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THE STUDY OF SYNTHETIC PEPTIDE LOADED PLGA NANOPARTICLES CYTOTOXICITY *IN VITRO*

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ABSTRACT

Canine parvovirus (CPV) is a viral disease which is seen widely worldwide and infects puppies. Current vaccines which developed against this infection have several disadvantages. The antigenic property of different peptide sequences on VP2 capsid protein which belongs to CPV has been shown in different studies. However, carriers or adjuvants are also required for peptide antigens which have small molecular size and weak immunogenicity.

Recently, nanoparticulate vaccine studies have begun against variety of infectious diseases. But, there is no vaccine study based on nanoparticle in CPV infections. In this study, cytotoxicity of W-1L19 peptide loaded poly(lactide-*co*-glycolide) (PLGA) nanoparticle systems was examined for the first time on mouse fibroblast L929 cell lines. A microculture tetrazolium salt reduction assay was used to determine the cellular toxicity of the CPV W-1L19 synthetic peptide and PLGA nanoparticles [empty (NP1), peptide loaded (NP2) and peptide+FITC dye loaded (NP3)]. The obtained results showed that the toxicity of free peptide is enhanced by loading to biocompatible PLGA nanoparticles. For the first time in this study it is shown that W-1L19 peptide loaded PLGA nanoparticle systems are non-toxic. Thus, these results suggest that it is possible for use these nanoparticular system as a vaccine candidate in future.

KEYWORDS:
Canine parvovirus, poly(lactide-co-glycolide) nanoparticles, L929 cell line, in vitro cytotoxicity, PLGA

INTRODUCTION

Canine parvovirus (CPV) remains a significant worldwide canine pathogen [1], is the cause enteric and myocardial disease in dogs [2,3]. In experimentally affected dogs, mortality without treatment has been reported as high as 91% [1,4].

Recently, Langeveld at all show that two peptides from VP2 capsid of Canine parvovirus, 1L15 (MSDGAVQPDGGQPAV) and 7L15 (QPDGGQPAVRNERAT), with the partially overlapping sequence have different potencies in inducing virus-neutralizing antibodies, produce good immune response in mice and immunogenic in several animal species [5,6].

Conventional CPV vaccines are based upon live or inactivated virus and recombinant vaccine [7], in contrast to some disadvantage of these vaccine, synthetic peptide vaccines are safe and easy to produce [6]. However, synthetic peptides are poorly immunogenic without adjuvant, in order to elicit a higher immune response and improve the efficiency of peptide-based vaccine, it is generally necessary to use a carrier system such as protein, polymer or nano-micro particles.

Nanoparticles (NPs) have been used as adjuvants in vaccine [8] and have novel physical/chemical properties as effective carriers [9]. In recent years, PLGA and PLA (poly lactic acid) micro-nanoparticles for administrations of vaccine antigens have received much attention.

In particular, biodegradable PLGA based polymeric nanoparticles are used as carriers for controlled delivery and targeting of anticancer drugs, analgesics, antibiotics and macromolecular drugs such as proteins, peptides, genes, vaccines, antigens, human growth factors, vascular endothelial growth factors etc. [10,11]. In this respect, nanoparticular carriers may offer some advantages for site-specific delivery, an increased intracellular penetration, a protection of the antigenic peptides against enzymatic and hydrolytic degradation [12,13]. PLGA or poly(dl,lactic-co-glycolic acid) is approved by Food and Drug Administration (FDA) and widely used copolymer for therapeutic devices, owing to its biodegradability and biocompatibility [14].

Cell viability assays are widely used to evaluate the safety of biologically applied materials [15]. Nanomaterials of the same composition would have different biologic responses for different morphologies. The advantages of the *in vitro* approach includes simplicity, consistency of the experimental setup, and the reproducibility of the

experimental results [10]. The colorimetric MTT [3-(4,5-dimehtylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is widely used *in vitro* for measuring cellular metabolic activity, proliferation of living cells, and cytotoxicity of new materials [16,17]. The MTT is a soluble yellow tetrazolium salt which is broken by the active mitochondrial dehydrogenase, forming insoluble, purple formazan crystals. The concentration of crystals can be read spectrophotometrically. The viable cells are detected through a colored signal depending on their mitochondrial activity compared with a non-irradiated control [18].

