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ANIMAL SCIENCE

Design, preparation, and characterization of bovine papillomavirus chitosan-based nanoparticles as a prototype for a bovine papillomatosis vaccine

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Abstract: The bovine papillomavirus (BPV) is known to cause lesions in the epithelial tissue of cattle, which causes bovine papillomatoses. This disease is characterized by wart lesions in different body parts, causing losses in the animal value. Despite the economic impact, there are no effective vaccines available at this moment. The present study aims to develop a nanoformulated autogenous vaccine using the inactivated virus present in warts to control the diseases associated with BPV. To this end, papillomavirus was collected from infected bovines on a farm in Goiás, Brazil. The warts containing the viruses were macerated to obtain the inactivated viruses to be formulated within chitosan nanoparticles. The chitosan nanoparticles containing the inactivated viruses presented 330.7 nm in size, a polydispersity index of 0.41, and a positive Zeta potential of 38.2 mV. The association efficiency was 58.3%. The nanoparticles containing the inactivated virus were stable for up to 30 days and presented no hemolysis toxicity. This could be an interesting approach to develop a vaccine to protect bovines from BPV infection.

Key words: bovine papillomatosis, BPV, cattle warts, cattle virus, biopolymer.

INTRODUCTION

The bovine papillomavirus (BPV) is a group of DNA viruses that cause bovine papillomatosis (Bauermann et al. 2017, Bertagnolli et al. 2020, Gharban et al. 2023). Considering that BPV affects farm animals, it can cause significant economic losses for livestock due to decreased leather value and cattle weight (Bertagnolli et al. 2020, Watanabe et al. 2020). These viruses present a tropism for epithelial cells and are associated with skin lesions such as warts and mucous papilloma. The course of the infection depends on the host's immune response and typically regresses but can progress to malignant tumors (Bocaneti et al. 2016, Módolo et al. 2017).

The bovine papillomavirus can be found worldwide in a diversity of known genetic types, infecting and co-infecting livestock in different countries (Bocaneti et al. 2016, Daudt et al. 2019, Gharban et al. 2023). The infection cycle of the papillomavirus is established in the stratified epithelium of the skin or mucosa and is closely associated with epithelial differentiation (Gharban et al. 2023). The transmission of BPV is facilitated by the presence of skin lesions or abrasions, which could be related to the genetic and immune host response (Bocaneti et al. 2016, Barreto et al. 2018).

Despite the significant economic impact caused by diseases associated with BPV, there

is currently no vaccine available to prevent its transmission. This is of great importance for the economies of several countries (Gharban et al. 2023). Given the impossibility of maintaining BPV in the laboratory to obtain virions or viral protein for use in serological tests, developing a vaccine strategy for this agent is challenging (Módolo et al. 2017).

Using the inactivated virus involving the entire viral particle is an intriguing approach to vaccine development. This is due to its ability to elicit a humoral immune response, producing specific B lymphocytes, memory cells, and antibody-secreting plasma cells (Nolte et al. 2001). These vaccines are safe because the BPV inactivation process enables the virus to be replicated in the host.

The mucosal route is an attractive option for vaccine administration due to its ease of handling and needle avoidance. It also reduces the risk of contamination, improves adherence to immunization, and is a less invasive method (Shakya et al. 2016, Santos Junior et al. 2023). This route of antigen administration has a higher uptake of the antigen via M cells and penetrates through the paracellular pathway (Dhakal et al. 2018, Lopes et al. 2018). However, it is necessary to protect the antigen against *in vivo* degradation. Despite the challenges posed by factors such as enzymatic degradation and low absorption, nanotechnology, along with its slow and sustained release mechanism, has been widely employed to develop new immunization strategies (Amaral et al. 2010, Santos Junior et al. 2020).

