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Immunomodulatory activity of polycaprolactone nanoparticles with calcium phosphate salts against *Leishmania infantum* infectionKübra Kelleci^{1✉}, Adil Allahverdiyev², Melahat Bağirova², Murat Ihlamur^{1,3}, Emrah Şefik Abamor¹¹Department of Bioengineering, Yildiz Technical University, Istanbul, Turkey²V. Akhundov National Scientific Research Medical Prophylactic Institute, Baku, Azerbaijan³Department of Electronics and Automation, Biruni University, Istanbul, Turkey

ABSTRACT

Objective: To prepare and characterize polycaprolactone (PCL) nanoparticles loaded with sonicator fragmented (SLA) and freeze-thaw *Leishmania* antigens (FTLA) and to investigate the *in vitro* immunogenicity of antigen-encapsulated nanoparticles with calcium phosphate adjuvant.

Methods: The water/oil/water binary emulsion solvent evaporation method was used to synthesize antigen-loaded PCL nanoparticles. Particles were characterized by scanning electron microscopy and zeta potential measurements. Their cytotoxicity in J774 macrophages *in vitro* was determined by MTT analysis. In addition, the amount of nitric oxide and the level of cytokines produced by macrophages were determined by Griess reaction and ELISA method, respectively. The protective effect of the developed formulations was evaluated by determining the infection index percentage in macrophages infected with *Leishmania infantum*.

Results: Compared to the control group, SLA PCL and FTLA PCL nanoparticles with calcium phosphate adjuvant induced a 6- and 7-fold increase in nitric oxide, respectively. Additionally, the vaccine formulations promoted the production of IFN- γ and IL-12. SLA PCL and FTLA PCL nanoparticles combined with calcium phosphate adjuvant caused an approximately 13- and 11-fold reduction in infection index, respectively, compared to the control group.

Conclusions: The encapsulation of antigens obtained by both sonication and freeze-thawing into PCL nanoparticles and the formulations with calcium phosphate adjuvant show strong *in vitro* immune stimulating properties. Therefore, PCL-based antigen delivery systems and calcium phosphate adjuvant are recommended as a potential vaccine candidate against leishmaniasis.

KEYWORDS: Leishmaniasis; Calcium phosphate; Polycaprolactone; Nanoparticle; Antigen delivery system; Adjuvant; Vaccine design

1. Introduction

Visceral leishmaniasis (VL) is a vector-borne infection caused by parasites of the *Leishmania* genus that can result in death. VL, seen in more than 90 countries, is estimated to cause the death

Significance

Although many new candidate vaccines for leishmaniasis are under development, no vaccine has successfully entered the market for human use. The use of adjuvant and carrier systems is important in vaccine development strategies. Polycaprolactone nanoparticles loaded with sonicator fragmented and freeze-thaw *Leishmania* antigens stimulated the immune system to secrete Th1 cytokines such as IFN- γ and IL-12. Moreover, antigen-encapsulated nanoparticles with calcium phosphate adjuvant provided high levels of protection against leishmaniasis.

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of approximately 20 000 individuals each year[1]. The disease is frequently seen in underdeveloped or developing countries, and it is known that factors such as irregular migration, war, and drought accelerate the spread of the disease[2]. In VL cases, the parasite affects the internal organs of the host organism such as the spleen, liver, and bone marrow. First-line anti-leishmanial drugs used in the treatment of the disease have disadvantages such as drug resistance, lack of specificity, and toxicity[3]. Increasing resistance to first-line drugs and the toxicity and side effects of second-line drugs have accelerated the search for vaccine candidates that are more effective in preventing the spread of the disease. However, despite intensive studies in this field, an effective and reliable vaccine suitable for use in humans has still not been formulated[4,5]. The increasing prevalence of VL with globalization has increased the need for new vaccine research to control the disease[6].

It is known that only first-generation vaccine candidates have been accepted into phase III clinical trials so far. Studies have proven that soluble *Leishmania* antigens are successful immune system stimulants and increase the Th1-mediated response[7–11]. It should not be forgotten that the balance between Th1 and Th2 is important for the development or elimination of leishmaniasis. First-generation vaccines have advantages such as containing different antigenic structures together, being simple, cheap, and controllable in production, and not requiring costly equipment and personnel. However, these vaccines require adjuvants to increase immunogenicity. Adjuvants can be found in particulate or non-particulate form[3].

Among the mineral salts in particulate form, calcium phosphate (CaP) salt is known to be biocompatible, biodegradable, non-toxic, and easily accessible since it is a part of the biological system[12,13]. It is known that CaP salts have been the subject of many vaccine studies as adjuvants, but they have not been used as adjuvants in research on leishmaniasis.