In this study, cytotoxicity of W-1L19 synthetic peptide loaded (PLGA) nanoparticle systems was examined first time in literature on mouse fibroblast L929 cell lines. Thus, in this study the effect of empty (NP1), peptide loaded (NP2) and peptide+FITC loaded (NP3) nanoparticles and W-1L19 peptide on L929 cell line examined by using light microscopy, cell survival-proliferation and standard cytotoxicity assays. Furthermore, the relative biocompatibility of these nanoparticles is discussed in comparison to peptide.

METHODS

Materials. Poly (DL-lactic-co-glycolic acid) (50:50) ([η] =0.45-0.60 dL/g, Mw ~ 38-54 kDa), 3-(4,5-dimetil triazol-2-il)-2,5-difeniltetrazoliumbromid (MTT), dimethyl sulfoxide (DMSO), Fluorescein isothiocyanate (FITC) and polyvinyl alcohol were purchased from Sigma Aldrich (St. Louis, USA), dichloromethane (DCM) was purchased Ridel de Haen. Synthetic peptide representing W-1L19 from the VP2 capsid protein of Canine Parvovirus (W-MSDGAVQPDGGQPAVRNERA) was synthesized via solid phase peptide synthesis method by Caslo Laboratory ApS (Denmark). The L929 mouse fibroblast cell line was kindly provided by the Department of Bioengineering, Ege University, Izmir, Turkey. Ultra-pure water was obtained from Millipore MilliQ Gradient system.

Preparation of PLGA Nanoparticles. PLGA nanoparticles [empty (NP1), peptide loaded (NP2) and peptide+FITC loaded (NP3)] were prepared with double emulsion solvent evaporation method. Briefly, 150 mg PLGA were dissolved in 2 ml DCM and emulsified with 200 µl of a peptide aqueous solution (5 mg/ml) in an ice-water bath to form primary oil-in-water emulsion (for NP1 200 µl ultra-pure water, for NP3 200 µl of a 5mg/ml peptide+0.1mg/ml FITC solution were used). Thereafter, the resulting emulsion (w/o) was poured into 4 ml PVA (% 2,5 w/v) aqueous solution and emulsified in an ice-water bath to form the double emulsion (w/o/w). The emulsifications were carried out using micro tip probe sonicator set at 55 W of energy output (Bandelin-sonopuls) for 2 min over an ice-water bath. The double emulsion (w/o/w) was diluted in 80 ml PVA (% 0.5 w/v) solution and the emulsion was stirred overnight on a magnetic stirrer plate at room temperature for evaporation of organic phase. The resulting dispersion of nanoparticles were centrifuged at 10.000 x g for 20 min (Sartorius-Biofuge), washed three times with ultra-pure water to remove PVA and then lyophilized for 2 days.

Morphologic Analysis Using an Inverted Microscope. Morphological investigations of nanoparticles and L929 mouse fibroblast cell line (treated with peptide, NP1, NP2 and NP3) were carried out using a phase-contrast inverted microscope (Olympus, CKX41, Germany) at 20x and 40x magnification to observe morphological properties of nanoparticle in solutions and morphological changes of cells such as; the cell shrinks, defect or deformation, differentiation and loses contact to its neighboring cells. Nanoparticles and peptide were used at 0.05; 0.1; 0.2; 0.4 and 0.5 mg/ml concentrations and the untreated cells served as the control. Photographs were taken with an Olympus C-5050 digital camera, using both light tubes.

Investigation of toxic effects of PLGA nanoparticles on L929 cells with MTT Assay. The cytotoxicity profile of peptide and the nanoparticles was studied by the colorimetric MTT assay which described by Mosmann [19]. In MTT assay, respiring cells (live cells) reduce the yellow 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) to purple formazan crystals by dehydrogenase enzyme secreted by the mitochondria of metabolically active cells [20].

