Hydrophobic gentamicin loaded nanoparticles are effective in \textit{Brucella melitensis} in mice

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\textbf{Running title:} GEN-AOT NPs are effective in \textit{Brucella}-infected mice

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Abstract:

The clinical management of human brucellosis is still challenging and demands in vitro active antibiotics capable of targeting the pathogen-harboring intracellular compartments. A sustained release of the antibiotic at the site of infection would make it possible to reduce the number of required doses and thus the treatment-associated toxicity. In this study, a hydrophobically modified gentamicin, gentamicin-AOT, was either microstructured or encapsulated in poly(lactic-co-glycolic acid) (PLGA) nanoparticles. The efficacy of the formulations developed was studied both in vitro and in vivo. Gentamicin formulations reduced Brucella infection in experimentally infected THP-1 monocytes (above 2 log_{10} unit reduction) when using clinically relevant concentrations (18 mg/L). Moreover, in vivo studies demonstrated that gentamicin-AOT loaded nanoparticles efficiently targeted the drug both to the liver and the spleen and maintained antibiotic therapeutic concentration for up to 4 days in both organs. This resulted in an improved efficacy of the antibiotic in experimentally infected mice. Thus, while 14 doses of free gentamicin did not alter the course of the infection, only 4 doses of gentamicin-AOT loaded nanoparticles reduced the splenic infection by 3.23 logs and eliminated it from 50% of the infected mice with no evidence of adverse toxic effects. These results strongly suggest that PLGA nanoparticles containing chemically modified hydrophobic gentamicin may be a promising alternative for the treatment of human brucellosis.

INTRODUCTION

Brucellosis remains one of the world’s most widespread zoonoses (1). It is an infectious disease caused by bacteria of the genus Brucella, a facultative intracellular pathogen localized predominantly in cells and organs of the mononuclear phagocytic system, such as
the macrophages of the liver and the spleen. Human brucellosis is usually manifested as a febrile illness which may persist and progress to a chronically incapacitating disease with severe complications, which is an important cause of morbidity worldwide (2). The chronicity of the infection results from the ability of the pathogen to survive within the phagocytic cells, which fail to eliminate the microorganism and act as a reservoir of the bacteria. Although it is a reportable disease, it is estimated that cases of human brucellosis are underdiagnosed and underreported and that the disease continues to be a major human health hazard (3, 4). The disease is also recognized as one of the most common laboratory-acquired infections (5, 6), and owing to its possible airborne transmission and its low infective dose it is considered a potential bioweapon for bioterrorism (7).

Concomitant with its pathogenicity, the clinical management of human brucellosis is also a difficult task. The intracellular location of the pathogen inside the phagocytic cells makes its eradication difficult to achieve, since many in vitro active antibiotics fail to reach Brucella-infected cells efficiently, lose their antimicrobial activity in the intracellular environment or do not persist enough time to produce a therapeutic effect (8). Therefore, the current treatment of human brucellosis requires a combination of antibiotics for long periods of time. World Health Organization guidelines recommend doxycycline with rifampicin for six weeks (9), but later recommendations also propose the use of doxycycline for six weeks with the aminoglycosides streptomycin for two-three weeks or gentamicin for one week (10). However, despite the reasonable efficacy of current treatment regimes, they often fail to eradicate the infection with relapse rates of about 5-10% (11). On the other hand, the combined therapies are more effective than individual, however, new therapies are inevitable due to the difficulties of patient adherence to
treatment itself together with the side-effects of combination therapy and the dangers of antibiotic resistance (12).

Nanotechnology has emerged as a promising approach for the treatment of intracellular infections by providing intracellular targeting and sustained release of the vehiculized drugs inside the infected cells (13, 14). These properties of drug delivery systems may lead to an improvement in drug cellular accumulation and a reduction of the dosing frequency which, in turn, will improve patient compliance and the efficacy of the antimicrobial therapy. The aminoglycoside gentamicin is a bactericidal antibiotic with a great in vitro activity against clinical isolates of Brucella, which has already been encapsulated into particulated systems for the treatment of experimental brucellosis with promising results (15, 16). However, the low encapsulation efficiency obtained limited the dose of particles that could be administered in vivo. We have recently reported that the hydrophobic ion pairing of gentamicin with the anionic surfactant AOT successfully improves the antibiotic payload in carriers without affecting its bioactivity (17).