It is also possible to use some synthetic polymers in vaccine carrier systems in particulate form. Approved by the Food and Drug Administration (FDA) for use in clinical studies, poly ϵ -caprolactone (PCL) is a biodegradable, biocompatible, and safe copolymer[14]. In addition to being used as an antigen delivery system, such as targeted and controlled release, PCL nanoparticles (PCL NPs) also exhibit adjuvant activity to ensure high antigen stability and immunogenicity. For this reason, it seems that the use of PCL NPs in vaccine studies is becoming more widespread.

When the literature was examined comprehensively, it was seen that antigen-encapsulated PCL NPs were not subject to vaccine research against leishmaniasis. It has been observed that PCL NPs have been used only in a limited number of studies as drug carrier systems in the treatment of leishmaniasis[15–18].

Considering the mentioned studies, to our knowledge, no study

to date has prepared a formulation containing freeze-thaw or sonicator-fractionated *Leishmania infantum* (*L. infantum*) antigens (FTLA or SLA) encapsulated in PCL NPs and CaP adjuvant. In this study we conducted to fill this gap, FTLA and SLA antigens were encapsulated into PCL NPs and then vaccine formulations were created at different concentrations by using CaP salt as an adjuvant. It aims to provide information about the *in vitro* immunostimulatory activity in macrophage cells with the developed formulations.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), phosphate-buffered saline (PBS), Roswell Park Memorial Institute (RPMI-1640) (GIBCO), penicillin, streptomycin, hemin, adenosine, NaOH, methyl thiazolyl tetrazolium (MTT), poly (vinyl alcohol) (PVA), PCL, dimethyl sulfoxide (DMSO) (Sigma-Aldrich), and brain heart infusion broth (Biolife) were purchased.

2.2. *L. infantum* parasite culture

L. infantum promastigotes (EP126) were planted in flasks containing RPMI-1640 medium supplemented with 10% FBS and 10 mg/mL gentamicin and incubated at 27 °C in a refrigerated oven. The development and morphological status of the parasites were examined under an inverted microscope (Olympus CKX41). Cell counts were performed with a hemocytometer (Menzel superior). Once the cells reached the required density, a gradual scale-up was started. Brain heart infusion broth was used as a medium for growing the culture. When the parasite culture in the 75 cm² flask reached 10⁷ parasites/mL, the parasites were transferred to a 1 L conical flask. Then 350 mL of brain heart infusion broth medium was added. Four days later, a fresh medium was added and the volume was doubled. During the incubation period, the parasite culture in the flask was incubated on the orbital shaker to ensure a homogeneous environment. In this way, we worked with 4 conical flasks at a time. The medium taken from the conical flasks was centrifuged in 50 mL falcons at 4 °C at 4000 rpm for 25 min. The pellet in the falcons was washed with 1 mL of PBS and collected in a 50 mL falcon[19]. Then, the supernatant was removed by centrifugation at 4000 rpm at 4 °C for 15 min. The resulting pellet was collected in a 50 mL falcon and stored at –20 °C until digestion.

2.3. Preparation of FTLA

In the freeze-thaw method, parasites removed from –20 °C were

thawed in a water bath at 37 °C by adding 1 mL of PBS in a 15 mL falcon. The thawed cells were then placed in a liquid nitrogen container to be covered with liquid nitrogen and the parasites were allowed to freeze for 15 min. The frozen parasites were thawed by keeping them in a water bath at 37 °C for 15 min. The process of freezing the parasites suspended in PBS in 15 mL falcons with liquid nitrogen and thawing them in a water bath was repeated 5 times, then centrifuged at 10000 rpm for 3 min and the supernatant was taken. Determination of the amount of protein in the lysate was carried out using the Warburg-Christian method on a UV spectrometer at wavelengths of 280 nm and 260 nm. The amount of protein in the lysate was calculated according to Equation 1[19].

The amount of protein = $[(A_{280} \times 1.5) - (A_{260} \times 0.75)] \times \text{dilution coefficient}$ (1)

2.4. Preparation of SLA

Parasite cells in a 15 mL falcon at -20 °C were vortexed. It was centrifuged at 1000 rpm for 5 min. After centrifugation, the supernatant was discarded. Cells were then suspended with 1 mL of PBS. A 15 mL falcon was connected to the sonicator probe and subsequently placed in a conical flask filled with ice water and surrounded by ice.

The sonicator was set for 40 s and 3 cycles. The power potentiometer of the sonicator generator was set to 40%. The sonication process was repeated 5 times. After sonication, the falcon was centrifuged at 10000 rpm for 3 min. Then the supernatant in the falcons was taken and the amount of protein in the antigen was measured using a spectrophotometer (Shimadzu) at 260 nm and 280 nm. The amount of protein was determined according to Equation 1.

2.5. J774 macrophage cell culture

J774 murine macrophage cell (ATCC) culture was carried out in 75 cm² (polystyrene surface, NEST) flasks using an appropriate medium. Stock medium was prepared by adding 1% penicillin-streptomycin into RPMI-1640 containing 1% *L*-glutamine. Cell culture was carried out by adding 10% FBS into the stock medium and under incubation conditions of 37 °C, 95% humidity, and 5% CO₂.