10^4 cells/mL were seeded into 96-well plates (Greiner Bio-One GmbH) and kept under 5% CO_2 at 37 °C for 24 h. After 24 h., cells were attached as controlled by microscopy and treated with the different compounds resulting in a final volume of 100 µL per well. All the compounds (Peptide, NP1, NP2, NP3) treated with the same concentrations of 0.5; 0.4; 0.2; 0.1; and 0.05 mg/mL. After 48 h. incubation, 50 µL of MTT solution, which prepared from thiazolyl blue tetrazolium bromide powder (Sigma M5655) (10 mg/mL) were added to each well. After 2-4 h incubation with MTT solution when the formazan needles were formed 150 µL of 100% dimethylsulfoxide (DMSO) were added to each well and incubated for 30 min in dark and room temperature. For evaluation of interaction of the compounds with the medium, the same procedure was conducted without cells. Optical density was measured at 540 nm wavelength with micro plate photometer (ThermoLabsystem, Multiskan Ascent).

All assays were performed in triplicate. The mitochondrial function was calculated, as a

percentage of the control group, considered as 100%. The percentage viability of cells was calculated according to following equation:

$$\%\ \text{Cell viability} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \text{x} 100$$

RESULTS AND DISCUSSION

Morphological investigation of nanoparticles. The morphology of nanoparticles was examined by using a phase-contrast inverted microscope. Light microscopic images for aqueous suspension of nanoparticles in solution shown Fig. 1.

In Fig. 1 (a, b, c), homogeneous nanoparticles dispersions in the aqueous solutions are seen clearly.

Morphological investigation of Mouse Fibroblast L 929 Cells and in vitro Cytotoxicity Study of Nanoparticles. The morphology of L929 cells treated with different concentration of (0.05; 0.1; 0.2; 0.4 and 0.5 mg/ml) free peptide, NP1, NP2 and NP3 nanoparticle solutions were examined by using a phase-contrast inverted microscope. All the morphological changes in L929 cells were investigated after incubation of cell with NP1, NP2, NP3 and free peptide by light microscope. Only the images which include highest and the lowest concentration values with comparatively to control are given below (Fig. 2, 4, 6, 8).

The MTT test is an assay for proliferation and cell viability by measuring the mitochondrial activity of cells. In this assay the live cells are able to convert the yellow water soluble tetrazolium salt to a water insoluble purple formazan [15]. Thus, the amount of formazan crystals formed is proportional to the number of viable cells. Cytotoxicity of NP1, NP2, NP3 samples and free W-1L19 peptide against L929 cells was evaluated for *in vitro* biocompatibility and the cell viability graphics are given below (Fig. 3, 5, 7, 9).

Morphology by inverted light microscopy of L 929 cells showed a characteristic spindle-shaped fibroblastic morphology (Fig. 2 a). In contrast, incubated with a solution of free peptide at the lowest concentration (0.05 mg/mL) morphological structure of cells was observed similar to the control group (Fig. 2 b), but at the highest concentration (0.5 mg/mL), unlike typical morphological image cells defects and spaces was observed. When the cytotoxic profile of the peptide was investigated, at the highest concentration (0.5 mg / mL) the cell survival is reduced to below 38 %, and this appears to be consistent with the morphological investigation (Fig. 3).



(a) **(b)** **(c)**

FIGURE 1

The light microscope (20x) image of aqueous suspension of nanoparticles NP1 (a), NP2 (b), NP3 (c)



(a) **(b)** **(c)**

FIGURE 2

Comparison of the cytotoxic effects of W-1L19 peptide solutions on cell viability of mouse fibroblast L929 cell line. Morphological changes of cells after treatment with different concentration of w-1l19 peptide solutions for 48 hours, control (a); 0.05 mg/ml (b); 0.5 mg/ml (c) with 20x magnifications