The aim of this research was to study the efficacy of gentamicin-AOT (GEN-AOT) loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles in both human macrophages and mice experimentally infected with B. melitensis. Moreover, the in vivo pharmacokinetics and toxicity of the formulations were also studied. The results demonstrated that the modification and encapsulation of gentamicin successfully improved its efficacy against brucellosis and reduced the associated toxicological profile.

MATERIALS AND METHODS

Materials
Gentamicin sulphate, doxycycline, polyvinyl alcohol (PVA) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT) from Sigma (Tres Cantos, Spain). PLGA 502H (Resomer® RG 502H, PLGA 50:50, 13.7 kDa) and 752H (Resomer® RG 752H, PLGA 75:25, 17 kDa) were supplied by Boehringer Ingelheim (Ingelheim, Germany). For bacterial growth, trypticase soy broth (TSB) was purchased from bioMérieux (Marcy l’Étoile, France), antibiotic medium 11 from Difco™ (Becton Dickinson, Franklin Lakes, NJ, USA) and American bacteriological agar from Pronadisa (Madrid, Spain). Reagents for cell culture were obtained from Invitrogen Inc. (Carlsbad, CA, USA) and other reagents were from Sigma-Aldrich (St. Louis, MO, USA) or Merck (Madrid, Spain).

**Preparation and characterization of microstructured gentamicin-AOT and gentamicin-AOT**

The modified gentamicin, gentamicin-AOT (GEN-AOT), was prepared by the hydrophobic ion pairing of gentamicin with the anionic surfactant AOT, as previously described (18). The obtained GEN-AOT complex was then either microstructured (PCA GEN-AOT) by a compressed fluid based technology called Precipitation with a Compressed Antisolvent (PCA) (17) or encapsulated into poly(lactic-co-glycolic) acid (PLGA) nanoparticles by an oil-in-water single emulsion formation solvent evaporation method, using a nominal drug loading of 20 mg per formulation, as previously reported (17). Briefly, GEN-AOT (20 mg) and the polymer (200 mg of PLGA 502H or PLGA 752H) were dissolved in 1 mL of ethyl acetate and mixed by ultrasonication with 2 mL of a 0.5% (w/v) PVA aqueous solution for 1 minute (15 W, Branson sonifier 450, Branson Ultrasonics Corp., Danbury, CT, USA). The resulting emulsion was poured into a 50 mL
solution of 0.2% (w/v) PVA and stirred for 3 h to allow solvent evaporation and nanoparticle formation. Particles were collected and washed with ultrapure water by three successive centrifugations at 21,000 x g for 10 min (Sigma Laboratory Centrifuges, 3K30, Osterode am Harz, Germany). Nanoparticles were lyophilized with 5% (w/v) of mannitol and characterized in terms of size, zeta potential and encapsulation efficiency (17).

**Cell culture and cellular differentiation**

Human myelomonocytic cell line THP-1 (ATCC TIB-202; American Type Cell Collection, Manassas, VA, USA) displaying macrophage-like activity (19) was cultured and differentiated into adherent macrophage-like cells as previously described (20).

**Bacterial strain and culture conditions**

*Brucella melitensis* 16M (ATCC 23456, biotype 1) smooth virulent strain was used for the different studies. Experiments were performed with fresh bacteria incubated in TSB medium at 37°C under shaking to the exponential growth phase (15).

**Macrophage infection with Brucella melitensis**

THP-1 macrophages (2 x 10^5 cells/well in 24-well cell culture plates) were infected with *B. melitensis* at a bacteria to macrophage ratio of 100 for 5 h at 37 °C with 5% CO₂. Cells were then washed with phosphate buffered saline (PBS) to remove the extracellular bacteria and incubated in complete RPMI medium. At 48 h post infection macrophages were washed with PBS and treated with the different gentamicin formulations at a 1 mg/L (2 times the minimal inhibitory concentration (MIC) of gentamicin against the tested strain (17)) or 18 mg/L of gentamicin (peak serum concentration in humans during conventional
gentamicin treatment, Cmax (21)). Control cells were incubated with 0.25 mg/L of gentamicin (0.5 x MIC) to prevent the extracellular bacterial growth.

After 24 h of incubation with the antibiotic treatments, cells were carefully washed with PBS and lysed. The remaining extracellular CFU in the last washing and the total CFU of *Brucella* in the lysates were determined by plating aliquots of 10-fold serial dilutions on TSA plates. The number of intracellular bacteria was calculated after incubating the plates at 37 °C by subtracting the extracellular CFU counts from the total CFU counts of each well. Results of CFU counts were log-transformed and expressed as the mean and standard deviation.