2.6. Preparation of antigen-loaded polymeric NPs

SLA or FTLA-loaded PCL NPs were prepared by the water/oil/water double emulsion solvent evaporation method. In brief, 10 mg of the antigens (SLA or FTLA) in pellet form were weighed and dissolved in PBS and 5 mL of 3% PCL solution (3 mg PCL/100 mL dichloromethane) was added. A sonicator (80% amplitude for 2 min,

100 W electrical input power) was used to emulsify the aqueous phase. The first emulsion was added to 15 mL of 1% PVA solution (1 mg PVA/100 mL Ultrapure sau) with the help of a syringe. It was emulsified under the same conditions to achieve double emulsion. A magnetic stirrer was used under a fume hood for 4 h at room temperature to remove dichloromethane (organic phase) from the environment. The resulting particles were centrifuged at 14000 rpm at 4 °C for 45 min and washed 3 times with PBS. Free NPs without antigen loading were similarly encapsulated in pure water (equal volume) to be used as a control group. All of the created NPs were dried in a lyophilizer for 48 h and stored at -80 °C for later use.

2.7. Characterization of NPs

2.7.1. Scanning electron microscope (SEM) analysis

SEM was used to determine the surface morphology and dimensions of NPs. For this purpose, a small number of NPs were placed on a double-sided carbon tape, coated with gold under a vacuum, and analyzed at 5-10 kV at different resolutions.

2.7.2. Zetasizer analysis

The zeta potential, particle size, and polydispersity index (PDI) of the obtained NPs were measured by dynamic light scattering (DLS) technique (Malvern Zetasizer, UK). During measurements, all samples were diluted 1/10 with ultrapure water. It was performed 3 times at room temperature.

2.7.3. Encapsulation efficiency and antigen loading capacity

Using the bicinchoninic acid assay method, antigen loading capacity and encapsulation efficiency were calculated according to Equation 2 and Equation 3[20]. Analyses were performed using the supernatants obtained in each NP formulation.

Encapsulation efficiency (%) = $\frac{\text{Amount of encapsulated antigen (mg)}}{\text{Amount of antigen used initially (mg)}} \times 100$ (2)

Antigen loading capacity (%) = $\frac{\text{Amount of encapsulated antigen (mg)}}{\text{Amount of NP produced (mg)}} \times 100$ (3)

2.7.4. In vitro antigen release test

To determine *in vitro* antigen (FTLA or SLA) release profiles from NP formulations, the method proposed by Arasoglu *et al.* has been modified[21]. Briefly, under physiological conditions (pH: 7.4), 15 mg of each antigen-loaded NP was added with 10 mL PBS and incubated in a horizontal shaking incubator at 37 °C at 150 rpm. At specified time intervals (1 h, 2 h, 4 h, 6 h, 8 h, and days 1, 2, 3, 4, 5, 6, 11, 16, 21, 26, 31, 36), the medium was completely removed and replaced with fresh PBS. The bicinchoninic acid assay was used to detect the amount of antigen.

2.8. In vitro analyses

2.8.1. MTT analysis

The toxicity of the developed NP formulations was determined according to MTT cell viability analysis. Briefly, 1×10^4 J774 macrophage cells were seeded in 96-well plates and incubated to adhere overnight at 37°C. Nonadherent cells were then removed and 100 µL of the nanovaccine formulation was added and incubated for 48 h.

Then, 10 µL of MTT salt was added to each well and the cells were incubated at 37°C for 4 h. A total of 100 µL DMSO was added to dissolve the formazan crystals formed in the living cell. Optical density (OD) values were determined at 570 nm in the ELISA reader (Thermo Scientific) by incubating for 30 min. Cell viability percentage was determined according to Equation 4. Since MTT salt is sensitive to light, the studies were carried out in a dark environment. Each run was validated in 3 replicates.

Cell viability = OD value in the experimental group / OD value in the control group $\times 100$ (4)

2.8.2. Nitric oxide (NO) production amount

Griess reaction was used to determine the effect of the developed nanovaccine formulations on the amount of NO secreted in macrophages. In brief, macrophages were seeded into 96-well well plates at 1×10^4 cells/mL and cultured at 37°C for 24 h. The cells were then incubated at 37°C for 2 d with suspensions containing the developed nanovaccine formulations in different concentrations. Supernatants were collected and NO determination was made. Nitrate solution (NaNO_2) was diluted with PBS into 96-well well plates. A diluted medium was used as a blank. Then 50 µL sample supernatants were added to the wells with 50 µL Griess reagents (1% sulfanilamide, 0.1% naphthyl ethylenediamine, and 2.5% H_3PO_4) and incubated at room temperature for 10 min. Absorbance values in each well were evaluated at 540 nm with the help of an ELISA reader (Thermo Scientific). The results were interpreted according to a standard curve constructed against known concentrations of NaNO_2 . Each run was validated in 3 replicates.

