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ORIGINAL ARTICLE / ÖZGÜN MAKALE

FORMULATION AND *IN VITRO - IN VIVO* EVALUATION OF TETANUS TOXOID-MANNITOL DRY POWDER INHALATION FOR PULMONARY DELIVERY

PULMONER VERİLİŞE YÖNELİK TETANOZ TOKSOİD-MANNİTOL KURU TOZ İNHALASYON FORMÜLASYONU VE İN VİTRO - İN VİVO DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: *As the conventional vaccines were accompanied by the limitations of pain, cold chain storage and sterility issues, a mucosal vaccine which is administered through pulmonary route was fabricated. A dry powder inhalation of tetanus toxoid (TT) and mannitol was prepared and evaluated for stability and immunogenicity in comparison to the conventional TT vaccine.*
Material and Method: *TT and mannitol dry powder inhalation was prepared and evaluated for particle size analysis, scanning electron microscopy, FTIR, flow properties, TT content estimation, flocculation, and in vitro drug vaccine release studies. Immunological studies of the formulation were performed on BALB/c mice.*

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Result and Discussion: *The powder blend of tetanus toxoid and mannitol remained stable under the process conditions and after storage. The result was confirmed through a flocculation test. The FTIR analysis indictated no interactions between the components. The homogenization process yielded a powder with a geometrical particle size diameter of 1312 ± 1310.9 nm which was found suitable for pulmonary administration. The zeta potential and polydispersity index {PDI} were found to be -22.6 ± 0.16 mV and 0.499 ± 0.015, respectively. The diffusion studies indicated immediate release of the TT with 82.4 ± 6.7% of drug released within 2 h following the diffusion mechanism and zero order kinetics and it was found that mannitol didn't retard the release of tetanus toxoid. Additionally, the flow properties of the dry powder inhalation were reported to have good flow properties. More importantly, the immunological studies inferred the induction of high systemic and mucosal immunity over conventional vaccines.*
Keywords: *Dry powder inhalation, mucosal immunity, Tetanus toxoid, pulmonary administration, vaccine*

ÖZ

Amaç: *Konvansiyonel aşılara ağrı sınırlamaları, soğuk zincir depolama ve sterilite sorunları eşlik ettiğinden, pulmoner yolla uygulanan mukozal bir aşı üretimiştir. Tetanoz toksoidi (TT) ve mannitolün kuru toz inhalasyonu hazırlanmış ve konvansiyonel tetanoz toksoid aşısına kıyasla stabilite ve immünojenisite açısından değerlendirilmiştir.*
Gereç ve Yöntem: *TT ve mannitol kuru toz inhalasyonu hazırlandı ve partikül boyutu analizi, FTIR, akış özellikleri, kapsülleme etkinliği, flokülasyon ve in vitro ilaç aşısı salım çalışmaları açısından değerlendirildi. Formülasyonun immünolojik çalışmaları, BALB/c fareleri üzerinde gerçekleştirildi.*
Sonuç ve Tartışma: *TT ve mannitolün toz karışımı, işlem koşulları altında ve depolama sonrasında stabil kalmıştır. Sonuç, bir flokülasyon testi ile doğrulanmıştır. FTIR analizine göre hiçbir etkileşim tespit edilmemiştir. Homojenizasyon işlemi ile, 1312 ± 1310.9 nm'lik geometrik partikül boyutuna sahip, pulmoner uygulama için uygun bulunan bir toz elde edilmiştir. Zeta potansiyeli ve polidispersite indeksi (PDI) sırasıyla -22.6 ± 0.16 mV ve 0.499 ± 0.015 olarak bulunmuştur. Difüzyon çalışmaları, difüzyon mekanizması ve sıfır derece kinetiği takiben 2 saat içinde açığa çıkan etkin maddenin %82.4 ± %6.7'si ile TT'nin derhal salındığını göstermiş ve mannitolün tetanoz toksoidinin salınımını geciktirmediği bulunmuştur. Ek olarak, kuru toz inhalasyonunun akış özelliklerinin iyi akış özelliklerine sahip olduğu rapor edilmiştir. Daha da önemlisi, immünolojik çalışmalar, geleneksel aşılara göre yüksek sistemik ve mukozal bağışıklığın indüklendiğini ortaya çıkarmıştır.*
Anahtar Kelimeler: *Aşı, kuru toz inhalasyonu, mukozal bağışıklık, pulmoner uygulama, Tetanoz toksoidi*