**In vivo Studies**

**Animals**

Female BALB/c mice (weight 20 g) were supplied by Harlan Interfauna Ibérica (Barcelona, Spain). Following acclimatization, mice were randomized into different groups and kept in cages with a 12-hour light-dark photocycle and water and food *ad libitum*. The experimental protocols were revised and approved by the animal experimentation ethics committee of the University of Navarra (protocol numbers 083-11 and 084-11).

**Tissue pharmacokinetics study**

Animals (n = 6 mice/group) received a single intraperitoneal dose of one of the different gentamicin formulations, equivalent to 5 mg/kg of gentamicin, in sterile 0.9 % saline. After 4 h, 8 h, 24 h, 48 h, 4 days or 7 days animals were sacrificed by cervical dislocation and their liver, spleen and kidneys were collected. Organs were then weighted, homogenized in 1 mL of sterile water (Mini-bead Beater, BioSpect Products, Inc., Bartelsville, OK, USA)
and centrifuged (10,000 x g, 10 min, 4 °C). Tissue supernatants were collected and stored at -80 °C until analysis. Prior to analysis, tissue samples were further processed for protein precipitation by the addition of a 10% trichloroacetic acid solution (1:10 v/v). Samples were then vortexed for 1 min and after centrifugation (10,000 × g, 10 min, 4 °C) protein free supernatant was collected.

Gentamicin content of the samples was analyzed by a microbiological assay using *Bacillus subtilis* ATCC 6633 as test organism. Fifty μl of the tissue samples were added into 6 mm diameter wells made in the inoculated antibiotic medium 11 agar and zones of inhibition were measured after incubation for 12 h at 4 °C and 24 h at 37 °C. In parallel, standard curves of gentamicin and GEN-AOT in control plasma and tissue homogenates were prepared using the same conditions.

Pharmacokinetic parameters were calculated by non-compartmental methods. All calculations were carried out using WinNonlin Professional Version 2.1 (Scientific Consulting, Inc., Mountain View, CA, USA). The area under the curve (AUC) for the time of administration to the last measured concentration (AUC₀₋ₜ) was calculated by trapezoidal integration. Maximum plasma and tissue concentration (Cₘₐₓ) and the time to attain peak (Tₘₐₓ) were obtained directly from the raw data.

**Toxicity studies**

For this study, mice were divided into six groups (n = 6 mice/group) that received for 14 days (i) a 0.9% saline solution, (ii) gentamicin, (iii) a combination of gentamicin and doxycycline, (iv) PCA GEN-AOT, (v) GEN-AOT loaded PLGA 752H nanoparticles, (vi) non-loaded PLGA 752H nanoparticles. Saline solution (100 μL/mice) was administered daily intraperitoneally, doxycycline (200 μg/mice, 10 mg/kg) was administered daily by
oral gavage, gentamicin (100 μg/mice, 5 mg/kg) and PCA GEN-AOT (360 μg/mice, equivalent to 100 μg of gentamicin/mice) were administered daily intraperitoneally and nanoparticles (4 mg/mice approximately, containing the equivalent to 100 μg of gentamicin/mice) were administered intraperitoneally every 4 days. At the end of treatment, overnight fasted animals were sacrificed and blood and tissue samples were collected. Hematological, biochemical and histological parameters were studied.

Blood samples were analyzed for routine hematological parameters – RBC (x10^6 cells/μL), WBC (x10^6 cells/μL), hemoglobin (g/dL), hematocrit (%), MCV (fL), MCHC (pg) and platelets (x10^3 cells/μL). Hemogram was investigated using a Roche Hematology Analyzer (Sysmex XT1800i). Biochemical parameters were investigated in serum sample – total bilirubin (mg/dL), creatinine (mg/dL) and urea (mg/dL) levels were studied by Roche semi auto analyzer (Hitachi 911) by using Roche analytical Kits.

The organs – liver, kidneys, spleen and lungs – were removed, weighed and preserved in 10% formalin for histological studies.

