of aggregates was evident at the end of the evaluation. When GA was included in the formulation, size was larger, indicating drug adsorption or chemical interaction with the nanocarrier surface. The initial and final Z-potentials were below 30 mV in all batches, a value associated with low dispersion stability for particles stabilised by ionic surfactants or charge repulsion.

Microscopic observation revealed that the nanoparticles had a solid spherical matrix structure without crystal formation on the surface, and a diameter of 100-200 nm (Fig. 5). These findings concurred with the results obtained by dynamic light-scattering.

Table 1. Results for mean particle size and Z-potential of SLN

	Batch number	MPS (nm), [IP]					Z-Potential (mV)[a]	
		Day 1	Day 2	Day 5	Day 8	Day 12	Day 1	Day 15
SLN with GA	1	96.3 [0.489]	95.8 [0.645]	98.2 [0.548]	102.6 [0.422]	135.4 [0.604]	10.83 ± 0.349	13.11 ± 0.461
	2	100.5 [0.325]	115.7 [0.342]	125.1 [0.657]	142.8 [0.564]	176.3 [0.821]	10.34 ± 0.027	13.98 ± 0.283
	3	101.5 [0.215]	113.5 [0.257]	128.4 [0.319]	155.9 [0.786]	181.7 [0.921]	10.97 ± 0.064	14.32 ± 0.139
	4	106.2 [0.561]	106.8 [0.543]	110.1 [0.684]	118.6 [0.624]	142.4 [0.776]	11.72 ± 0.192	14.67 ± 0.139
	5	119.4 [0.314]	120.2 [0.363]	147.5 [0.455]	166.4 [0.759]	180.3 [0.913]	11.93 ± 0.896	15.45 ± 0.391
	6	108.2 [0.385]	110.5 [0.472]	121.4 [0.483]	129.7 [0.698]	138.2 [0.991]	11.59 ± 0.735	15.84 ± 0.892
SLN without GA	7	90.4 [0.122]	93.2 [0.425]	94.1 [0.356]	95.7 [0.779]	96.7 [0.732]	22.16 ± 0.330	24.49 ± 0.675
	8	89.4 [0.231]	85.2 [0.564]	91.7 [0.678]	92.3 [0.345]	93.9 [0.689]	22.89 ± 0.682	24.60 ± 0.753
	9	90.2 [0.764]	90.5 [0.598]	92.4 [0.728]	93.8 [0.643]	95.3 [0.892]	22.32 ± 0.740	23.61 ± 0.831

[a] *Reported as mean ± standard deviation; n= 3*

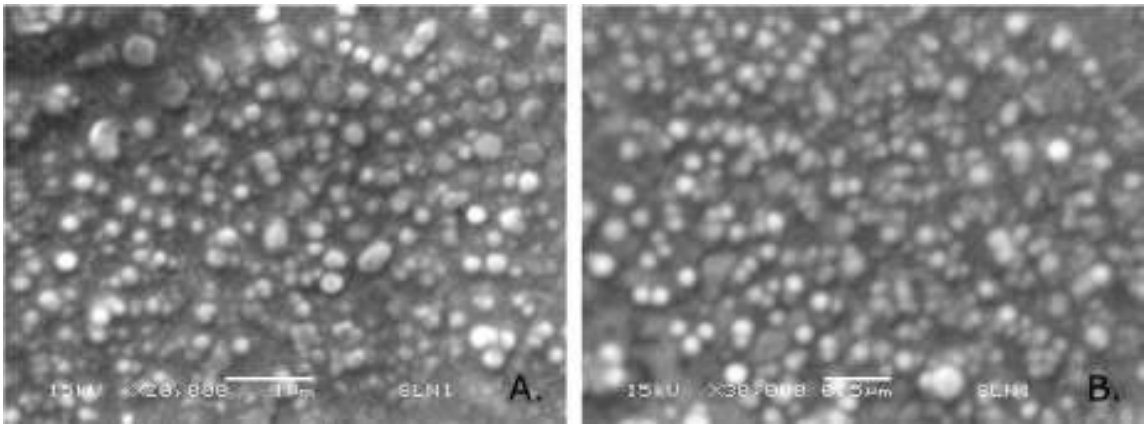

Fig. 5. SEM images of SLN of Gelucire®. SLN at t= 0 (A), and at t= 15 days (B). Drops of dispersed nanoparticles were placed on a slab. The dried samples were coated with gold (~20 nm thickness). The bars represent 1 µm or 0.5 µm, as specified

After 24 h of treatment with SLN, cell morphology revealed drastic changes; namely, loss of confluence, cell-rounding and vacuole formation, when compared to the positive and negative controls, which conserved their characteristic morphology. Direct cell-counting by the TB assay revealed a significant decrease in viable cells (60-70% *vs*. controls) at that time.