Polymeric nanoparticles based on biodegradable and biocompatible biopolymer chitosan, which has mucoadhesive properties, are suitable for vaccine delivery via nasal administration (Santos Junior et al. 2023). Chitosan also acts as an adjuvant, enhancing cellular and humoral immune responses by stimulating Th1 responses (Amaral et al. 2010, Lopes et al. 2018, Santos Junior et al. 2023).

In this context, this study aimed to create chitosan nanoparticles containing the inactivated virus for use as a vaccine against bovine papillomatosis.

MATERIALS AND METHODS

Research ethics

The Committee for Animal Care and Use of the Universidade Federal de Goiás, under registration #036/2017, approved all animal handling and experimental procedures in this study.

Bovine papilloma processing

Six warts of bovine papilloma from two Girolando breed were collected by a veterinarian using aseptic and surgical techniques and transported in ice to be processed in the Laboratório de Nano&Biotecnologia (LANAB) of the Instituto de Patologia Tropical e Saúde Pública of the Universidade Federal de Goiás (IPTSP/UFG), according to the protocol previously described by Inayat et al. (1999) and Mayilkumar et al. (2014). Briefly, after being washed with ether and weighted, 10 mL of 50% glycerin and 50% physiological saline solution was added to each 1 g of wart. The warts were macerated, and the mixture was kept refrigerated for 24 hours and then filtered using 0.45 µm nitrocellulose membrane (Millipore™). To the filtrate was added 0.5% of formalin 40% (125 µL) and 2 mg/mL of a combination of the antibiotics Benzylpenicillin Procaine 7.500.000 UI, Benzylpenicillin Potassium 2.5000.000 UI, and Streptomycin 500 mg. The wart filtrate containing the virus (now wart-BPV) was refrigerated until it was processed. A sample (100 µL) of the wart-BPV was seeded on blood agar and incubated for 48 hours at 37 °C to certify no contamination.

Protein inactivation

The protein in the wart-BPV was inactivated using 0.01% trypsin (Gibco®), following the method described by Araldi et al. (2014). The trypsin was added in varying volumes to the virus macerate (2 mL, 4 mL, 6 mL, 8 mL, and 10 mL) and then incubated for 2 h at 37 °C in a shaker at 250 rpm. The enzymatic activity was then deactivated by incubating the mixture in a water bath at 100 °C for 1 h. The protein concentration was analyzed using the Molecular Probes® (Labelling & Detection) kit. A sample of wart-BPV without the enzyme was used as a control.

Nanoparticles preparation

The inactivated wart-BPV virus was encapsulated within chitosan nanoparticles using the ionic gelation method, as adapted from Calvo et al. (1997). First, low-weight chitosan (Sigma Aldrich), deacetylation degree ≥ 75%, and viscosity 20-300 cps, was dissolved in 0.1 M acetic acid using the Ultra-turrax equipment at 15,500 rpm for 40 minutes. The nanoparticle formation containing the wart-BPV with the inactivated virus (named BPV-CS) was formed by adding 8 mL of pentasodium tripolyphosphate (TPP, Sigma Aldrich) (1 mg/mL) and 1.5 mL of the wart-BPV dropwise in 15 mL of chitosan solution (2 mg/mL) under gentle agitation. The empty chitosan nanoparticles used as blank (E-CS) were prepared using the same protocol, adding 1.5 mL of ultrapure water instead of the equal volume of wart-BPV.

The BPV-CS and E-CS were transferred to 1.5 mL tubes and centrifuged at 13,200 rpm for 40 minutes. All formulations and their characterization were prepared in triplicate. The supernatant was collected to determine the wart-BPV association efficiency, and the pellet was re-suspended in 1 mL of ultrapure water and stored for physical-chemical characterization.

Nanoparticles physical-chemical characterization

Inactivated virus association efficiency

The association efficiency (AE%) of wart-BPV containing the inactivated virus entrapped within chitosan nanoparticles was determined indirectly by quantifying the wart-BPV in the nanoparticles supernatant after centrifugation using the Molecular Probes® (Labelling & Detection) kit according to equation 1 (I).