**Experimental infection of the mice with *Brucella melitensis***

Animals were housed in microisolator cages and handled under sterile conditions in biohazard level 3 facilities. Mice were infected with 10^5 CFU of *B. melitensis* 16 M in 0.1 mL of 0.9 % saline solution. At day 14 after infection a group of mice was sacrificed and their spleens were aseptically removed for viable bacteria count. This group of mice was used as a control for baseline infection. Of the other groups of mice, one group was kept untreated and the others received (i) free gentamicin, (ii) a combination of free gentamicin and doxycycline, (iii) PCA GEN-AOT, (iv) GEN-AOT loaded PLGA 752H nanoparticles,
(v) non loaded PLGA 752H nanoparticles. The doses were the same as those described in the toxicity studies. One and 3 weeks after the last antibiotic dose (corresponding to days 35 and 49 post-infection) animals were sacrificed and their spleens were aseptically collected. Organs were homogenized with 1 mL of 0.9% sterile saline solution and centrifuged for 10 min at 10,000 x g. The efficacy of the treatments was determined by counting the number of CFU per spleen after spreading 0.1 mL of neat supernatant and 10-fold serial saline dilutions on TSA plates.

**Statistical analysis**

Data analysis and graphical presentations were done using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Comparison of means between groups was performed by Mann-Whitney U-test. Statistical significance level was defined as $p < 0.05$.

**RESULTS**

**PCA gentamicin-AOT and gentamicin-AOT nanoparticles**

PCA-processed GEN-AOT was obtained as white powdered solid formed by micron-sized particles with a mean diameter of 1 μm and a zeta potential of around -1 mV. On the other hand, GEN-AOT loaded PLGA 502H and PLGA 752H nanoparticles presented mean diameters of 289 ± 15 nm and 299 ± 23 nm, with a polydispersion index of 0.10 ± 0.07 and 0.08 ± 0.09 and a zeta potential of -3.7 ± 0.4 mV and -3.6 ± 0.7 mV, respectively. Encapsulation efficiencies of 100% were obtained for both types of formulations with drug loadings of 23.8 ± 0.5 and 24.1 ± 0.6 μg of gentamicin/mg of nanoparticle for PLGA 502H and PLGA 752H nanoparticles, respectively.
Efficacy of gentamicin formulations against *Brucella melitensis*-infected macrophages

Under the experimental conditions, the reduction in the intracellular *Brucella* obtained by GEN-AOT formulations was studied after the treatment of the cells with an equivalent dose of 1 mg/L or 18 mg/L of gentamicin for 24 h and compared to that obtained with free gentamicin. As shown in Fig. 1, regardless of the concentration used, free gentamicin did not reduce bacterial growth. The same results were obtained for non-loaded PLGA nanoparticles. In contrast, all GEN-AOT treatments significantly reduced the intracellular infection \((p < 0.01)\) as compared to the control cells \((1.23-1.41 \log_{10} \text{ unit reduction})\). On the other hand, although no statistically significant differences were found between the different GEN-AOT formulations at 1 mg/L, treatment with 18 mg/L of GEN-AOT 752H nanoparticles was significantly more effective \((p < 0.05)\) than the other gentamicin treatments, achieving a 2.35 \(\log_{10} \text{ unit reduction}\) of the intracellular *Brucella*. Therefore, and considering previous studies with GEN 502H and 752H microparticles (15), GEN-AOT 752H nanoparticles were selected for *in vivo* studies. For comparison, the non-encapsulated form PCA GEN-AOT was also assayed. This particulate formulation was preferred over the as-synthesised GEN-AOT because of its more favourable physico-chemical properties, as PCA GEN-AOT could be prepared as a suspension in aqueous media after ultrasonication.

Pharmacokinetics study

Pharmacokinetics studies of free gentamicin, PCA GEN-AOT and GEN-AOT loaded nanoparticles were performed after a single intraperitoneal administration of a dose
equivalent to 5 mg/kg of gentamicin for each formulation. The concentrations of the antibiotic in the liver and spleen (target organs of *Brucella*) and kidney (since gentamicin is nephrotoxic) were determined. After the administration of free gentamicin no antibiotic was detected either in the spleen or in the liver (limit of detection 0.125 μg/g and 0.25 μg/g, respectively) (Figs 2A and B). In contrast, a high accumulation of gentamicin was detected in the kidneys, with measurable gentamicin concentrations for at least one week after the administration of the dose (Fig. 2C).

The AOT-coupling and encapsulation markedly altered the distribution of the antibiotic, reducing its accumulation in the kidneys and increasing it in both the liver and the spleen. Cmax and AUC values were decreased in kidneys by 3.5-fold after PCA GEN-AOT administration with respect to the non-modified free gentamicin (table 1), whereas relevant concentrations were measured in liver and spleen up to 7 days. PCA GEN-AOT presented similar AUC values in the liver and the spleen, indicating that the drug distributed equally to both tissues. However, the Cmax values achieved in the liver and the spleen with the dose administered were below the MIC against *Brucella melitensis* (1 mg/L). As *Brucella* infection is localized in those tissues, a daily administration schedule was established for PCA GEN-AOT therapy.

