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Short Communication

EVALUATION OF TISSUE REACTION AND FTIR SPECTROSCOPY OF CALCIUM PHOSPHATE ADJUVANTED OUTER MEMBRANE PROTEINS VACCINE OF *SALMONELLA TYPHI*

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ABSTRACT: In the present study, the potential of calcium phosphate nanoparticles was assessed to work as a nano-adjuvant system for vaccine of *Salmonella* Typhi. The tissue reaction analysis and Fourier Transform Infra-red (FTIR) spectroscopy was carried out to evaluate the toxic effects and stability of the calcium phosphate adjuvanted outer membrane proteins nanoparticles. The findings of the study revealed no tissue reaction in the rat model and calcium phosphate adjuvanted outer membrane proteins nanoparticles were found to be stable in the FTIR spectroscopy. The observed results showed that the calcium phosphate nanoparticles may be employed as an adjuvant in the vaccines administered through subcutaneous route.

Keywords: Adjuvants, Calcium phosphate nanoparticles, *Salmonella* typhi, Wistar rats.

Vaccination has been proven as an effective tool for the control and eradication of infectious diseases. Vaccines comprise two major components: antigens and adjuvants. Antigens are molecules that trigger an immune response, whereas adjuvants are substances used to enhance the immune response in the presence of an antigen. Few conventional adjuvants like Alum, Freund's incomplete adjuvant (FIA), Freund's complete adjuvant (FCA) etc. have been extensively used in different vaccine formulations for humans and animals. Conventional adjuvants have few limitations as sometimes may cause swelling, redness at the injection site, fever body aches [1]. Moreover, in some cases, conventional adjuvants fail to produce a balanced Th1 and Th2 immune response. Therefore, efforts have been made develop new generation adjuvants. Inorganic nanoparticles like calcium phosphate nanoparticles have been targeted for the delivery of DNA and protein antigens. These nanoparticles are highly biocompatible, biodegradable and safe [2].In our recent study, calcium phosphate adjuvanted outer membrane proteins (Omps) vaccine was developed, which induced an effective humoral, cellular and protective immune response [3] and also revealed no long term oxidative stress in the vital organs of mice [4]. In present study, the vaccine formulation was studied for the tissue reaction and stability using Fourier Transform Infra-red (FTIR) spectroscopy.

The study

FTIR spectroscopy is a unique technique, involved in observing and describing the changes in vibrational modes of functional groups. The functional groups responsible for stability and capping of synthesized calcium phosphate adjuvanted Omps nanoparticles (CaPNPs-Omps) were identified by

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FTIR spectroscopy[5]. The infra-red (IR) spectra of the CaPNPs-Omps was obtained by using ALPHA spectrometer (Bruker Optics, GmbH, Ettlingen) in the wave number range from 400 to 4000 cm^{-1}. It consists of low voltage air cooled IR Source, Zn Se Beam Splitter, Zn Se window and Detector. Sample (calcium phosphate adjuvanted Omps nanoparticles) was spread over ATR crystal and scanned. The obtained sample IR spectrum was displayed in the computer with OPUS software (version 6.5 service pack).

Rats have been used as an animal model for toxicity and tissue reaction studies from centuries as they are physiologically and genetically similar to humans making them a preferred and appropriate model for analyzing wide variety of diseases and conditions. Wistar rats (6-8 weeks of age) were procured from the Indian Veterinary Research Institute, Izatnagar and reared in small animal house, College of Veterinary and Animal Sciences as per the Institutional animal ethics committee guidelines. The rats were vaccinated subcutaneously with calcium phosphate adjuvanted Omps nanoparticles (treated group) and void calcium phosphate nanoparticles (control group). The muscle tissue samples were collected aseptically from the site of immunization of calcium Phosphate nanoparticles-Omp injection and control group of rats. These tissues were fixed in 10% neutral buffered formalin for 48 hours. The fixed tissues were washed overnight under running tap water, dehydrated by ethanol (*viz*. 50%, 70%, 80%, 90%, 95% and two changes in 100%) for one hr in each solution and cleared by using xylene-Alcohol (1:1), Further the tissue samples were treated with Xylene-I and Xylene-II for one hour each to remove alcohol from dehydrated tissues and were impregnated with liquid paraffin wax in 3 stages (I, II and III) for 1 hr each at 60º C in the wax chamber followed by embedded with paraffin wax at 60ºC. Sections of approximately 3-4 micron thickness were cut in the microtome (Leica RM 2125RT) and further stained by Haematoxylin and Eosin method [6]. The stained sections were mounted with DPX mountant (Molychem) in clean glass slides and examined under Labomed microscope (USA) for photomicrography. The gross pathological study of muscle tissue was done from both the groups i.e. treated groups (vaccinated subcutaneously with calcium phosphate adjuvanted Omps nanoparticles) and control group (void calcium phosphate nanoparticles) after sacrifice of the rats at 14th day and necropsy examination was done for recording gross pathological changes.