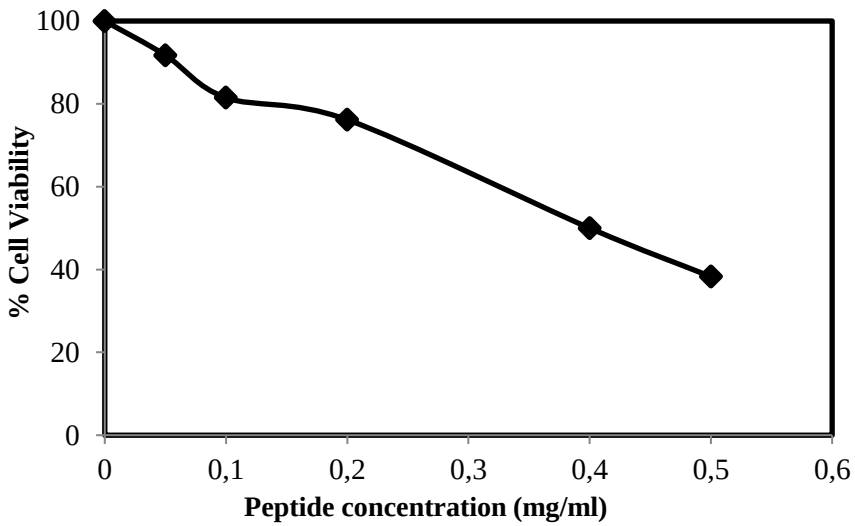


FIGURE 3
Cytotoxicity tests: cell viability of L929 cells (n =3) treated with different concentrations (0.5 mg/mL; 0.4 mg/mL; 0.2 mg/ml; 0.1 mg/mL; 0.05 mg/mL) of W-1L19 synthetic peptide solutions



FIGURE 4
Comparison of the cytotoxic effects of NP1 solutions on cell viability of mouse fibroblast L929 cell line. Morphological changes of cells after treatment with different concentration of NP1 solutions for 48 hours, control (a); 0.05 mg/ml (b); 0.5 mg/ml (c) with 20x magnifications

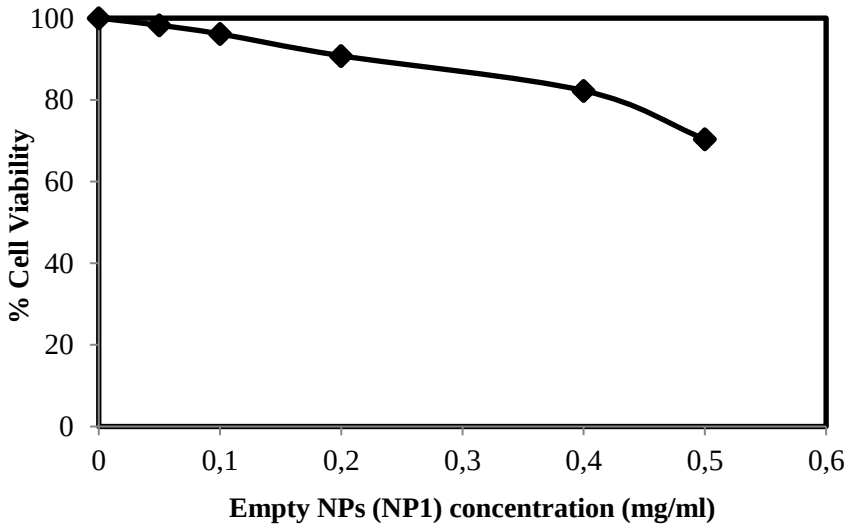


FIGURE 5
Cytotoxicity tests: cell viability of L929 cells (n =3) treated with different concentrations (0.5 mg/mL; 0.4 mg/mL; 0.2 mg/ml; 0.1 mg/mL; 0.05 mg/mL) of NP1 solutions

L929 cells incubated with NP1 (empty nanoparticles) solution and the morphological characteristics shown similar result to the control (Fig. 4) both of morphological investigation and cytotoxic analysis. And even at high concentrations the cell viability was observed as 70 % (Fig. 5).

The cells which were incubated with NP2 (peptide loaded nanoparticles) solution at lowest concentrations (0.05 mg/mL) the morphological structure was similar to the control group (Fig. 6 b), and at the highest concentrations (0.5 mg/mL) it seem different from morphological image but compared with free peptide less cell defects and spaces were observed (Fig. 6 c).