Encapsulation of GEN-AOT further enhanced the antibiotic tissue accumulation, especially in the spleen. Nanoparticles increased GEN-AOT’s AUC values by 10.7 and 2-fold in the spleen and the liver, respectively and Cmax by 24.4 and 4.7-fold. Moreover, antibiotic concentrations above the MIC were maintained in both organs for up to 4 days and so a therapeutic schedule of one dose every 4 days was selected for the efficacy evaluation of the formulations against mice experimentally infected with *Brucella melitensis*. 
Toxicity of the formulations in healthy mice

In all the treated groups, there was no significant alteration in hematological and biochemical parameters as compared to the saline control group, whatever the parameters measured (data not shown).

The organ’s weights were normal and no significant differences were observed among the different groups. Histological analysis of the liver, the spleen and the lungs revealed no treatment-related alterations. However, kidney examination revealed significant differences among the different groups. In the group treated with gentamicin plus doxycycline all animals presented small foci of tubulonephrosis and one animal showed focal necrosis (Fig. 3B). Moreover, the mice treated with free gentamicin presented a slight tubular lipidosis (Fig. 3A). However, no alterations were found in mice treated with PCA GEN-AOT or GEN-AOT loaded PLGA 752H nanoparticles (Figs 3C and 3D).

Efficacy of the formulations against Brucella melitensis-infected mice

The efficacy of PCA GEN-AOT and GEN-AOT nanoparticles was studied in B. melitensis-infected mice and compared to that of free gentamicin or the combination of gentamicin plus doxycycline. The administered Brucella inoculum produced a baseline splenic infection of 4.91 log$_{10}$ CFU/spleen and after the two-week treatment regimen the course of the splenic infection was monitored. The results are summarized in table 2.

In comparison with the non-treated group, no significant ($p > 0.05$) reduction of the infection was obtained in the groups of mice receiving free gentamicin and non-loaded nanoparticles. On the other hand, treatment with PCA GEN-AOT, GEN-AOT nanoparticles and gentamicin plus doxycycline resulted in a significant reduction in the bacterial load in
the spleen. Daily treatment with PCA GEN-AOT significantly reduced the splenic infection at both 1 and 3 weeks after the end of the treatment. However, this treatment was not able to sterilize the infected spleens. Regarding the combination of gentamicin and doxycycline, one week after the end of the treatment it produced a $5.2 \log_{10}$ reduction and sterilized 80% of the infected spleens (less than 10 CFU/spleen). However, thereafter, the infection reemerged and a therapeutic failure of 83% was observed at the third week after treatment. Conversely, GEN-AOT nanoparticles exerted their action more progressively, showing improved therapeutic activity over the time of the study. Thus, after 3 weeks post-treatment with the nanoparticles a $3.23 \log_{10}$ unit reduction was obtained and 50% of the animals presented no bacteria in the spleen, a percentage much higher than the 17% presented by the combined therapy.

**DISCUSSION**

Standard therapy for human brucellosis is based on six-week administration of a combination of doxycycline with rifampicin or an aminoglycoside, such as streptomycin or gentamicin, which often lead to poor patient compliance, frequent relapses and serious side effects (22). The doxycycline plus rifampicin regimen has the advantage of being a completely oral regimen, unlike aminoglycoside-containing regimens that require intramuscular administration. However, treatments including rifampicin have been shown not to be as effective as the regimens that include an aminoglycoside, and present higher relapse rates (23-26). On the other hand, although monotherapy could prevent at least some of the side effects and improve compliance with treatment, it has been associated with therapeutic failure and high relapse rates (27-29). As a consequence, there is a recognized need to improve the current treatment for human brucellosis. Appropriate antibiotics should
achieve therapeutic concentrations at the sites of infection while maintaining their activity. We have previously reported that hydrophobic modification and nanoencapsulation of gentamicin enhance its cellular accumulation and improve its efficacy against intracellular bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes* (20). Therefore, encouraged by previous results with gentamicin loaded microparticles *in vivo* (15), we evaluated the suitability of a monotherapy with hydrophobic gentamicin formulations such as PCA GEN-AOT and GEN-AOT-loaded PLGA nanoparticles.