Results and discussion

The functional groups associated with CaPNPs-Omps were identified by FTIR spectroscopy (Fig.1). The major absorption bands have been found at 689, 858, 1021 and 1644 cm^{-1}.The band at 689 cm^{-1}corresponds to the O-P-O bending vibration of phosphate group $(PO_4^{3})^{-}$in CaPNPs-Omps. The ion stretching vibration around 858 cm^{-1} resembles the asymmetric P-O stretching vibration of the phosphate band (PO_4^{3-}). The peak at 1021 cm^{-1} confirms the presence of PO_4^{3-} group and the peak at 1644 cm^{-1} signifies to C=O stretching of β-keto ester group.

During necropsy examination, there were no observable external lesion and no gross lesion at injection site in muscle tissues. Histopathological study of muscle tissues from different groups at 14^{th} day after sacrifice of the rats was carried out with the help of light microscope using formalin fixed tissue sections and stained with Hematoxylin & Eosin (H&E). Microscopically, the muscle sections of treated group showed no significant histopathological lesions (Fig. 2). There were no significant inflammatory changes and necrosis at injection site in muscle tissue.

The findings of the earlier workers who used FTIR spectroscopy for characterization of calcium phosphate nanoparticles are in the agreement with our findings. Studies revealed the sharp peaks at 594, 698, 878, 1095 and 3450 cm^{-1} attributing to the O-P-O bending vibration of PO_4^{3-}, P-O stretching vibration of the PO_4^{3-} band, presence of PO_4^{3-} and stretching vibration of the lattice OH^{-} ion respectively [7]. Another study revealed the major peaks for calcium phosphate nanoparticles at 1393, 1470, 1529 and 1622 cm^{-1} indicating to CH_3bending, COO^{-} symmetric stretching of beta-keto ester respectively [8].

From the present findings, we observed the major absorption peaks of calcium phosphate nanoparticles revealing the presence of different functional groups (C=O and PO_4^{3-}), which have been considered to be important for stabilization of the calcium phosphate adjuvanted Omps nanoparticles. Histopathological studies were conducted to observe the site-specific effects of CaPNPs-Omps. There were no signs of necrosis and inflammatory reactions observed in the muscle tissue of rats. Our results were in accordance with the findings of earlier studies. Earlier workers have also evaluated the site-specificreaction of calcium phosphate nanoparticles and also assessed calcium phosphate nanoparticles for delivery of recombinant Omp87 and conducted the histopathological analysis of muscle tissue [9]. They also reported no significant tissue reaction at the site of injection. Similar studies