Cell viability at 72 h by MTT assay of the SLN-treated cells showed non-significant differences for all virus concentrations *vs*. the positive control, Tukey $P > .05$, with high percentages of viable cells (>90%) for all treatments (Fig. 6), though a slight increase in viability was observed as the virus titer decreased (reaching 100% for 1 x 10^8 $TCID_{50}$/ml). TB results at that time indicated a significant decrease in the number of viable cells $P < .05$.

Furthermore, after 48 h, all cells treated with SLN containing GA evidenced the formation of needle-like structures that increased in number at the end of treatment (Fig. 7). After adding MTT, some wells became completely filled with these needle-like crystals, apparently attributed to formazan. Formation of formazan crystals was less evident in the control wells.

4. DISCUSSION

PRRS causes enormous economic losses in hog-producing countries, so it is urgent to find new alternatives for treating this infection. Vaccination is important because of the partial protection it has shown against PRRS. While modified live vaccines have demonstrated effectiveness against homologous strains, results against heterologous strains have varied and,

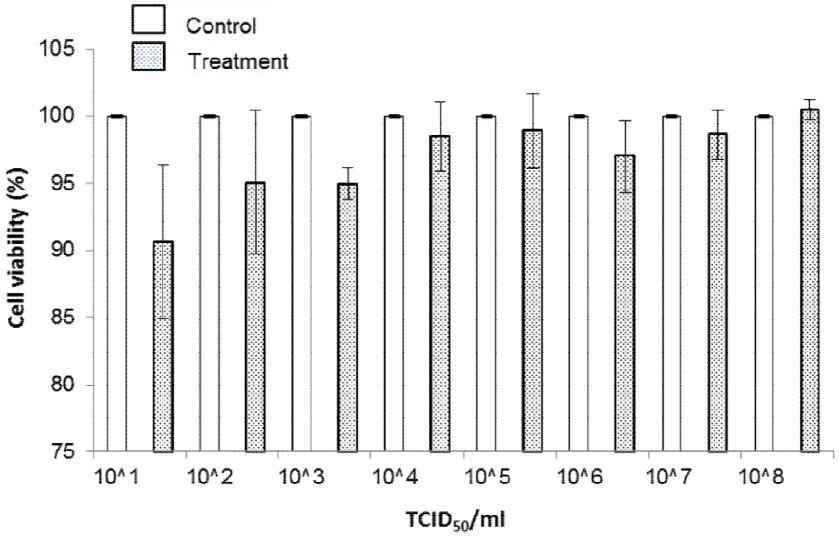

Fig. 6. Cell viability obtained by MTT assay expressed as the percentage of control. Evaluated after 72 h of exposure time of MARC 145 cells to SLN containing GA and previously infected with various virus titers. n= 3

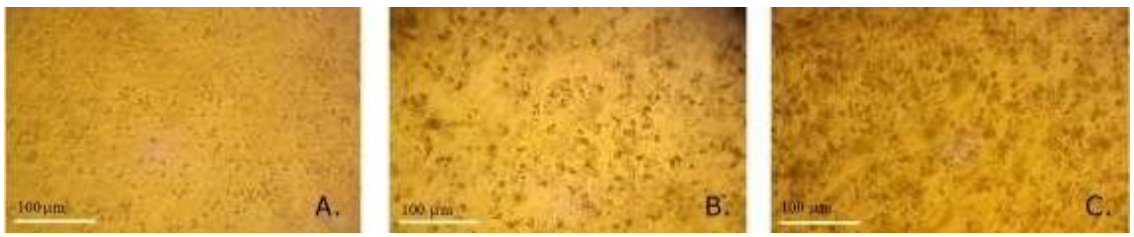

Fig. 7. SEM of control cells (A); SLN-treated cells simultaneously infected with PRRS (1 x 10^5 $TCID_{50}$/ml); the presence of needle-like structures is observed (B); SLN-treated cells after 72 h of exposure (C). MTT assay was performed and showed an increase of needle-like structures under inverted microscope