Intracellular activity studies were carried out in human macrophages, which are the target cells of human brucellosis, using two different concentrations: 1 and 18 mg/L, corresponding to 2 times the MIC of gentamicin against the tested *B. melitensis* strain and the Cmax of gentamicin in human serum, respectively. Regardless of the concentration used, free gentamicin was not able to reduce the intracellular *Brucella* infection. This lack of efficacy was attributed to the low gentamicin accumulation inside the cells and the major distribution of the drug into the lysosomes that impedes the final encounter between bacteria and drug (20). Studies examining the intracellular fate of *Brucella* in phagocytic cells indicate that the *Brucella* organisms reside in acidified phagosomes that fuse with components of the early endosomal pathway but not with the lysosomes (30). It has also been suggested that these replicative phagosomes arise through continual interactions with the endoplasmic reticulum (31). Hydrophobic modification and encapsulation of gentamicin significantly enhanced the intracellular killing of *Brucella*, reducing the intracellular infection below the baseline control infection. Such a finding has also been previously reported for *Staphylococcus aureus* and *Listeria monocytogenes*-infected cells and was attributed to the higher cellular accumulation achieved by GEN-AOT formulations, particularly by GEN-AOT 752H nanoparticles, and the altered subcellular
distribution of the encapsulated antibiotic, which may enable it to reach the pathogen-harboring compartments at therapeutically relevant concentrations (20). In addition, it has been demonstrated with gentamicin PLGA formulations that particle uptake stimulates the oxidative burst of macrophages, which may also account for the better efficacy of the encapsulated GEN-AOT in comparison to the free form (32, 33).

On the other hand, after their entrance in the host, *Brucella* cells are taken up by the local tissue lymphocytes and transferred into the general circulation, whence they are disseminated throughout the body, with special tropism for the cells and organs of the mononuclear phagocytic system, such as the macrophages of the liver and the spleen (34). In sharp contrast, most gentamicin injected into the body is eliminated by renal clearance without being metabolized, and the rest of the dose accumulates mainly in the renal cortex, leading to the well-known nephrotoxicity of aminoglycosides (35). Consistently with these observations, after the administration of 5 mg/kg of free gentamicin no antibiotic was detected in the liver or the spleens of the mice. In contrast, a high drug accumulation was observed in the kidneys (30 μg/g).

Both conjugation of gentamicin with AOT and its encapsulation manage to increase the gentamicin concentration level in the liver and more pronouncedly in the spleen, while decreasing antibiotic accumulation in kidneys. Aminoglycosides present a concentration-dependent antimicrobial activity and thus require elevated peak levels to reach the pharmacodynamic objectives. The plasma Cmax to MIC ratio has been shown to be a good predictor of aminoglycoside therapeutic efficacy, and it is generally accepted that optimum bactericidal activity is achieved when the peak concentration is approximately 10 times the MIC (36, 37). Taking into account the *in vivo* distribution of the bacteria, special attention was paid to the ratio Cmax/MIC in the liver and the spleen.
As previously observed in cell culture models (20), nanoparticles yielded larger antibiotic levels in all the tissues studied, which was translated into higher Cmax and AUC values when compared to PCA GEN-AOT. At equivalent drug doses, nanoparticles achieved a Cmax to MIC ratio of 9 and 109 in the liver and the spleen, respectively, while these ratios were below 1.5 in both tissues for PCA GEN-AOT. Moreover, as a result of nanoparticle treatment, sustained drug levels above the MIC were achieved for 4 days in the liver and the spleen, which formed the basis for designing a treatment schedule based on a lower dosing frequency. This is an important issue to take into account, as several studies have demonstrated that extended-interval dosing, or administration of aminoglycosides in larger and less frequent doses, allow for increased bacterial killing while minimizing the associated nephrotoxicity (38-40). The toxicological and therapeutic aspects of the PCA GEN-AOT and GEN-AOT loaded nanoparticles were therefore studied and compared to those observed for gentamicin and the reference treatment of gentamicin plus doxycycline. Along the treatment, no hematological or biochemical alterations were observed. However, histological examination revealed characteristic aminoglycoside-induced renal alterations such as tubular lipoidosis and tubulonephrosis foci in those mice that had received a gentamicin solution, alone or in combination with doxycycline, respectively (35, 41). Conversely, no alterations were found in those mice receiving PCA GEN-AOT or the GEN-AOT loaded nanoparticles. Aminoglycoside-derived nephrotoxicity has been associated with the accumulation of a small percentage of the administered dose in the proximal renal tubular epithelial cells. Because of its polycationic properties, gentamicin binds to the negative charges of membrane phospholipids and enters tubular cells via endocytosis mediated by the megalin/cubilin complex (42). Therefore, the complexation of gentamicin with the anionic AOT surfactant may decrease the affinity of
gentamicin to the membrane phospholipids and endocytic receptors and thus, the uptake of GEN-AOT by these cells, and may reduce its interaction with the lysosomal or endosomal phospholipids, inhibiting the phopholipidosis, as has also been observed after the coadministration of gentamicin with some polyanions (43). Regarding nanoparticles, both a different uptake mechanism and the extended dosing interval used could protect the tubular cells from being continuously exposed to high antibiotic concentrations, minimizing the chances of renal damage.