AE% = {[(total wartBPV – wartBPV in the supernatant)] ÷ (total wartBPV)} x 100 (I)

Spectroscopy analysis of the photon correlation

The zeta potential and the mean size and size distribution of the nanoparticles containing the wart-BPV with the inactivated virus (BPV-CS), given respectively as Z-average and polydispersity index (PdI), were determined by dynamic light scattering (angle of 90º at 25 °C) were performed in a Zetasizer Nano ZS ZEN3600 equipment (Malvern Instruments, UK). The nanoparticles' stability was assessed 30 days after storage in ultrapure water under refrigeration (4 °C).

Stability of the nanoparticles

The BPV-CS nanoparticle's stability was determined after 30 days of storage in ultrapure water under refrigeration (4 °C). For this, 1 mL of the BPV-CS dispersion was collected at times 1, 7, 15, and 30 days and characterized in function of size, PdI, and zeta potential. An equal volume of ultrapure water was added to the BPV-CS nanoparticle tubes to maintain the initial storage conditions.

Scanning Electron Microscopy

The scanning electron microscopy images were obtained by dispersing one drop of BPV-CS or

E-CS nanoparticles over coverslips. After drying, the images were collected using an electron microscope (Jeol, model JSM–6610, Thermo Scientific NSS Spectral Imaging). The analysis was done in the Multiuse High-Definition Microscopy Laboratory (LabMic) at the Universidade Federal de Goiás.

Toxicity investigation

The hemolysis test was done by collecting bovine blood, utilizing tubes treated with EDTA, and adapting the protocol previously described (Nahar et al. 2008, Italia et al. 2009). The tubes were centrifuged at 5,000 rpm for 10 minutes, the supernatant was discarded, and the cells were washed three times with PBS 1× and diluted in PBS in a 3:11 (v/v) proportion. 10 µL of this solution was used for each 100 µL of the final reaction in 96 "V" bottom well plates. The test was performed by adding the BPV-CS and E-CS in serial dilutions (factor 2) from 258 µg/ mL to 0,50 µg/ mL. The cells were treated with distilled water for the positive control and PBS for the negative control.

The plates were incubated for 1, 3, and 6 hours and then centrifuged at 4,500 rpm for 10 minutes. The supernatant was collected and kept at room temperature for 30 minutes for hemoglobin oxidation. After that, it was transferred to another plate to measure the absorbance (A) at 540 nm. The test was done in triplicate, and the data was expressed as mean ± SD. The hemolysis was calculated according to equation 2 (II).

% hemolysis = {[(sample A – negative control A)] ÷ (positive controlA)} x 100 (II)

Where A= absorbance.

Statistical analysis

The results were expressed by mean ± SD (Standard Deviation). The statistical analysis was done using the ANOVA one-way test. Results were considered significant when $p< 0.05$.

Results

Nanoparticles characterization

The BPV-CS and E-CS nanoparticles characterization are shown in Table I. The diameter of BPV-CS presented 330.73 nm ± 36.08, a positive Zeta potential of 38.2 mV ± 5.48, and the polydispersity index of 0.413 ± 0.08. The empty chitosan nanoparticles (E-CS) parameters are also presented in Table I.

The wart-BPV association efficiency (AE%) within chitosan nanoparticles was obtained indirectly by quantifying the amount of filtrated wart macerate containing the BPV. The initial protein concentration in the wart-BPV after

Table I. Characterization of chitosan nanoparticles containing the inactivated bovine papillomavirus obtained from the Girolando warts.

	BPV-CS	E-CS
Size (nm)	330.73 ± 36.08	384.43 ± 41.30
PDI	0.41 ± 0.08	0.44 ± 0.06
Zeta potential (mV)	38.2 ± 5.48	25.83 ± 4.48
Association Efficiency (%)	58.3% ± 0.76 (367.3 µg)	-

BPV-CS: chitosan nanoparticles containing inactivated bovine papillomavirus. E-CS: blank chitosan nanoparticles. PDI: polydispersity index.

filtration and trypsin treatment, which was added to the nanoparticle preparation, was 420 µg/ mL. The calculated AE was 58.3% ± 0.76, corresponding to 367.3 µg (Table I).