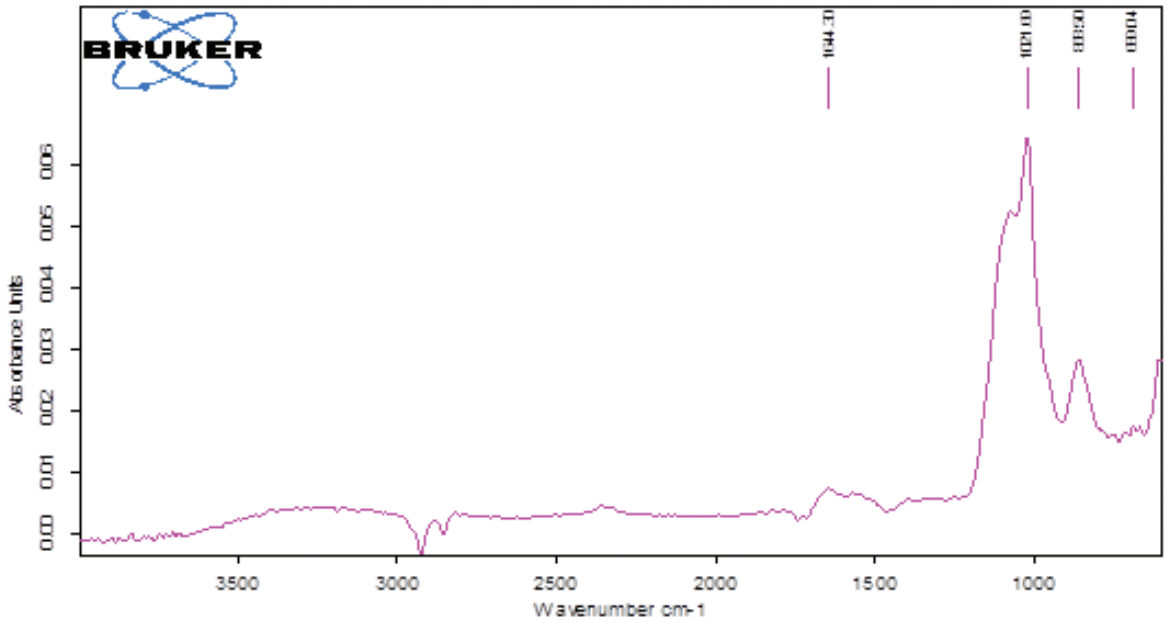

Fig. 1. FTIR spectra of calcium phosphate adjuvanted Omps nanoparticles.

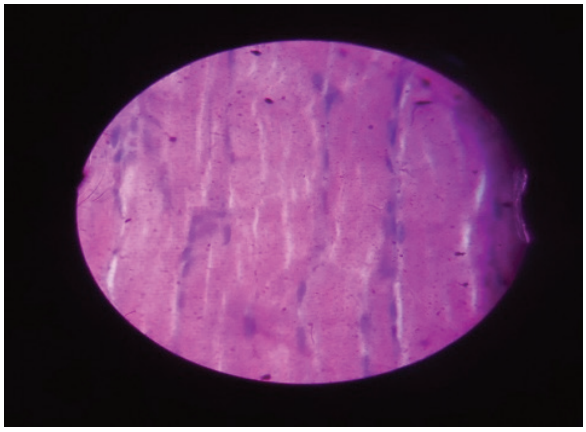

Fig. 2(a)

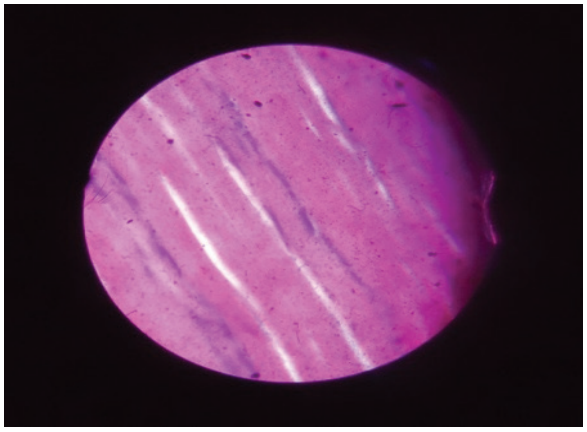

Fig. 2(b)

Fig. 2. (a) Histopathological slide of muscle tissue of Control group of rats and (b) histopathological slide of muscle tissue of Calcium phosphate adjuvanted Omps nanoparticles vaccinated group of rats.

have been reported with no significant abnormalities or histological changes in the organs of rats injected with calcium phosphate nanoparticles [10]. Therefore, we could presume that calcium phosphate nanoparticles did not cause adverse tissue reactions as adjuvant, which has been observed with many other conventional adjuvants like Freund's complete adjuvant [11], Alums [12], Aluminium hydroxide [13]. Therefore, It can be a suitable for humans and animals' vaccine adjuvant.

Conclusion

On the basis of our study, we could conclude that CaPNPs-Omps was found to be stable. Moreover the vaccine formulation did not caused any tissue reaction in rats. Therefore, calcium phosphate nanoparticles may be proven as an efficient adjuvant system for delivery of vaccines by subcutaneous route.

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