2.8.3. Determination of cytokine production

Commercial ELISA kits (Ebioscience, Inc.) were used to determine cytokine levels produced in J774 macrophage cells treated with NP formulations. Cells were seeded at 10^4 cells/100 µL in each well of 96-well well plates and incubated for 24 h. Then, vaccine formulations at a concentration of 200 µg/mL were added to the cells and incubated for 3 d. The concentrations of IFN- γ , IL-12, IL-10, and IL-4 in the supernatant were determined according to the manufacturer's instructions.

2.8.4. In vitro macrophage infection by *L. infantum*

Sterile coverslips with a diameter of 12 mm were placed in each well of the plates. In each well with a coverslip placed, 2×10^4

J774 macrophage cells were seeded in RPMI 1640 medium and incubated for 24 h at 37°C in an oven containing 5% CO_2 . The next day, the macrophage cell/parasite ratio was 1/10 log. Then 2×10^5 *L. infantum* promastigotes in the phase were added to the macrophage cells. The plates prepared in this way were incubated for 4 h at 37°C in an oven containing 5% CO_2 to support the transformation of promastigotes into intracellular amastigotes and to ensure infection of macrophages. All wells were washed three times with 1 mL of PBS to remove uninfected promastigotes. To support the proliferation of *L. infantum* amastigotes in infected macrophages, 1 mL of fresh medium was added to each well and incubated at 37°C for 48 h. Then, infection index analysis was performed to determine the anti-leishmanial effects of the developed vaccine formulations on *L. infantum* amastigotes.

2.8.5. Infection index analysis

Giemsa staining method was used to evaluate the infection index analysis. Non-toxic concentrations of the developed vaccine formulations were transferred to wells containing infected macrophages and incubated at 37°C for 48 h. After incubation, all media in the wells were removed and the wells were washed three times with 1 mL of PBS. Then, the wells were washed with 500 µL of PBS-methanol (1:1.5) solution, and 250 µL of methanol was added to each well to fix the macrophages and incubated for 10 min at room temperature. After incubation, the methanol in the wells was removed from the medium and the macrophages were exposed to Giemsa dye for 5 min. Approximately 200 µL of Giemsa dye was added to cover the entire surface of the coverslips. After 5 min, the dye was removed from the medium and all wells were washed with 1 mL PBS. The 12 mm lamellae in the wells were removed and examined under an inverted microscope (Olympus CKX41) with an immersion structure at 100 $\times$ magnification.

To measure the infection index rates of all samples, a certain number of macrophages were determined in each coverslip and the number of macrophages infected with *L. infantum* parasites among these selected macrophages was counted under the microscope.

Calculation of the % infectivity value was obtained by dividing the number of infected macrophages by the number of all macrophages in the examined coverslip. Then, the average number of amastigotes in a macrophage was evaluated by calculating the number of amastigotes in macrophages. The infection index of macrophages was calculated by multiplying the average number of amastigotes per macrophage and the % infection rates.

2.9. Statistical analysis

Data were statistically analyzed for significance levels using ANOVA analysis of variance. Differences between means were determined using Tukey's *post hoc* test. It was considered significant if the probability level was $P < 0.05$. Repeated measurements are given as mean $\pm$ standard deviation.

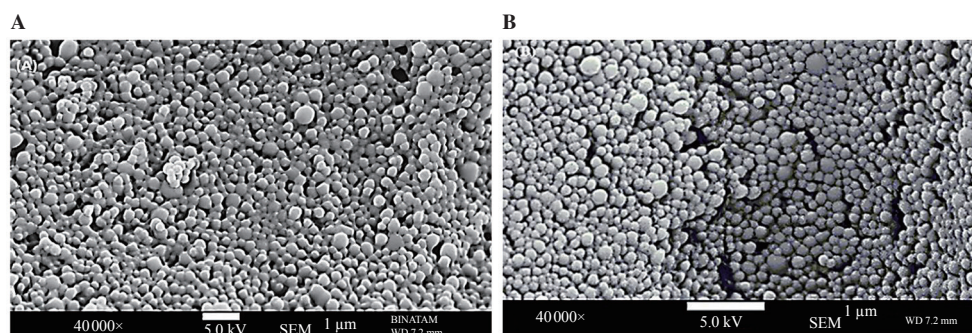


Figure 1. Scanning electron microscope images of *Leishmania* antigen-encapsulated polycaprolactone nanoparticles prepared by (A) sonicator (SLA) and (B) freeze-thaw method (FTLA).

3. Results

3.1. Characterization of NPs

3.1.1. SEM analysis results

SEM analysis showed that antigen (SLA or FTLA) encapsulated PCL NPs have spherical, homogeneous dimensions and smooth surface morphology. SEM images are shown in Figure 1.