Nanoparticles scanning electron microscopy

The scanning electron microscopy images of the chitosan nanoparticles containing the inactivated bovine papillomavirus macerate (Figure 1a) presented a homogeneous shape in a dispersion pattern. In Figure 1b, it is possible to note the agglomeration of the empty chitosan nanoparticles.

Nanoparticles stability

The stability profiles of both BPV-CS and E-CS nanoparticles are shown in Figure 2. The results indicate the stability of the nanoparticles considering the size, PdI, and zeta potential until 30 days of storage. No significant differences were observed during the time investigated.

Nanoparticles cytotoxicity

The hemolysis test was done to verify the nanoparticle's hemolytic activity 1, 3, and 6 hours of incubation with bovine erythrocytes. No toxicity was observed after the incubation times for both nanoformulations (Figure 3).

DISCUSSION

This study attempted to propose a vaccine for bovine papillomatosis by encapsulating inactivated bovine papillomavirus in wart macerate within chitosan nanoparticles. Chitosan is a natural biopolymer obtained by the deacetylation of chitin, a linear polymer formed by N-acetylglucosamine. It has considerable potential in drug and vaccine delivery due to its biocompatibility, biodegradability, and, most importantly, its mucous-adhesive properties (Mikusova & Mikus 2021). This polymer can enhance the immune response when used to deliver antigens, as it also acts as an adjuvant in delivering bioactive molecules. Considering the mucous characteristics of the respiratory tract, chitosan-based nanoparticles are interesting for intranasal delivery of vaccines (Wei et al. 2021, Lin et al. 2022, Santos Junior et al. 2023).

The prepared nanoparticles have a diameter of 330.73 ± 36.08 nm and homogeneity, as observed in the polydispersity index of less than 0.5 and the scanning electron microscopy images. This size makes them suitable for easy phagocytosis by dendritic cells and M cells in the mucous (Hiremath et al. 2016, Dhakal et al. 2017). Chitosan nanoparticles carrying a

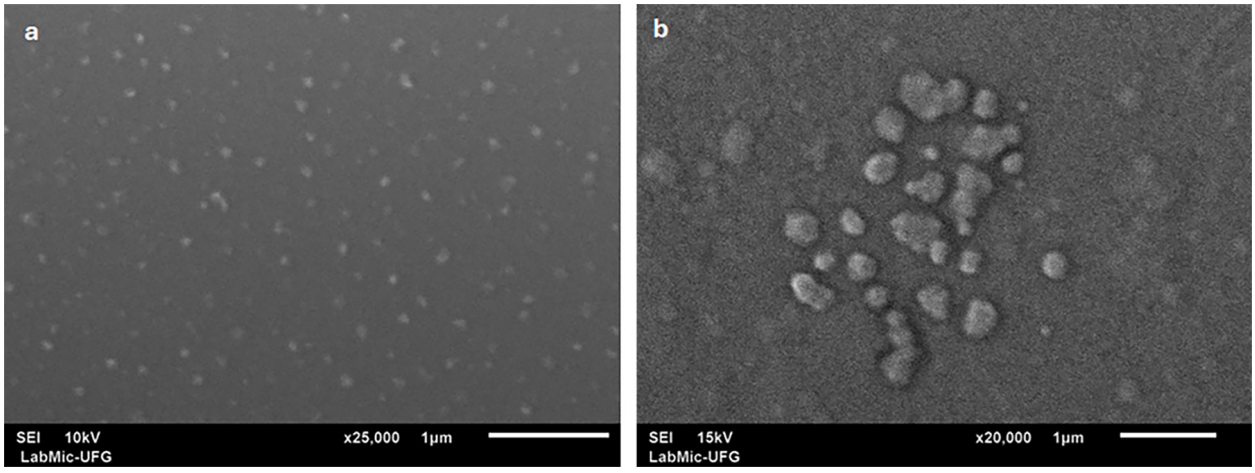

Figure 1. Scanning electron microscopy of the chitosan nanoparticles containing the inactivated bovine papillomavirus within chitosan nanoparticles (a, BVP-CS) and blank chitosan nanoparticles (b, E-CS).