INTRODUCTION

Tetanus is a deadly sickness that produces convulsions (seizures) and severe muscle spasms, which can result in spinal bone fractures. In 30% to 40% of instances, tetanus results in mortality. Tetanus vaccination is advised for all newborns 6 to 8 weeks of age and older, as well as all children and adults [1, 2]. It has been an integral part of maternal care for the prevention of neonatal tetanus [3]. A series of three to four shots is given during maternal care. It is advised during minor cuts and surgeries. Tetanus toxoid (TT) vaccination is known to induce short-lived immunity in patients [2]. As it is apparent, the introduction of the vaccination programmes was found to be effective in controlling the disease [2]. Despite the benefits of the intra muscular tetanus toxoid vaccine, it is also accompanied by limitations of sterility issues, pain, cold chain storage, and minimal induction of mucosal immunity. Mucosal linings are exposed to a wide variety of microorganisms and are naturally provided with immune components to restrict microbial entry [4-9]. It indicates the potential for the administration of vaccines through mucosal ports. In this regard, mucosal vaccines are available on the market, which are also known for their non-invasive approach. Oral, nasal, sublingual, vaginal, and pulmonary vaccine delivery routes have been investigated and reported for conferring mucosal and systemic immunity [10-13]. Mucosal immune responses are increasingly being recognised as critical for disease protection [14]. Vaccines administered through mucosal ports produce mucosal immune

responses most effectively, whereas vaccines injected are generally poor inducers of mucosal immunity and hence less effective against infection at mucosal surfaces [14]. Nonetheless, clinical vaccination development has relied heavily on antigen injection, and the majority of vaccines currently in use are given intramuscularly or subcutaneously. Several observations have implied a paradigm shift for mucosal vaccine delivery [15]. When compared to other delivery routes, pulmonary delivery has a number of advantages, including rapid drug uptake, a wide surface area for solute transport, and good bioavailability, as well as its non-invasive nature, rich vasculature, and minimum proteinase activity [16]. Additionally, pulmonary routes have evidenced the induction of mucosal antibodies in the bronchial associated lymphoid tissue (BAL) fluids [17]. Although the benefits of the pulmonary tract are appealing, product deposition in the alveoli appears difficult. It is to be borne in mind that 2-5 μ particles are reported for efficient deposition in the lower respiratory tract [18,19]. In earlier studies, the micronization method was found to have a good chance of making content that could be used for deep lung delivery [20].

Previous studies reported the fabrication of particulates to micron size through homogenization [21]. Pulmonary products available on the market are metered dose inhalers (MDIs), soft mist inhalers (SMIs), nebulisers, and dry powder inhalers (DPIs) [22]. Metered dose inhalers have long been popular due to their simple design and low cost, but they are reported for their inefficient drug delivery and use of propellants [23]. On the other hand, soft mist inhalers are expensive [24]. Although nebulisers are often used in hospitals, they require repeated heating of the product, which might damage the heat sensitive contents [25]. Among different inhalation products, dry powder inhalation is sought because of the benefits of drug administration efficiency, avoidance of propellants, and availability of the product form as a solid, further contributing to its stability [11]. Owing to its advantages, DPI is believed to be the right option for the administration of vaccine constituents. The DPI formulations are required to possess suitable parameters for effective deposition of the particles in the lungs. Changes in the shape, size, surface energy, and surface content of the drug particles can have a considerable impact on the aerosol performance of dry powder inhalation. In order to improve powder aerosolization, the physico-chemical properties of the powders can be optimised by adopting suitable techniques and formulation compositions [26]. It was observed that homogenisation of the admixture of powders yielded micron-sized particles [26]. Mannitol is known for its wide use in DPI's and has also been reported for its use in market formulations [27-30]. Due to its established toxicity profile and blends of DPI's with sufficient fine particle fraction, it is selected for the given study for the preparation of DPI's of mannitol and TT. Additionally, mannitol has been reported for its promising features for its use in dry powder inhalation. Its non-hygroscopic nature makes it a suitable carrier for the administration of proteins. Mannitol's sweet aftertaste serves as an indicator of dose deposition. Because conventional vaccines had disadvantages such as discomfort, cold chain storage, and sterility, a mucosal vaccine that is delivered via the pulmonary route was developed. In comparison to the standard TT vaccination, a dry powder inhalation of tetanus toxoid and mannitol was developed and tested for stability and immunogenicity.

MATERIAL AND METHOD

Mannitol was purchased from Sigma Aldrich chemicals, Bengaluru, India. Dano Vaccines and Biologicals Pvt. Ltd., Hyderabad, India, generously donated 1500 Lf/ml tetanus toxoid and commercial tetanus toxoid (5 ml vials each containing 5-25 Lf/ml, Batch No. 1923). Premium Serums and Vaccines Pvt. Ltd., Mumbai, India, provided Tetanus Antitoxin I.P. (3000 I.U./ml, Batch No. 141016). Bradford reagent was obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Mannitol was procured from Sigma Aldrich, Mumbai, India. The ELISA kit was purchased from Elabscience, Houston, USA (Catalog No: E-EL-M0692). The remaining chemicals were all of analytical quality.

Formulation of Mannitol-TT Microparticles

Based on a previous report on DPI formulation, the quantity of mannitol was selected [31]. An accurate quantity (7.5 g) of mannitol, 300 limit of flocculation (Lf) of TT was added to 50 ml of

distilled water and stirred by homogeniser for a period of 15 min. The preparation was subjected to cool centrifugation for 30 min at 1000rpm. The supernatant was separated and the pellet was dried in a desiccator to obtain a dry powder of the formulation [21]. Three repetitions were procedure was repeted three times.