3.1.2. Particle size, PDI, and zeta potential results

The average particle sizes of blank, SLA, and FTLA encapsulated PCL NPs were (229.5 ± 1.4) , (217.0 ± 12.1) , (237.7 ± 3.8) nm, respectively, and PDI values were (0.12 ± 0.01) , (0.08 ± 0.21) , (0.10 ± 0.11) , respectively. In addition, zeta potentials were (-5.00 ± 0.20) , (-13.7 ± 2.0) , and (-13.8 ± 1.2) mV, respectively.

The results show that the obtained particles were nano-sized and had a narrow size range with a homogeneous structure. According to the zeta potential values, it was seen that the particles had high stability.

3.1.3. Encapsulation efficiency and antigen loading capacity

The encapsulation efficiency was found to be 51.3% and 56.6% for SLA and FTLA, respectively. The loading efficiency was determined to be 5.50% and 8.62%, respectively. According to the results, both values of FTLA were higher than those of SLA.

3.1.4. In vitro antigen release efficiency

To determine antigen release profiles, a calibration graph was created using absorbance values versus antigen concentration. Figure 2 shows the two-phase release graph of SLA PCL NPs and FTLA PCL NPs. At the end of the first day, a rapid release of 18.0% and 13.7% occurred in SLA PCL NPs and FTLA PCL NPs, respectively, while a continuous and slow release occurred in the following periods. At the end of the 864th hour, a release of 83.15% was achieved in SLA PCL NPs and 85.30% in FTLA PCL NPs.

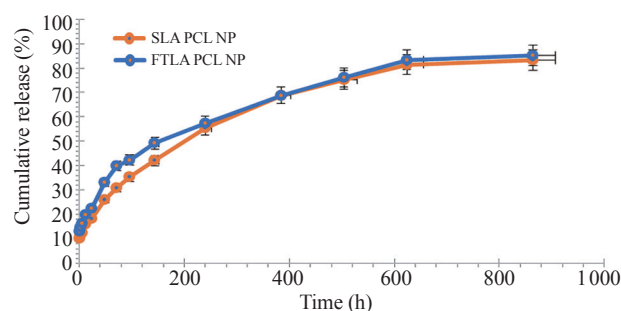


Figure 2. Antigen release graph in PCL NPs. Data are expressed as mean $\pm$ SD of three experiments. SLA PCL NP: *Leishmania* antigen-encapsulated polycaprolactone nanoparticles prepared by sonicator method. FTLA PCL NP: *Leishmania* antigen-encapsulated polycaprolactone nanoparticles prepared by freeze-thaw method.

3.2. In vitro analysis results

In vitro analyses were performed with both antigen + adjuvant formulations and antigen-loaded NPs and adjuvant formulations.

3.2.1. MTT results

Cell viability results are shown in Figure 3 and Table 1. The empty and antigen-loaded PCL NPs did not cause significant toxic effects on macrophage cells. The IC_{50} value for each NP was found to be over 1000 μ g/mL. In addition, there were slight changes in the cell viability of macrophages exposed to PCL NPs at concentrations ranging from 25 to 200 μ g/mL.

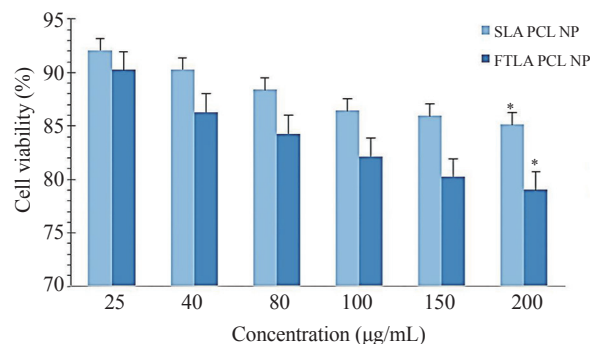


Figure 3. Cell viability of antigen-loaded PCL NPs at different concentrations. Data are expressed as mean $\pm$ SD of three experiments. * $P < 0.05$ compared to the control group.

3.2.2. NO production

Figure 4 shows that NO production increased with increasing concentration with the highest value obtained at 200 µg/mL (approximately 2.25 times for SLA PCL NPs and 2 times for FTLA PCL NPs). However, there was no noticeable variation at 25 µg/mL of both SLA and FTLA PCL NPs compared to the control group.

In Table 1, the amount of NO released by macrophages in the control group was 4.28 pg/mL. For free PCL NPs, NO level was 6.58 pg/mL, which had no significant difference in contrast with the control group. The SLA PCL NP + CaP and FTLA PCL NP + CaP vaccine formulations caused an approximately 6- and 7-fold increase in the amount of NO secreted from macrophages, respectively, as compared to the control group ($P < 0.01$). Moreover, the SLA + CaP and FTLA + CaP groups demonstrated an approximately 2-fold increase in NO level compared to the control group. These results show that the immunomodulatory performance of antigen-loaded NPs was strong.