peptide antigen resulting in 338 nm were able to elicit a mixed pattern of Th1 and Th2 immune response and efficiently protect animals against pulmonary fungal infection (Santos Junior et al. 2020). Encapsulating the inactivated antigen of the H_1N_1 flu (swine flu) in chitosan nanoparticles with a mean size of 571.7 nm was able to deliver the antigen not only to the upper and lower respiratory mucosa but also reach the blood, which increased the immune response (Dhakal et al. 2018).

In the present work, the chitosan nanoparticles associated with the inactivated BVP virus presented a positive zeta potential of 38.2 mV ± 5.48, which is a critical parameter since it represents the surface charge of the nanoparticles. A zeta potential of around 30 mV (negative or positive) is suitable for nanoformulation stability, which means that the nanoparticles preserved the physical-chemical characteristics during storage (Vaghasiya et al. 2020). Then, due to the high zeta potential, the maintenance of the nanoparticle's size, PdI, and zeta potential were observed for up to 30 days of storage. Also, the higher zeta potential of chitosan nanoparticles associated with the BVP inactivated virus than blank nanoparticles was indicative that corroborates the formation of the nanoparticles containing the inactivated virus.

The stability of nanoparticles containing the virus was investigated for up to 30 days. This short-term stability study is adequate to identify issues such as nanoparticle aggregation, which may increase their size and disrupt the formulation's stability (Jonassen et al. 2012). In

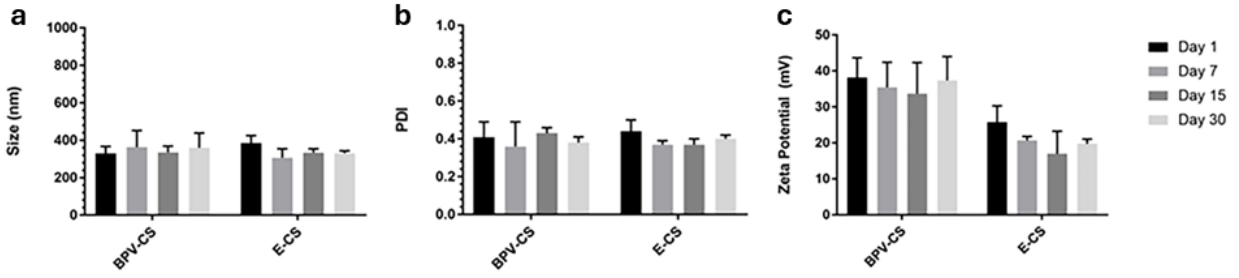

Figure 2. Nanoparticles stability profile. The stability of the chitosan nanoparticles containing the inactivated bovine papillomavirus macerate (BPV-CS) and blank chitosan nanoparticles (E-CS) was assessed over 30 days in terms of size (a), polydispersity index (PDI, b), and Zeta potential (c). The results are expressed as mean ± standard deviation of three independent experiments. $p < 0.05$.