Finally, in accordance with the undetectable gentamicin levels in the spleen, it was observed that gentamicin monotherapy did not affect the course of the murine infection. On the other hand, despite initially the combined therapy promisingly sterilized 80% of the infected mice spleens, 83% of of the animals became reinfected at the end of the study. Previous studies by Shasha, Lang and Rubinstein showed sterile spleen in 100% of the infected animals after a 14 days’ treatment with doxycycline (6-10 mg/kg) combined either with streptomycin (44) or rifampicin (6 mg/kg) (45). However, these results were obtained just after the conclusion of the treatment period. Further data at different times of sacrifice will be required in order to compare the efficacy of the different combined therapies.

Interestingly, both GEN-AOT treatments significantly reduced the splenic infection, particularly GEN-AOT loaded nanoparticles. Remarkably, with 4 nanoparticle doses the splenic Brucella infection was reduced by 3.23 log_{10} units and eliminated in 50% of the mice. Furthermore, the trend towards a greater therapeutic efficacy observed over the time of the study could indicate that the peak efficacy of the nanoparticles may not have been reached. The gentamicin-AOT loaded nanoparticles developed had superior efficacy to other drug delivery systems, such as gentamicin PLGA microparticles (15) and polymeric nanoparticles containing streptomycin and doxycycline (46), and some free antibiotics,
such as quinolones, co-trimoxazole (47) and ciprofloxacin plus streptomycin combination (44) against *Brucella melitensis* in mice.

5. Conclusions

The present study demonstrates the potential of PLGA nanoparticles for delivering sustained therapeutic GEN-AOT concentrations in the liver and the spleen, the target organs for intracellular infections such as brucellosis, with no associated toxicity. These high and sustained tissue concentrations resulted in reduced dosing frequency and improved therapeutic efficacy when compared to the free drug. Therefore, the polymeric nanoparticles developed in this study emerge as promising tools to meet the current challenges in the treatment of human brucellosis. This result, alongside the well accepted use of PLGA by medical regulatory agencies, opens promising perspectives for this novel nanomedicine. Presently, research is being carried out to optimize the dose and the duration of the nanoparticle therapy with the aim of maximizing its therapeutic efficacy. Importantly, these nanoparticles may also be useful platforms for the encapsulation of other antibiotics and the treatment of diseases caused by intracellular bacteria, such as tuberculosis.

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Figure legends:

**Fig 1:** Efficacy of 1 mg/L (horizontal lines) or 18 mg/L (diagonal lines) of gentamicin in the different formulations against intracellular *Brucella melitensis* infection in THP-1 human macrophages. Results are expressed as log_{10} of intracellular CFU per well and represented as the mean value ± S.D. of at least three independent assays made in triplicate. Dotted line indicates the intracellular *Brucella* inoculum at the beginning of the treatment. Statistical analysis: **p < 0.01 compared to the control cells; a = p < 0.05 and b = p < 0.01 compared to gentamicin-treated cells; c = p < 0.05 compared to cells treated with gentamicin-AOT, microstructured gentamicin-AOT or gentamicin-AOT PLGA 502H nanoparticles. Mann Whitney U test. Abbreviations: GEN= gentamicin, GEN-AOT= gentamicin-AOT, PCA GEN-AOT =PCA microstructured gentamicin-AOT, GEN-AOT 502H NP = gentamicin-AOT loaded PLGA 502H nanoparticles, GEN-AOT 752H NP = gentamicin-AOT loaded PLGA 752H nanoparticles, 502H NP = PLGA 502H nanoparticles, 752H NP = PLGA 752H nanoparticles.