Particle Size Analysis

The TT formulation was dispersed in de-ionized water to achieve a 1% concentration and agitated at 100 rpm at 37°C. Using a 532 nm laser and a Zetasizer Nano Instrument (Malvern Instruments, Nano ZS, ZEN3600, UK), the particle size, zeta potential value, and polydispersity index of the DPI formulation were detected [32,33]. Samples are taken in triplicate.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy [33] was used to investigate the size and surface morphology of nanoparticles. The images were captured using a 5.0 kV field emission scanning electron microscope (Philips CM12, Eindhoven, Netherlands) at a 9.7 mm working distance. Tetanus toxoid nanoparticles were dispersed in *n*-hexane and sonicated for 20 min at a concentration of 50 µg/ml and placed on a specially polished sample grid for sample preparation. Before microscopical analysis, the samples were vacuum-dried for 18 h and then sputter-coated with a 20 nm gold layer.

Fourier Transform Infrared Spectroscopic Analysis (FTIR)

A Shimadzu 435 U-04 IR spectrophotometer, Japan, was used to analyse FTIR spectra for pure tetanus toxoid and the formulation as KBr disc. The characteristic peaks obtained in the optimised formulation and the pure samples were recorded [33].

Determination of Flow Properties

The powder flow properties were determined as per the standards of United States Pharmacopoeia and mentioned by Lau. [34]. All the tests were performed in triplicate.

Bulk density is a parameter that indicates the degree of lightness of a powder. DPI requires being light and having a low bulk density. When the particles are loosely packed, it is characterised by high bulk volume and low bulk density. Powders with low bulk density are designated as "light powders". The cylinder is filled with a powdered substance. Tapping was done 50 times. The bulk volume was measured from the initial and final volumes after tapping. The measurements were substituted in the formula [34].

$$Bulk\ density = weight\ of\ powder/bulkvolume$$

When voids or air gaps between particles are removed, tapped density is often expressed in terms of mass per unit volume. Tapped density is an important parameter which indicates the flow properties. To determine the same, the powder was tapped 1250 times and the volume tapped was measured. The measurements determined are substituted in the formula [34].

$$Tapped\ density = Weight\ of\ powder/Tapped\ volume$$

The flow property of the DPI was determined by the angle of repose. The frictional forces determine the flow properties. The frictional forces are quantified by the angle of repose. It is the angle formed by the powder heap and the horizontal plane. For the determination of the same, an appropriate amount of the powder blend is allowed to form a pile on the horizontal plane by passing the mixture through a funnel fixed with a clamp to a stand. The height and radius of the pile were measured and the angle of repose was determined by the given formula. [34].

$$Tan\ \theta = h/r$$

Where h is the height (cm) of the pile, and R is the radius (cm) of the pile.

The Hausner ratio is a measure of interparticulate friction, and it is used to rate the flow character. A powder with a lower Hausner ratio has better flow characteristics. A Hausner ratio of > 1.25 indicates ‘poor’ flow. The Hausner ratio is calculated by the following formula.

$$H = \rho T / \rho B$$

Where, ρB is the freely settled bulk density of the powder, and ρT is the tapped bulk density of the powder.

TT Content Estimation in Microparticles

The amount of tetanus toxoid in micropartiles was measured by decanting the supernatant after centrifugation and quantifying the TT using the Bradford reagent UV-Visible spectrophotometric technique [35]. The binding of protein molecules to Coomassie dye under acidic circumstances resulted in a colour change from brown to blue in this experiment.

An appropriate amount of the given powder sample was transferred into a mortar and distilled water was added and rendered at 1 mg/ml and triturated. From the mixture, 30 µl of sample was mixed with 1.5 ml of Bradford reagent and assayed by a UV-Visible spectrophotometer at 295 nm [35,36].

Flocculation Test

The flocculation test was performed in triplicate to determine the loaded TT's structural integrity. The TT-mannitol formulation equivalent to 50 Lf was placed into a series of 6 flocculation tubes to conduct the test. Antitoxin was added to these flocculation tubes in graduated doses that differed by 10%, and the mixtures were incubated at 50 ± 1°C in a contanst water bath (Joanlab, Huzhou, China). The flocculation duration and antitoxin concentration at which the toxoid's initial flocculation was seen during incubation determine the toxoid's Lf value. The length of the flocculation (Kf) was recorded. This can be used to assess the toxin's effectiveness. The antigen quality is good if the Kf is low, and vice versa [37,38].

***In Vitro* TT Release Studies**

To simulate the conditions in the pulmonary region, the optimised nanoparticle formulations were put on a filter paper between the donar and receiver compartments of a Franz diffusion cell apparatus [13] containing pH 7.4 phosphate buffer. The apparatus was put on a magnetic stirrer and swirled continuously for 4 h, after which the samples were extracted at regular intervals and analysed using UV-Visible spectrophotometry at 295 nm with Bradford reagent.