more importantly, shown a tendency to revert to virulence. On the other hand, with killed vaccines, it was observed less effectives in preventing the disease and infection [28]. Other options available to treat PRRS infection include: administering quinolones [29]; probiotics [30]; chimeric vaccines [31]; peptide-conjugated morpholino oligomer [32]; and montmorillonite-bamboo vinegar [33]. Unfortunately, some of these methods are prohibitively expensive for large-scale production operations. GA has been used extensively for many years in the traditional medicine of several cultures, and its antiviral activity has been tested both *in vitro* and *in vivo*. Nevertheless, GA (as monoammonium salt) has rarely been studied in relation to cytotoxicity and antiviral activity against PRRS.

According to Andrighetti-Fröhner et al. [34], an antiviral compound should act selectively on specific virus processes with little or no interference in cell metabolism. Cytotoxicity tests *in vitro* are based on measuring changes in cell function or structural damage as a result of the toxic effects produced by a compound. Such changes can be evaluated using cell viability or mitochondrial function assays. Moreover, microscope observation of morphological changes in cells has been used as an indicator of compound toxicity. In the present study, GA toxicity to MARC 145 cells was assessed by direct microscope observation and cell viability tests using the TB dye exclusion test and colorimetric MTT assay.

The cytotoxic concentration (CC_{50}) of GA was above 4.2 mg/ml. The CC_{50} reported by other authors are >20 mg/ml, >3mg/ml and 3.78 mg/ml (5, 6, 10), while Wang, *et al.* [35] reported a CC_{50} >4 mg/ml for dipotassium glycyrrhetate, a finding very similar to our result.

Although the percentage of viable cells found by MTT exceeded 80% in all concentrations tested, the TB assay showed that cell viability decreased in a concentration-dependent manner. Visual examination of the cell culture showed damage to cell morphology from 0.9 mg/ml. The decreased viability observed in the TB dye exclusion test results is probably due to a cytostatic effect and not cell death. This difference between the results of MTT and TB emphasises the need to use distinct methods of assessing toxicity to reach solid conclusions concerning the performance of compounds, because in some cases in which cells present morphological changes dye uptake may indicate values equivalent to those of untreated cells.

The morphological changes in cell monolayers can be explained by the fact that GA is a saponin, so it possesses surface activity and is capable of solubilising hydrophobic compounds. Since the structure of the cell membrane includes such molecules it is susceptible to suffering damage by the drug. Moreover, it has been found that dipotassium glycyrrhizinate alters and fluidises the lipid membrane of liposomes. Because the chemical structure of GA is similar to that of dipotassium glycyrrhizinate, it may act in a similar way on cell membranes [36,37].

Another finding was that the effective concentration of GA required to inhibit the cytopathic effect to 50% (EC_{50}) was relatively low, compared to levels reported in previous studies [5,9,11]. Since the cytopathic effect was inhibited to 50% at a concentration of just 0.48 mg/ml, we can suggest that even low concentrations of GA can achieve effective responses. Surprisingly, the TB dye exclusion test results showed a high percentage of viable cells for all concentrations tested, while the MTT assay showed no statistical difference in cell viability among groups.

The selectivity index was 8.7, which leads to the conclusion that GA is active against PRRS. Crance et al. [6] reported selectivity index values above 4 for viruses of the *Flaviviridae* family. He tested four antivirals and found that while GA had the lowest selectivity index it was a potent inhibitor of all flaviviruses. With respect to the SARS-associated coronavirus, the selectivity index was >8.33 [5]. It is important to note that the selectivity index of a compound may be moderately influenced by the strain of the virus tested.

As these results show, GA reduced the virus titer by two logarithms, indicating good antiviral activity against PRRS. Previously, Wolkerstorfer et al. [14] reported a $CCID_{50}$ titer reduction of 90% for human lung cells infected with influenza A virus.