**Fig 2:** Pharmacokinetics profile. Gentamicin concentrations (mean ± standard deviation, n = 6) in the spleen (A), liver (B) and kidneys (C) after the administration of a single intraperitoneal dose of gentamicin (GEN), PCA microstructured gentamicin-AOT (PCA GEN-AOT) or gentamicin-AOT loaded PLGA 752H nanoparticles (GEN-AOT 752H NP) equivalent to 5 mg/kg of gentamicin.
Fig 3: Kidney section obtained after administration (n = 6) of gentamicin (A), a gentamicin and doxycycline combination (B), PCA gentamicin-AOT (C) or gentamicin-AOT PLGA 752H nanoparticles (D) during 14 days (H&E, x400). The kidney showed cytoplasmic vacuolation of the tubular cells (arrows) (A), degeneration and focal tubular necrosis with cellular debris in the lumen of the renal tubules (arrows) (B) or no histopathological changes, with normal structure for the glomerulus and renal tubules (C and D).
Tables:

Table 1: Mean pharmacokinetics parameters of gentamicin and gentamicin-AOT treatments in mouse tissues after a single intraperitoneal antibiotic administration.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment</th>
<th>Cmax (μg/g)</th>
<th>Tmax (h)</th>
<th>AUC (h* μg/g)</th>
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</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>GEN</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>PCA GEN-AOT</td>
<td>1.12</td>
<td>8</td>
<td>47.04</td>
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<tr>
<td></td>
<td>GEN-AOT 752H NP</td>
<td>27.32</td>
<td>8</td>
<td>504.24</td>
</tr>
<tr>
<td>Liver</td>
<td>GEN</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td></td>
<td>PCA GEN-AOT</td>
<td>0.48</td>
<td>48</td>
<td>34.80</td>
</tr>
<tr>
<td></td>
<td>GEN-AOT 752H NP</td>
<td>2.25</td>
<td>8</td>
<td>73.92</td>
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<tr>
<td>Kidney</td>
<td>GEN</td>
<td>30.76</td>
<td>4</td>
<td>1407.12</td>
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<tr>
<td></td>
<td>PCA GEN-AOT</td>
<td>8.87</td>
<td>8</td>
<td>403.44</td>
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<tr>
<td></td>
<td>GEN-AOT 752H NP</td>
<td>23.60</td>
<td>8</td>
<td>1297.92</td>
</tr>
</tbody>
</table>

Abbreviations: AUC= area under the curve, Cmax = maximum concentration, Tmax = time to maximum concentration, GEN = gentamicin, PCA GEN-AOT = PCA microstructured gentamicin-AOT, GEN-AOT 752H NP = gentamicin-AOT loaded PLGA 752H nanoparticles, n.d. = not detectable.

Table 2: Antibacterial efficacy of the different gentamicin formulations in *Brucella melitensis* 16M-infected BALB/c mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1 week post-treatment</th>
<th>3 weeks post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log CFU/Spleen (mean ± SD)</td>
<td>Reductiona (log)</td>
</tr>
<tr>
<td>Untreated</td>
<td>5.54 ± 0.50</td>
<td>-</td>
</tr>
<tr>
<td>GEN+DOX</td>
<td>0.37 ± 0.83**, d</td>
<td>5.17</td>
</tr>
<tr>
<td>GEN</td>
<td>5.10 ± 0.25</td>
<td>0.44</td>
</tr>
<tr>
<td>PCA GEN-AOT</td>
<td>4.85 ± 0.10**</td>
<td>0.69</td>
</tr>
<tr>
<td>GEN-AOT 752H NP</td>
<td>3.69 ± 1.86**</td>
<td>1.85</td>
</tr>
<tr>
<td>752H NP</td>
<td>5.49 ± 0.30</td>
<td>0.05</td>
</tr>
</tbody>
</table>
a statistical analysis: * $p < 0.05$ and ** $p < 0.01$ compared to the untreated group at the same time of sacrifice. c = $p < 0.05$ and d = $p < 0.01$ compared to the untreated group 2 weeks post-infection (baseline infection, 4.91 log CFU/spleen). Mann-Whitney U test. b less than 10 CFU/spleen (limit of detection). Abbreviations: CFU, colony forming units; GEN, gentamicin; DOX, doxycycline; PCA GEN-AOT, PCA microstructured gentamicin-AOT, GEN-AOT 752H NP, gentamicin-AOT loaded PLGA 752H nanoparticles; 752H NP, PLGA 752H nanoparticles.