Stability Studies

Stability studies were conducted to see how storage conditions and shelf life affected the prepared product. These experiments were carried out for a year at 4°C/0% RH and 25°C/60% RH, and flocculation tests were conducted to determine the stability [39].

***In Vivo* Efficacy Studies**

BALB/C mice weighing 18 to 25 g were chosen for this investigation and maintained in a HEPA-filtered environment with a 12 h light/12 h dark cycle and a constant temperature of 22°C. A normal diet was provided, as well as unrestricted access to water. Animals were separated into three groups (each with eight animals) and vaccinated with a dry powder mucosal vaccine inhalation formulation of 1 mg, equivalent to 0.5 Lf, and a traditional intramuscular immunization. Mice are initially anaesthetized with diethyl ether. The animals were quickly secured on the platform's 45° slant. Intubation was used to administer a tetanus toxoid dry powder formulation to anaesthetized animals. Each animal's tongue was gently taken out using blunt plastic-tipped forceps, and an otoscope was used to find the tracheal aperture in order to enter the cannula tube with the dry powder inhalation. A plastic syringe was filled with 1 ml of air and carefully attached to the top of the cannula tube to blow out the loaded dry particle inhalation. DPIs containing TT were breathed into the lungs of the animals [40]. The negative control group received saline solution intraperitoneally, while the standard group received the market formulation by intraperitoneal immunization. The animals received the original dose during the first week and the booster dose during the fourth week. Blood samples were obtained before immunisation and after 4, 6, 8, and 10 weeks. The sample tubes were allowed to clot at room temperature for 1 h before being centrifuged at 1000 rpm for ten minutes.

Serum samples were isolated and maintained at -20°C for the ELISA test to determine immunoglobulin G (IgG) titers [41].

ELISA Analysis

The total IgG titres in serum samples were determined using the Elabscience Mouse IgG ELISA kit and analysed using the sandwich method. The diluted standard, blank, and sample each had their own set of wells. In each well, 100 µl of standard dilution, blank, serum, and Bronchoalveolar lavage (BAL) samples were introduced to the respective wells. The plate was then incubated at 37°C for 90 min after the sealer was placed on it. After removing the liquid, 100 µl of Biotinylated Detection Ab working solution was added to the well, which was then incubated at 37°C for 60 min. The solution was decanted from each well, and 350 µl of wash buffer was added. The solution was allowed to sit on the plate for 1 min before being aspirated three times. After filling each well with a 100 l Horseradish peroxidase (HRP) conjugate working solution, the plate was capped and incubated at 37°C for 30 minutes. The liquid was decanted, rinsed, and aspirated five times. After that, a new sealer was applied to the plate, and 90 µl of substrate reagent was added to each well. The plates were kept dark and the wells were incubated at 37°C for around 15 min. After 50 µl of stop solution was added to each well, the plates were read at 450 nm. The standard was used to determine the concentrations of IgG in the samples.

RESULT AND DISCUSSION

DPIs are believed to be the right option for delivering vaccine constituents to the lungs. This is due to their stability, ease of use, and non-invasive administration [11]. In the present research, tetanus toxoid-mannitol microparticles were successfully prepared.

Particle Size Analysis Results

The geometric particle size distribution's volume weighted median, d(0.5), was 776.4 ± 4.2 nm. The size of a 10% volume of particles with a diameter less than d(0.1) was 348.9 ± 6.3 nm, while a 90% volume of particles with a diameter less than d(0.9) was 3099.4 ± 9.4 nm. Polydispersity index (PDI) is a measure of the variance in the molecular chain lengths and weights in a polymer. The PDI obtained was 0.499 ± 0.02, which indicate that monomer units of TT were arranged in chains of different length which will influence its antigenicity. This could be the reason why the commercial formulations of TT are available in 5-25 Lf/ml.

It was observed that the particles lying in the micron range find effective deposition for deep lung delivery. To achieve peripheral drug penetration in the event of pulmonary drug delivery for systemic absorption, fine particle size aerosols would be necessary. Particles smaller than 3 µ have an approximate 80% chance of reaching the lower airways, with 50–60% landing in the alveoli [42]. Smaller sizes below the micron range possess a high amount of free energy and form aggregates. They are characterised by poor flowability and aerosolization performance, and tend to stick to the inhaler. The negative charge of the particulates is believed to prevent the formation of aggregates due to the existence of repulsive forces. In terms of aerosol quality and efficiency, particle size distribution is critical [43]. It is frequently assessed using PDI. A higher PDI value for the carrier (mannitol) suggests a wider particle size distribution, resulting in a more heterogeneous drug combination. This may result in more variability in drug deposition in the lungs after inhalation [44]. The PDI obtained was 0.499 ± 0.02, which indicates partially homogenous dispersion of the particles.