All of these findings confirm that GA has a high potential for treating PRRS infections. The mechanism through which GA exerts its antiviral activity is not yet clearly understood and is beyond the scope of this research. However, Harada [8] demonstrated that adding GA to an

enveloped virus (like PPRS) modified the plasmatic membrane and the envelope of HIV, thus inhibiting virus infection by decreasing the fluidity of the lipid bilayer membranes and, consequently, preventing the formation of the fusion pores that enveloped viruses require to achieve penetration.

Another important aspect to consider is GA's capacity to form a gel structure; because GA has a hydrophobic and hydrophilic moiety that interacts to form micelles that initiate the formation of the gel [38]. Gel formation was observed at concentrations above 10 mg/ml, which impedes application to the cell monolayer. For this reason, we limited our tests to concentrations below 1 mg/ml. New assays are being performed to study GA as a carrier or support material in a gel structure for other drugs, or alone as a new pharmaceutical formulation for various diseases of veterinary and human interest. This is the first time that an *in vitro* evaluation of the antiviral activity of GA has mentioned the capacity of solutions of this drug to form a gel (at concentrations above 10 mg/ml).

Finally, the preparation and characterisation of the lipidic nanoparticles indicated that submicron carriers with good stability were obtained. A polydispersity index value below 1 indicates that size distribution is narrow and homogeneous. The Z-potential value (below 30 mV in all batches) may be indicative of low dispersion stability [39], but in this case, the stabilisation of the particles was due to the steric repulsion exerted by the non-ionic three-block copolymer Pluronic® F127. Morphology studied by scanning electron microscopy revealed spherical particles of nanometric size without crystal formation on their surface.

Our interest in using SLN as carriers of GA is based on the advantages they present such as low toxicity and biocompatibility; furthermore, an SLN dispersion can be easily administered e.g. by nebulization to pig exploitations avoiding handling of the animals that can cause them injuries and is also more time-consuming. The assay of SLN with and without GA showed inconsistent results. Morphological changes in monolayers after 24 h of SLN treatment were noted. Apparently, a cytotoxic effect could be responsible for this result, as shown by the results of the TB assay, which revealed a significant decrease in viable cells $P < .05$, (60-70% *vs*. control) at termination (72 h).

However, cell viability at 72 h by MTT assay of SLN-treated cells showed non-significant differences for all virus dilutions *vs*. the positive control, Tukey $P > .05$. Furthermore, at 48 h, all cells treated with SLN containing GA showed the formation of needle-like structures which increased in number towards treatment end. This phenomenon was observed in rat hepatocytes incubated with palmitic acid, which after a rapid conversion to triglycerides, and due to a combination of factors, resulted in saturation of the cytosolic surface of the endoplasmic reticulum with triglyceride molecules initiating needle-like or spinnacle formation [40]. Gelucire® is a mixture of mono-, di- and triglycerides, so it may be that saturation of these molecules triggers the formation of the needle-like crystals observed in our assay.

After MTT addition, some wells filled completely with these needle-like crystals, apparently attributed to formazan. In the control wells, the formation of formazan crystals was less evident. This behaviour has also been observed with empty liposomes on macrophage-like P388D1 cells. In their research, Angius and Floris [41] observed needle-like structures after 120 min of MTT treatment, indicative of formazan/lipid saturation, and concluded that liposomes caused an increase of formazan storage in cells. It is well-known that formazan has high affinity to lipids; it tends to accumulate in cell membranes and lipid droplets. In this way, formed formazan can accumulate in SLN, resulting in an apparent increase of cell viability. In contrast to this result, the reduced number of viable cells found by the TB test after 24 h of SLN treatment is probably the result of a cytostatic effect rather than cell death. Considering these results, it can be concluded that cells treated with SLN interfere with the MTT assay, so it is advisable to combine different methods of assessing viability, including direct counting or dye- exclusion tests, or even more sophisticated, but reliable, methods.

5. CONCLUSIONS

Considering the results obtained, it is clear that GA included in aqueous solutions has antiviral activity *in vitro* against PRRS. Due to the lack of consistent results regarding the treatment of virus-infected cells with SLN containing GA it is necessary to perform more assays to determine if there is an improvement in the antiviral activity of this drug when administered in nanoparticles. Our results reveal that GA may

be a promising option for treating PRRS infections. Currently, we are performing assays to study GA activity *in vitro* against other viruses of veterinary interest.

GA's ability to form a gel under certain conditions is also being studied by our group in order to identify possible new veterinary applications.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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