(a) **(b)** **(c)**

FIGURE 6

Comparison of the cytotoxic effects of NP2 solutions on cell viability of mouse fibroblast L929 cell line. Morphological changes of cells after treatment with different concentration of NP2 solutions for 48 hours, control (a); 0.05 mg/ml (b); 0.5 mg/ml (c) with 20x magnifications

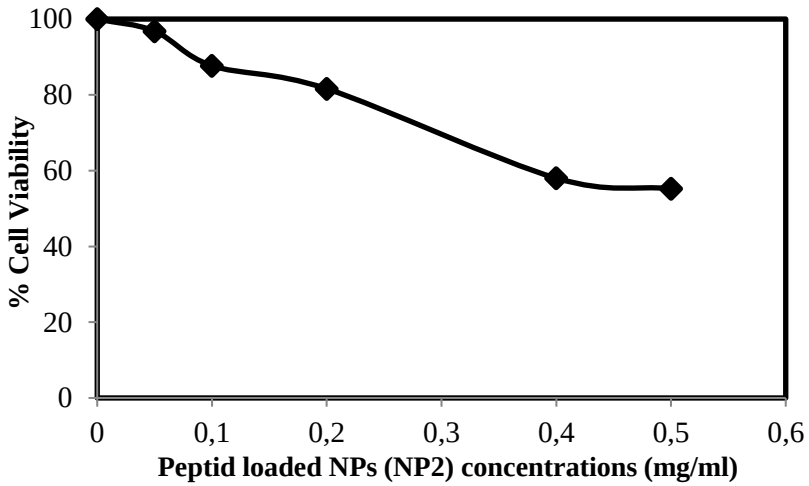
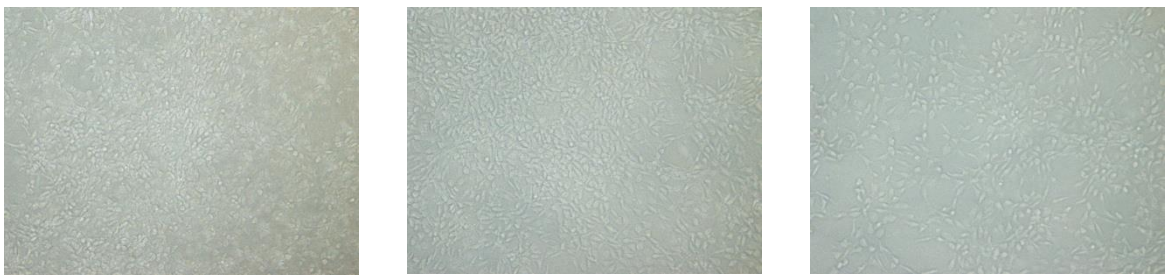


FIGURE 7

Cytotoxicity tests: cell viability of L929 cells (n =3) treated with different concentrations (0.5 mg/mL; 0.4 mg/mL; 0.2 mg/ml; 0.1 mg/mL; 0.05 mg/mL) of NP2 solutions



(a) **(b)** **(c)**

FIGURE 8

Comparison of the cytotoxic effects of NP3 solutions on cell viability of mouse fibroblast L929 cell line. Morphological changes of cells after treatment with different concentration of NP3 solutions for 48 hours, control (a); 0.05 mg/ml (b); 0.5 mg/ml (c) with 20x magnifications