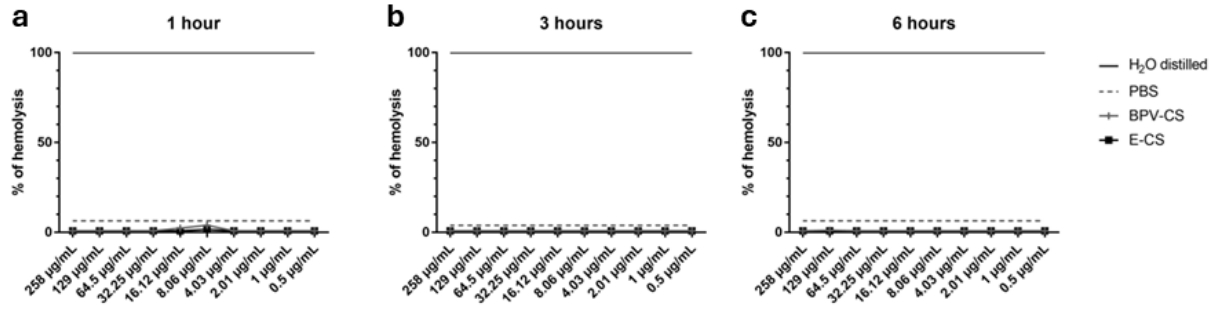

Figure 3. Hemolysis cytotoxicity of nanoparticles. Hemolysis percentage after 1 (a), 3 (b), and 6 hours (c) of the incubation of the bovine erythrocytes with chitosan nanoparticles containing the macerate inactivated virus (BPV-CS) and blank chitosan nanoparticles (E-CS). The distilled water was the positive control, while PBS was the negative control. The results are expressed as mean ± standard deviation of three independent experiments.

early studies of preclinical formulations using polymeric nanoparticles, the maintenance of size and zeta potential is typically observed for up to 30 days (Maruyama et al. 2016, Gimondi et al. 2023, Zashikhina et al. 2023). Since this is a preliminary study, further tests, including animal trials, will be conducted. In case of positive *in vivo* results, long-term stability and storage studies for this vaccine prototype will be performed, extending up to 90 days (Pozo-Rodriguez et al. 2009).

Considering the use of a nanoparticle as a strategy for vaccine development, a positive value for zeta potential is associated with its potential to adhesion to biological surfaces (Singh & Lillard 2009), including for mucosal administration, influencing the interaction with the cell membrane (Duan et al. 2010, Santos Junior et al. 2020). Chitosan nanoparticles entrapping plasmidial DNA for nasal immunization against hepatitis B produced immune responses even with a low but positive zeta potential of 13.8mV ± 1.5 (Khatri et al. 2008).

In the present work, 58.3% ± 0.76 of the wart-BPV was entrapped within chitosan nanoparticles. This association efficiency follows previous works that used chitosan nanoparticles to encapsulate antigens for mucous immunization. Dhakal et al. (2018) achieved 67% encapsulation of the inactivated antigen of the H_1N_1 flu in pigs in chitosan nanoparticles, resulting in reduced nasal viral shedding. Vila et al. (2004), encapsulating a tetanus toxoid antigen within nanoparticles to nasal immunization, achieved 55.1%, and the zeta potential of 37.1 mV, and following intranasal administration, the nanoparticles induced an increasing and long-lasting immune response.

The obtained chitosan nanoparticles entrapping the BVP inactivated virus were non-toxic in the hemolysis test, an essential parameter to evaluate erythrocyte toxicity (Gomathi et al. 2017, Chen et al. 2019). In the current work, the toxicity was below the negative control (PBS) and had a 5% hemolytic relationship with biomaterials (Gomathi et al. 2017). This suggests the formulation is hematological biocompatible since it does not induce oxidative processes in the cellular membrane, making it safe to be administered in blood contact (Sarangapani et al. 2018).

CONCLUSIONS

The results indicate the capacity of the chitosan nanoparticles to encapsulate the wart macerated containing the inactivated bovine papillomavirus. The formulation was stable for up to 30 days and presented no erythrocyte hemolysis. These elementary parameters indicate the potential of this formulation to be evaluated *in vivo*. Efforts are being made to evaluate this vaccine prototype in cattle affected by bovine papillomatosis.

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RAB: conceptualization, data curation, methodology, writing. CAS: conceptualization, data curation, methodology, supervision. ACA: conceptualization, funding acquisition, project administration, resources, supervision, writing.