Scanning Electron Microscopy (SEM) Results

The SEM images of pure TT showed descrete crystalline forms, whereas the TT-mannitol formulation showed the crystalline TT particles (white color) uniformly dispersed in mannitol crystals (grey color) as shown in Figure 1.

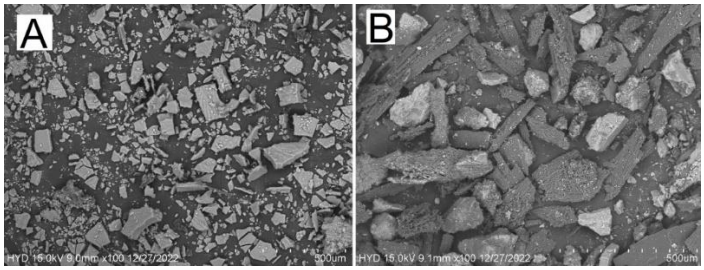

Figure 1. SEM images of (A) tetanus toxoid and (B) TT-mannitol formulation

Fourier Transform Infrared Spectroscopy (FTIR) Results

The FTIR spectra of pure TT and TT formulation were given in Figure 2, and the peaks were observed at 3416, 1646.2, and 629.6 cm^{-1}, while the formulation resulted in peaks at 3400 and 1600 cm^{-1}. The results of FTIR analysis suggest that tetanus toxoid remained intact during the preparation. The additional peaks indicate the molecular dispersion of mannitol and tetanus toxoid.

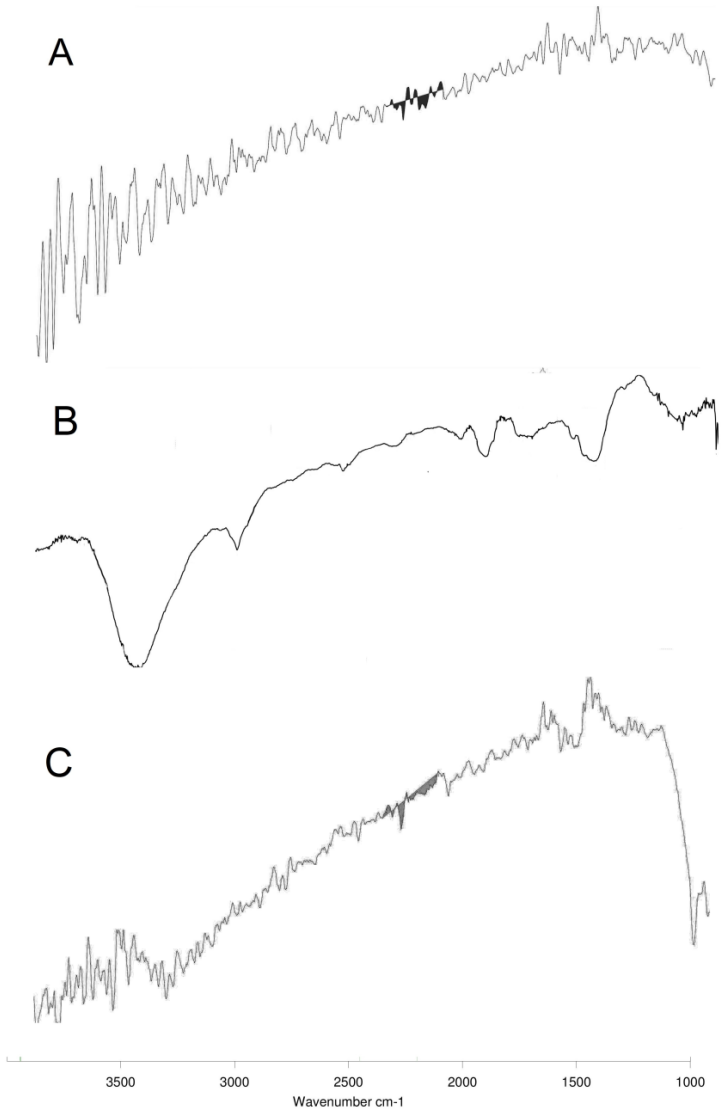

Figure 2. FTIR spectra of (A) mannitol, (B) tetanus toxoid and (C) TT-mannitol formulation

Flow Properties and TT Content Estimation Results

The flow properties of the powder blend are characterised by bulk density, tapped density, and angle of repose. The parameters should report less value for efficient flow. The dry powder inhalations should possess good flow properties to deagglomerate and disperse during breath actuation. The formulation of mannitol and tetanus toxoid dispersion has reported good flow properties as shown in Table 1. The TT content of the given formulation was found to be 95.3 ± 3.1 % and the drug content of the sample indicated 94 ± 1.8 %. Furthermore, non-hygroscopic nature of mannitol contributes to the free flowing properties of the TT-mannitol formulation.