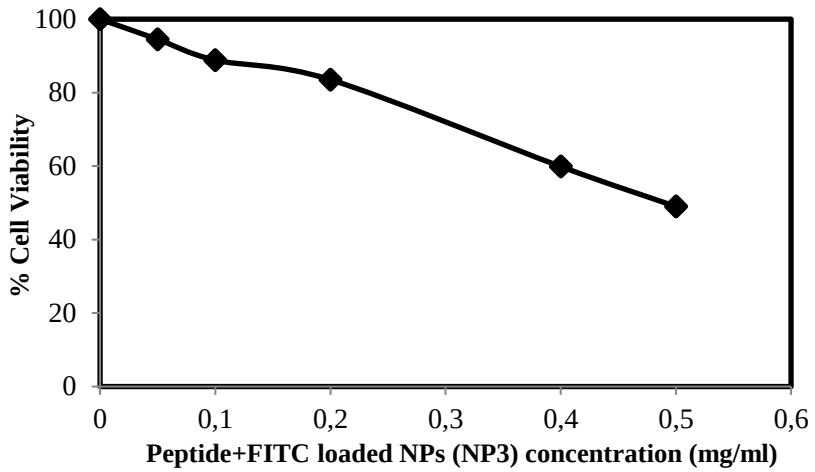


FIGURE 9
Cytotoxicity tests: cell viability of L929 cells (n =3) treated with different concentrations (0.5 mg/mL; 0.4 mg/mL; 0.2 mg/ml; 0.1 mg/mL; 0.05 mg/mL) of NP3 solutions

The cytotoxicity assay results of NP2 (peptide loaded nanoparticles), the highest concentration (0.5 mg / mL) the cell viability was 55 % and seems compatible with morphological investigation (Fig. 7).

As a result of microscopic examination at the cells incubated with NP3 (Peptide + FITC loaded nanoparticles) solution, the cell morphology (Fig. 8) and cytotoxicity analysis (Fig. 9) gave similar results with the cells which were incubated with NP2 solution.

This condition could describe by that FITC has not got a significant effect at very low concentrations.

In conclusion; cytotoxicity profile of the nanoparticles on mouse fibroblast L929 cell line was studied using MTT Assay. Five different concentrations of each samples (free W-1L19 peptide, empty nanoparticle, peptide loaded nanoparticle and peptide FITC loaded nanoparticles) were used for the cytotoxicity studies. After 48 hours of incubation at the highest concentration (0.5 mg/mL) of the all nanoparticles showed viability more than % 55 on the other hand the peptide showed only % 38 viability (Fig. 3). Similarly, there were no significant differences at the lowest concentrations (0.05 mg/mL) of these samples. This could be explained by increase in the biocompatibility of peptide which is encapsulated into FDA approved PLGA nanoparticles.

Thus, in this study, with the aim of developing peptide-based nanoparticulate vaccine against CPV, the peptide sequence of CPV W-1L19 (W-MSDGAVQPDGGQPAVRNERA) was loaded into PLGA nanoparticles. It has been determined that these nanoparticles are not toxic on the L929 cell line and are suitable for *in vivo* investigation.

In studies the sequences 1L15 (MSDGAVQPDGGQPAV) and 7L15 (QPDGGQPAVRNERAT) at VP 2 capsid proteins which belongs to canine parvovirus was shown to be immunogenic and created an immune response [5,6]. However, the uses of synthetic peptides as vaccine candidates and for increasing their immune response various carrier molecules are needed. PLGA is the most commonly used polymer in the nanoparticulate systems for loading of weak antigenic peptide, drug, or protein. And also, PLGA is biocompatible, biodegradable and approved by FDA. In literature many infections vaccine candidates based on the nanoparticulate system is seen to be studied [8,21,22]. However, in contrast to this, there has not been any study in literature about nanoparticulate-based vaccines in the CPV infection.

Therefore, in this study for the first time the biocompatibility of CPV W-1L19 (W-MSDGAVQPDGGQPAVRNER A) peptide loaded nanoparticulate systems were investigated on L929 mouse fibroblast lines. According to literature, from the obtained results it was found that empty PLGA nanoparticles is non-toxic [23]. The cytotoxic effect of free peptide and also peptide-loaded PLGA nanoparticles were investigated on the L929 mouse fibroblast lines and results showed that cell viability and proliferation was not significantly affected to addition of peptide-loaded PLGA nanoparticles.

The enhancement of peptide biocompatibility by loading PLGA nanoparticles suggests the effectiveness of PLGA as a delivery system for development of Canine Parvovirus vaccine candidate. Studies are currently in progress to test the immunogenicity and efficiency of W-1L19 loaded nanoparticles as a vaccine candidate against Canine parvovirus infections.

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