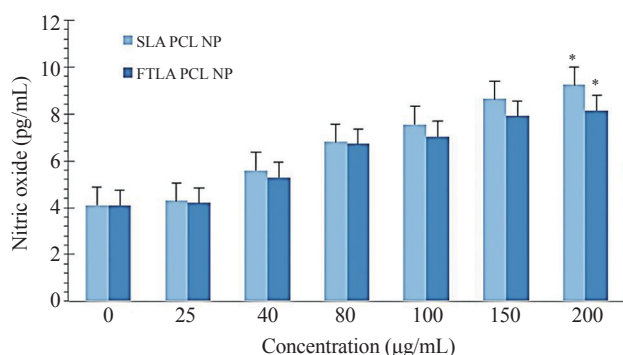


Figure 4. Nitric oxide level of antigen-loaded PCL NPs at different concentrations. Data are expressed as mean $\pm$ SD of three experiments. * $P < 0.05$ compared to the control group.

Table 1. NO amount and % cell viability values in macrophage cells treated with different formulations.

Groups	NO (pg/mL)	% Cell viability
Control	4.28 $\pm$ 0.21	100.00 $\pm$ 0.35
Free PCL	6.58 $\pm$ 0.95	91.02 $\pm$ 0.14
FTLA	6.55 $\pm$ 1.24	82.26 $\pm$ 0.01
SLA	6.12 $\pm$ 0.17	85.74 $\pm$ 0.11
FTLA+CaP	7.94 $\pm$ 0.03	73.34 $\pm$ 0.08
SLA+CaP	8.10 $\pm$ 1.15	83.02 $\pm$ 0.10
SLA PCL	9.24 $\pm$ 0.46	86.16 $\pm$ 0.34
FTLA PCL	8.16 $\pm$ 2.04	78.94 $\pm$ 1.01
FTLA PCL+CaP	29.31 $\pm$ 0.23*	82.75 $\pm$ 0.03
SLA PCL+CaP	26.10 $\pm$ 0.07*	85.34 $\pm$ 0.72

NO: nitric oxide, PCL: poly ϵ -caprolactone, FTLA: freeze-thaw-fractionated *Leishmania infantum* antigens, SLA: sonicator-fractionated *Leishmania infantum* antigens, CaP: calcium phosphate adjuvant. Data are expressed as mean $\pm$ SD of three experiments. * $P < 0.05$ compared to the control group.

3.2.3. Amount of cytokines produced by macrophages treated with different formulations

In macrophages, antigen-loaded PCL NPs induced significant increases in IL-12 and IFN- γ levels as compared to the control

group. As seen in Figure 5, IL-12 levels produced by macrophages exposed to both unencapsulated and encapsulated SLA and FTLA antigens were significantly higher than the control group. It was also observed that the level of IL-12 in macrophage cells treated with free or encapsulated antigens was higher than that in cells treated with empty PCL, and the highest IL-12 level was in the groups treated with SLA and SLA+CaP. SLA and FTLA antigens increased the amounts of IL-12 approximately 6-fold in contrast to the control ($P < 0.01$). Antigen-loaded PCL NPs triggered macrophages to produce approximately 4-fold higher levels of IL-12 in contrast to the control ($P < 0.01$). A similar trend was observed in IFN- γ production. Vaccine formulations developed with the use of only antigen produced more IFN- γ than nano vaccine formulations containing the antigen. The highest IFN- γ production was found in the SLA + CaP group.

On the other hand, there was no significant change in IL-10 and IL-4 levels in macrophages treated with free antigens or nanoparticle formulations. The highest level of IL-10 was obtained in the SLA + CaP formulation, while the highest level of IL-4 was found in cells treated with FTLA alone.

3.2.4. Infection index values of macrophages

Microscopic images of J774 murine macrophages infected with *L. infantum* promastigotes at 24 and 48 h are shown in Figure 6. The results showed that 80% of the macrophages were infected at the end of the 24th hour. In the control group, the macrophage infection index was found to be 550. This value was found to be 234 and 260 in the SLA + CaP and FTLA + CaP groups, respectively, and 43 and 50 in the SLA PCL NP + CaP and FTLA PCL NP + CaP groups, respectively.

The SLA + CaP and FTLA + CaP formulations caused a 2-fold reduction in infection index compared to the control group. In addition, SLA PCL NP + CaP and FTLA PCL NP + CaP formulations caused a decrease of approximately 13 and 11 times, respectively, compared to the control group. Therefore, it can be said that nanoparticle formulations show approximately 5 times more anti-leishmanial effect than formulations in which only antigen is used.

4. Discussion

In this study, FTLA PCL NP + CaP and SLA PCL NP + CaP formulations, whose effectiveness against VL was investigated *in vitro*, were developed. It was proven that both SLA and FTLA encapsulated PCL NPs and CaP adjuvant could be used to induce immunity against leishmaniasis. The study results exhibited that macrophage cells treated with SLA and FTLA-encapsulated PCL NPs combined with CaP adjuvant had strong NO production ability.