Table 1. Determination of flow properties

Parameter	Formulation
Angle of repose (θ)	18.1 ± 1.6
Bulk density (gm/cm^3)	0.51 ± 0.01
Tapped density (gm/cm^3)	0.65 ± 0.01
Hausner ratio	1.27 ± 0.01

All values are average of three determinations

Flocculation Test Results

Limes flocculation means, when the concentration of toxin, or toxoid, is held constant while the concentration of antitoxin is altered in constant volume mixtures, the mixture that flocculates first includes the most nearly equivalent quantities of toxin, or toxoid, and antitoxin [44]. The antigenicity of tetanus toxoid was confirmed by the development of flocculation with antitoxin, as shown in Figure 3. The presence of flocculation was found after 14 ± 0.5 min. The World Health Organization's Expert Committee on Biological Standardization determined that flocculation tests using reference toxoids are extremely reliable and reproducible [37]. The flocculation test determines the sample's stability and antigenicity. Moreover, the observed Kf indicates the toxoid's purity and stability in the formulation. Furthermore, the flocculation test was carried out after the material had been kept at room temperature. As a result, the tetanus toxoid dry powder inhalation can be stored at ambient temperature without the need for cold chain storage. The presence of flocculation found after 14 ± 0.5 min indicated that the sample was stable. Similar results were obtained for tetanus toxoid vaccine formulation with chitosan microspheres in earlier study [45].

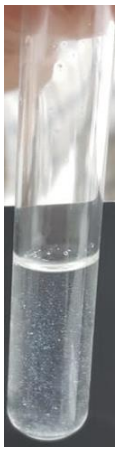

Figure 3. Flocculation test of Mannitol-TT formulation

In Vitro TT Release Studies Results

In vitro diffusion studies were conducted using the Franz diffusion cell apparatus. As observed

in Figure 4, there was immediate release of TT and 82.4 ± 6.7% of the drug was released within 2 h. The zero-order kinetics and Higuchi plot showed r^2 of 0.869 and 0.958, respectively. Similar release of pure TT was observed with and 88.3 ± 4.15% of the drug was released within 2 h. The zero-order kinetics and Higuchi plot showed r^2 of 0.84 and 0.952, respectively. Student t-test was performed on the drug release patterns, in whicih which the *t*-value obtained was -0.16155 and the *p*-value as 0.43684. Therefore the TT release from pure TT and formulation was *not* significant at $p < 0.05$.

The results of *in vitro* TT release studies indicated that mannitol didn't significantly interfere in the release of TT. From the above results, the release was found to follow zero-order kinetics according to the r^2 values. Also, the Higuchi plot's r^2 values showed that the way TT was released from the formulation was through diffusion.

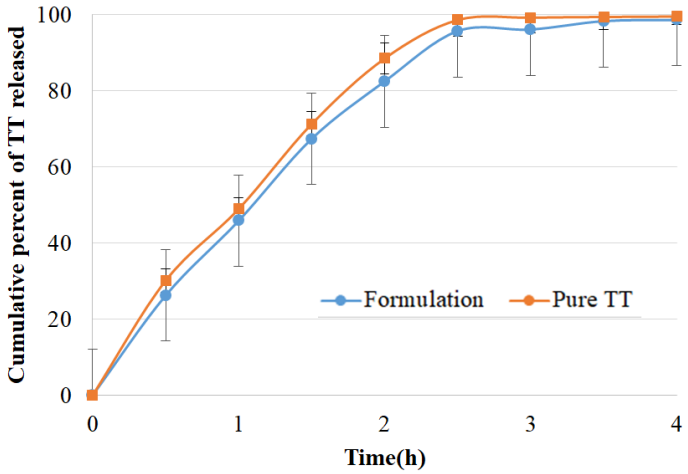

Figure 4. Diffusion analysis of mannitol-TT formulation

Stability Studies Results

The flocculation test was used to determine the stability of the TT-mannitol formulation for dry powder inhalation. The flocculation was observed at 14 ± 0.5, 14.5 ± 0.25, and 15.5 ± 0.5 min respectively at 3, 6 and 12 month period. This means as long as the Lf is maintained, the stability of vaccine is assured. The vaccine was shown to be stable at 25ºC and 60% RH for 12 months without the need for refrigeration. The product's results remained unchanged when stored at 4°C with 0% RH. The TT DPI formulation, stored at 25ºC and 60% RH for 12 months, showed stability using a flocculation test. This indicates that, the dry powder inhalation of tetanus toxoid can be stored at ambient temperature without any need for cold chain storage. Furthermore, the data points to the dry powder vaccine's commercial viability.

In Vivo Efficacy Studies Results

BALB/c mice were used to compare the dry powder inhalation of the formulation with the traditional vaccination. The existing tetanus toxoid vaccine was given via intramuscular injection. An ELISA test was used to determine the vaccine formulation's immunogenicity. The total IgG concentration in the mice's serum and BAL fluids was calculated. The results showed that antibodies were present before the vaccine was given and that the antibody level had decreased by week four. In addition, Table 2 and Figures 5 and 6 showed an increase in antibody levels in the tenth week. The negative and positive control groups both demonstrated a gradual decline in serum IgG titers after 10 weeks of the trial. The serum IgG level was marginally enhanced to 14.4 ± 2.8 ng in those mice that received the booster dosage. The developed TT formulation, on the other hand, showed a nearly two-fold increase in IgG titer, going from 48.4 ± 7.4 to 105.5 ± 8.1ng. In BAL fluids, a similar trend was seen for IgG titer. Table 2 shows a two-fold increase in IgG titers before and after the booster dosage.

According to previous research, conventional vaccines cause systemic immunity and poor mucosal immunity. Because infections enter the body through mucosal ports, developing a mucosal vaccination that provides both systemic and mucosal immunity is necessitated. For this reason, mucosal routes such as the oral and nasal have been investigated, with encouraging results [13,46]. The pulmonary pathway was investigated in this study to see if it might be used to induce systemic and mucosal immunity. The pulmonary route's near closeness to the blood vascular system suggests that it could be preferred over other non-invasive ways.

Table 2. Determination of IgG concentration in serum and BAL fluids

Group	IgG in serum (ng/ml)						IgG in BAL fluids (ng/ml)	
	0 w	4th w	8 weeks (ng/ml)		10 weeks (ng/ml)		BAL fluids (ng/ml)	
			8th w		10th w		10th w	
			BB	AB	BB	AB	BB	AB
Negative control	36.2 ± 6.9	2.25 ± 1.4	0.6 ± 0.1	0.8 ± 0.2	1.12 ± 0.4	-	34.4 ± 5.2	-
Positive control	54.6 ± 7.8	5.43 ± 0.87	7.23 ± 1.2	9.6 ± 2.1	10.8 ± 2.4	14.4 ± 2.8	38.1 ± 4.7	42.5 ± 4.8
Formulation	48.4 ± 7.4	8.1 ± 1.5	16.3 ± 2.4	29.52 ± 4.4	45.4 ± 5.6	105.5 ± 8.1	60.2 ± 5.1	102.5 ± 8.4

All values are average of three determinations. BB = before booster dose, AB = after booster dose, w = week

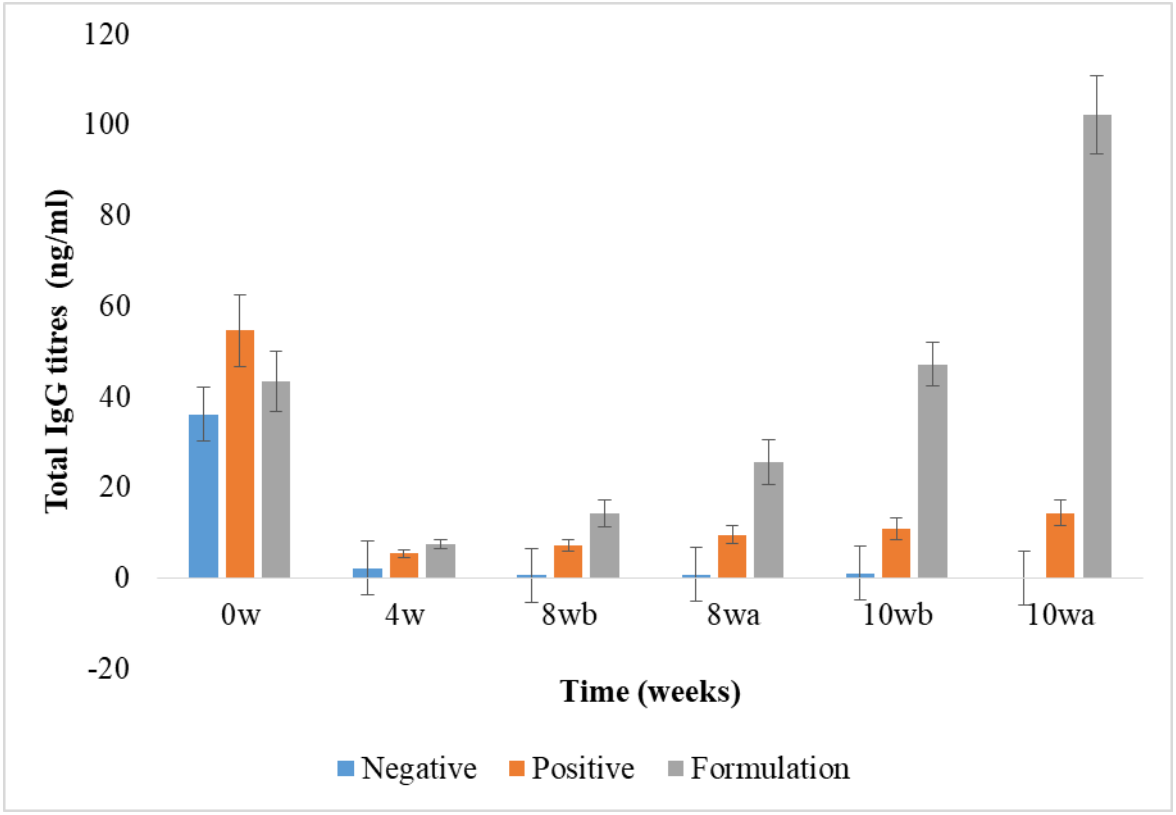

Figure 5. Serum immunity studies

Neg-Negative; Pos-Positive; 0w:0 week; 4w:4th week; 8wb:8th week before booster; 8wa:8th week after booster; 10wb:10th week before booster; 10wa:10th week after booster