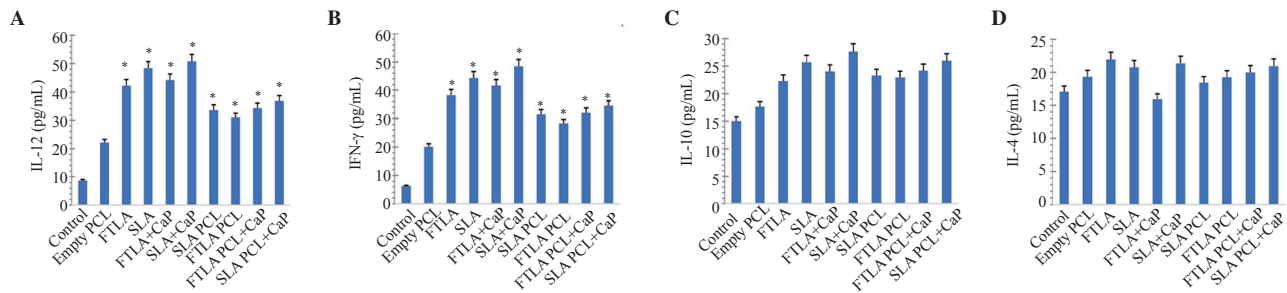


Figure 5. Levels of (A) IL-12, (B) IFN- γ , (C) IL-10, and (D) IL-4 secreted from macrophages treated with different formulations. Data are expressed as mean $\pm$ SD of three experiments. * $P < 0.01$ compared to the control group.

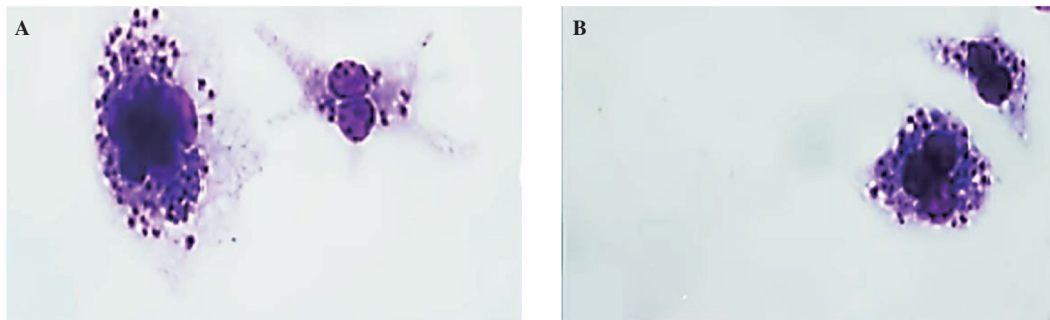


Figure 6. Images of infected macrophage cells at (A) 24 and (B) 48 h (magnification: 100 $\times$).

It resulted in a 6- and 7-fold increase, respectively, compared to the control group.

Additionally, the developed formulations supported a strong Th1-related cytokine production. The IL-12 and IFN- γ production of the SLA + CaP formulation is 6 and 8 times higher, respectively than the control group. SLA PCL NP + CaP formulation provided a 4- and 5.5-fold increase, respectively. While IL-12 and IFN- γ production in macrophage cells treated with FTLA + CaP formulation increased 5- and 7-fold, respectively (compared to the control group), a 4- and 5-fold increase was obtained with FTLA PCL NP + CaP. There was no significant change in IL-4 and IL-10 levels in all formulations.

Although many new candidate vaccines have been developed, no vaccine has successfully reached the market for human use. Among all these studies, it is known that the first-generation vaccine candidates are much more controllable, reliable, and cheaper. It has been observed that there are a limited number of studies in which *Leishmania* antigens obtained by freezing-thawing and sonicator disruption were used as vaccine candidates against VL[22–25]. There are some basic reasons for the failure of vaccine studies against leishmaniasis. The first of these reasons is that the correct adjuvant cannot be selected[26,27]. CaP is a tolerable, biocompatible, and biodegradable mineral salt found in particulate form[28]. Although many studies have been conducted on the adjuvant feature of CaP, none of them have been conducted to determine its protective ability against leishmaniasis. In this study, we demonstrated the immunogenicity of CaP adjuvant towards VL.

Another reason is that antigens cannot be transported to the correct

cells (antigen-presenting cells and dendritic cells)[29,30]. Very small-sized NPs can be easily phagocytosed by these cells. Therefore, NPs are thought to play a key role in transporting the relevant antigen to target sites[31,32]. PCL polymer is a biocompatible, biodegradable, easily obtainable polymer that is approved for clinical use by the FDA. Due to its slow degradation profile, PCL provides a long-term degradation profile with drug/antigen release reaching up to several days/months.

It is known that NPs will stimulate the immune system for a long time and create a strong immune response due to their continuous and long-term antigen-release properties. For this reason, PCL NPs interact with antigen-presenting cells in the long term and have adjuvant properties, providing durable protection against leishmaniasis. When the literature was examined, we could not find any previous study examining the transport of *Leishmania* antigens by PCL NPs.