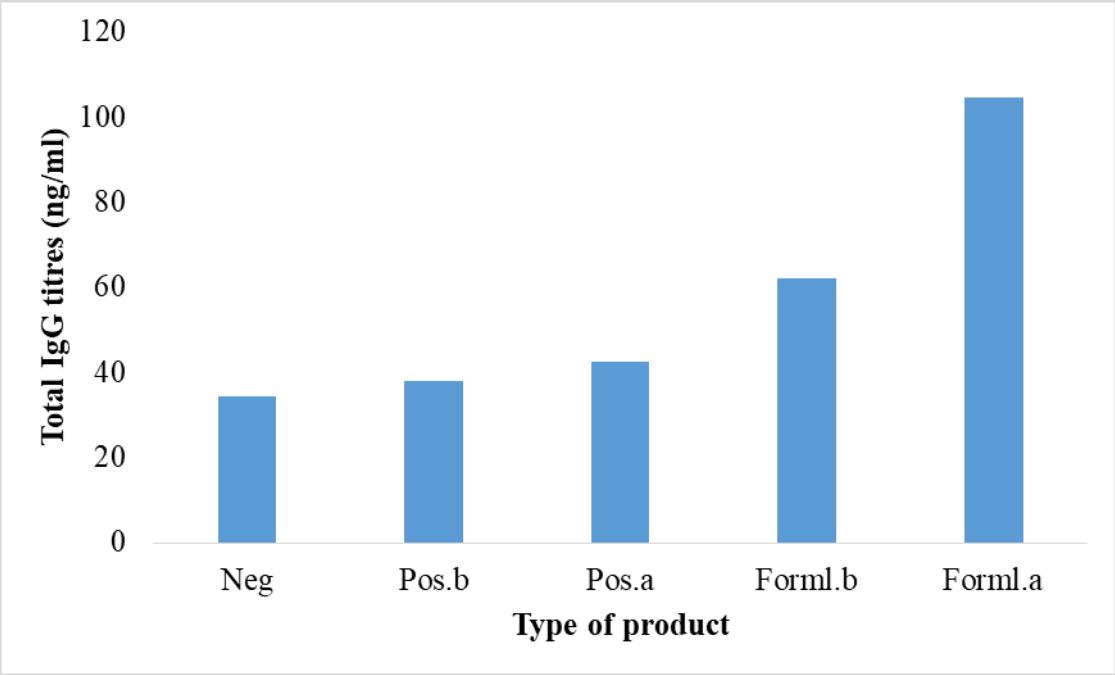

Figure 6. Mucosal (BAL) immunity studies
Neg: Negative control; Pos.b: Positive group before booster dose; Pos.a: Positive group after booster; Formln.b: Formulation before booster dose; Formln.a: Formulation after booster dose

The results of the ELISA test showed that antibodies were present before the vaccine was given, and that the antibody level had decreased by week four. Table 2 shows a two-fold increase in IgG titers before and after the booster dosage, indicating that the formulation was capable of producing mucosal antigenicity in the mice. The dry powder vaccination group imparted a considerable boost in systemic and mucosal immunity as compared to the positive control group, which had received regular conventional immunization. Furthermore, mucosal immunity was much stronger than systemic immunity, indicating that vaccinations may be delivered to fight respiratory diseases. It was shown that the pulmonary mucosal vaccination proved effective.

Owing to its suitable particle size and good flow properties, the TT-mannitol formulation was found to be an efficient carrier to be used as a dry powder inhalation for pulmonary administration. The geometrical particle size of the particles was within the micron range, which indicates that they can be well absorbed by alveoli. Further, the non-hygroscopic nature of mannitol contributes to the free flowing properties of the TT-mannitol formulation. The immunogenicity of the solid formulation infers the potential of the pulmonary TT-mannitol formulation. Future detailed *in vivo* studies with other animal models can show that this formulation works for both systemic and mucosal immunity.

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AUTHOR CONTRIBUTIONS

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M.M.A., P.K., S.I.; Manuscript Writing: M.M.A, N.V.K.A., J.D.V., S.M.; Critical Review: M.M.A., P.K.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that this work involved animal research, and the protocol for this investigation was approved by the Institutional Animal Ethics Committee in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals' disciplinary principles and guidelines CPCSEA proposal number RB-9/5/2021, dated March 28, 2021.

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