No study has been conducted to examine the *in vitro* immunostimulatory activity of FTLA and SLA encapsulated PCL NPs isolated from *L. infantum* parasites and combined with a mineral salt such as CaP, whose immunogenicity towards leishmaniasis has not been previously investigated, and to evaluate their protective effect as a vaccine candidate.

In this study, the double emulsion solvent evaporation method was used to encapsulate *L. infantum* antigens, which were fragmented in the sonicator and obtained by the freeze-thaw method, into PCL NPs. The properties of the synthesized NPs were defined according

to empty NPs. The particle size of SLA NPs and FTLA NPs were (217.0 ± 12.1) and (237.7 ± 3.8) nm, and the encapsulation efficiency was 51.3% and 56.6%, respectively. The measured negative zeta potential of the synthesized NPs shows that they have high stability. When the antigen release profiles are examined, it is seen that at the end of the 36th day, 83.15% release was achieved in SLA PCL NPs and 85.30% release was achieved in FTLA PCL NPs. It can be said that the release difference between the two particles is due to factors such as particle size, low swelling of the polymer, and diffusion in the release medium.

NO is a powerful killer that prevents the proliferation of microorganisms[33]. NO production is stimulated by Th1-mediated IFN- γ . If the infection spreads, NO production is inhibited by suppressing IFN- γ . Therefore, knowing the amount of NO in macrophages will provide information about the intracellular fate (development or elimination of infection) of the *Leishmania* parasite. The vaccine formulation to be developed is also required to support high amounts of NO production. In previous studies, the NO production abilities of soluble *Leishmania* antigens were investigated[34–37].

In a study, autoclaved (ALA) or soluble *Leishmania* antigens (SLA) and lipophosphoglycan antigens were encapsulated in PLGA NPs and their antileishmanial activity was investigated both *in vitro* and *in vivo*. As a result, antigen-loaded NPs increased the amount of NO released from macrophages by 14 and 18 times compared to the control group. It has been reported to provide approximately 80% protection against VL[36].

In our previous study[19], the *in vitro* antileishmanial activity of *L. infantum* antigen obtained by the freeze-thaw method was investigated by using Freund's adjuvant (complete and incomplete), which is a classical adjuvant, by determining NO activity. It was found that there were 78%–89% cell viability in J774 macrophage and L929 fibroblast cells. NO production abilities (a 37% increase compared to the control group) were obtained with incomplete Freund's adjuvant (40 μ g/mL formulation).

In this study, we also performed *in vitro* cytokine determination. It is known that the Th1-Th2 balance is important to ensure effective immunity against leishmaniasis. Th1 immune response-mediated cytokines (IL-12 and IFN- γ) are associated with the elimination of the disease, and Th2 immune response cytokines (IL-4 and IL-10) are linked with the development of the disease. Therefore, an ideal vaccine candidate would be required to stimulate Th1 cytokines[38,39].

According to our results, both vaccine formulations (SLA + CaP or FTLA CaP) and nano vaccine (SLA PCL NP + CaP or FTLA PCL NP + CaP) formulations significantly increased IL-12 and IFN- γ (Th1 immune response) production. However, no significant change was observed in IL-4 and IL-10 cytokine levels in all formulations.

Therefore, the formulations we developed further induce Th1 cytokine production, which can be targeted for the elimination of leishmaniasis.

The study also determined the infection index value in macrophages. For this purpose, macrophage cells infected with *L. infantum* were treated with the developed formulations. As a result of the treatment, effective protection was provided with both vaccine formulations CaP (SLA + CaP or FTLA + CaP) and nano vaccine formulations (SLA PCL NP + CaP or FTLA PCL NP + CaP). A 2-fold protection was provided with vaccine formulations, and 13-fold and 11-fold protection was provided with nano-vaccine (SLA PCL NP + CaP or FTLA PCL NP + CaP) formulations, respectively. Therefore, an average of 5-fold protection has been developed by using antigen carrier vehicles such as PCL. Thus, we have shown that the use of polymeric NPs, which not only carry antigens but also have adjuvant properties, can increase the immunogenicity of *Leishmania* natural molecules and can be used as a potential vaccine candidate. Further studies can be conducted on preparing antigen cocktails into polymeric NPs and further improving the adjuvant properties of CaP salts to increase immunity against leishmaniasis in the future.

In conclusion, the nano vaccine formulations we developed increased the amount of NO produced by macrophages *in vitro* and promoted the production of Th1 cytokines. In infected macrophage cells, significant protection was provided with nano-vaccine formulations. All these results show that the formulations we developed are powerful immune system stimulants, suggesting the development of a nanoparticle-based adjuvant and antigen delivery system to overcome the disadvantages of current *Leishmania* vaccine development formulations.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

KK and MI conducted the experimental study and analyzed the data. KK prepared the draft and revised the manuscript. EŞA and AA developed the work. EŞA supervised the study. AA and MB reviewed the draft text. All authors read and approved the final version of the manuscript.

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