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ORIGINAL ARTICLE

Immunoprotective Effect of Red Blood Cell Membrane-Coated Nanoparticles Against *Clostridium Perfringens* Epsilon Toxin

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ABSTRACT

Background: ***Clostridium perfringens*** **epsilon toxin is a highly potent pore-forming toxin responsible for fatal enterotoxemia in livestock. Current vaccines require complex production steps and may induce suboptimal immune responses. This study aimed to develop RBC membrane coated PLGA nanoparticles loaded with ETX toxoid (RVNTs), evaluate their immunogenicity, specificity, and protective efficacy in rabbits, and compare them with bare nanoparticles (NTs) and a commercial vaccine.**

Methods: **Toxin-loaded poly (lactic-co-glycolic acid) nanoparticles and RBC membrane-coated NTs were synthesized and characterized by quantitative and qualitative assays. RVNTs were prepared by fusing purified rabbit RBC membranes with ETX-loaded PLGA nanoparticles and characterized for safety, residual toxicity, and potency. Thirty-five rabbits were immunized under single- or three-dose regimens with NTs, RVNTs, heat-treated NTs alongside commercial vaccine and PBS controls. Anti-ETX titers were quantified over 150 days using an optimized indirect ELISA and validated by mouse neutralization tests. Protective efficacy was assessed via subcutaneous challenge with 100 MLD of activated ETX.**

Results: **The iELISA showed high sensitivity, specificity, and reproducibility, with no cross-reactivity to other clostridial toxins. Three-dose regimens induced significantly higher and more sustained antibody titers than single-dose regimens ($p < 0.05$). RVNTs elicited stronger responses than NTs, with RVNTs-exp2 achieving the highest titers and complete survival post-challenge. Heat treatment did not impair NTs' immunogenicity. The commercial vaccine showed rapid antibody decline and failed to protect beyond 24 hours, whereas RVNT-treated groups maintained elevated titers and protection for 150 days.**

Conclusions: **RBC membrane-coated PLGA nanotoxoids provide enhanced stability, prolonged circulation, and potent, long-lasting immunity against ETX, outperforming bare nanoparticles and conventional vaccines. RVNTs represent a promising next-generation nanovaccine platform for preventing clostridial enterotoxemia, though further multi-species and long-term studies are warranted.**

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KEYWORDS

Clostridium perfringens, epsilon toxin, RBC-coated nanoparticles, nanovaccine, immunization

INTRODUCTION

Epsilon toxin (ETX) is the third most potent toxin produced by Clostridia species [1]. This toxin is an aerolysin-like pore-forming toxin produced by *Clostridium perfringens* (*C. perfringens*) toxinotypes B and D. It plays a key role in causing severe and rapidly fatal enterotoxemia in young livestock, particularly affecting goats, calves, and foals worldwide [2]. Vaccines are the most effective way to prevent clostridial diseases, and vaccines of varying quality are available to combat ETX enterotoxemia. The production of current clostridial vaccines involves expensive, time-consuming, and hazardous steps such as detoxification, purification, and antigen concentration processes [3]. Nanovaccines are a promising strategy for addressing clostridial infections that focus on facilitating the targeting and sustained and prolonged release of antigens to antigen-presenting cells (APCs), which could also reduce immunization frequency [3]. Antigens are coated onto nanoparticles to improve stability, immunogenicity, and targeted delivery [4,5]. It is essential to develop safe and effective adjuvants that can elicit robust humoral and cellular immune responses [6]. Indicator properties such as biocompatibility, validated formulations, FDA-approved excipients, antigen protection from degradation, potential for sustained release, and the ability to release entrapped antigens over an extended period have contributed to the widespread use of poly (lactic-co-glycolic-acid) (PLGA) as a popular biodegradable polymer [7]. However, one deficiency of nanosystems is that nanoparticles suffer from delivery obstacles, including immune clearance, biological adhesion, and targeting specific sites [8]. A bio-interfacing strategy that enhances existing synthetic nanosystems, enables immune evasion, and extends their circulation duration should be developed [9]. Nanoencapsulation for *in vivo* use protects substances, improves absorption and bioavailability, prolongs circulation, and reduces toxicity. Cell membrane-coated nanoparticles mimic natural membranes, enhancing biocompatibility and reducing immune rejection. Considering these attributes, cell membrane-coated nanocarriers have emerged as an innovative platform for antigen delivery in modern vaccinology [10]. The integration of nanoencapsulation, which confers enhanced stability, controlled release, and targeted delivery with the biomimetic and immune-evasive properties of natural cell membranes, provides a synergistic strategy to improve vaccine performance [11,12]. Such a system holds the potential to overcome key challenges associated with conventional ETX vaccines, including limited stability, rapid clearance, and suboptimal immune activation [13]. Consequently, the development of a PLGA-based nanoparticle coated with a biocompatible cell membrane may represent a next-generation immunization approach, offering improved safety, enhanced immunogenicity, and prolonged protective immunity against clostridial enterotoxemia [14]. This study developed RBC membrane-coated PLGA nanoparticles loaded with *C. perfringens* epsilon toxoid and evaluated their immunogenicity, specificity, and protective efficacy in rabbits, compared to conventional vaccines. An indirect ELISA was also optimized to monitor antibody responses over 150 days.

MATERIALS AND METHODS

Experimental materials and biological resources

New Zealand White rabbits, NMRI mice, and native Iranian sheep were obtained from the RVSRI, Iran, and housed under controlled conditions of temperature, light, and odor. All animals underwent a quarantine period prior to experimentation. The sample size for each test was determined based on statistical validation and prior research. *C. perfringens* type D (CN_{409}), *C. perfringens* type C (CN_{301}), *C. novyi* (CN_{901}), *C. chauvoi* (Iranian strain), and *C. septicum* (CN_{913}) bacterial strains were obtained from the bacterial archive of RVSRI. The strains were initially confirmed by microbiological tests and then confirmed by molecular methods. Reference toxins, antibodies, and the control vaccine were sourced from NIBSC, Sigma-Aldrich, Abcam, and RVSRI, respectively.

Preparation and processing of epsilon toxoid-loaded nanoparticles

Lyophilized vials containing epsilon toxoid/PLGA nanoparticles were obtained from a previous research project [8]. The RBC membrane-coated NTs (RVNTs) were prepared using a previously described protocol [8]. Briefly, rabbit RBCs underwent hypotonic treatment to isolate purified RBC membrane ghosts, which were then fused with preformed NTs at a 1:10 volume ratio. The mixture was sonicated and extruded 11 times through a 200 nm polycarbonate membrane using an Avanti mini extruder (Avanti Polar Lipids, Alabaster, AL, USA). NTs and RVNTs were subjected to comprehensive quality control testing, including sterility, safety, residual toxicity, and potency assays, as described in established protocols for biological therapeutics [15,16]. For preparation of heat-treated NTs, 150 µL of PBS containing 200 µg of NTs was pre-incubated at 70°C for 60 minutes before administration [17].

In vivo immunogenicity assessment of NTs and RVNTs

Thirty-five female New Zealand White rabbits (3 - 4 months old, 1,400 - 1,700 g) were used for *in vivo* evaluation. Animals received subcutaneous injections (1 mL) of NTs or RVNTs into the dorsal neck region, following two immunization regimens (Figure 1). In Experiment 1 (single-dose scheme), 10 rabbits were randomly allocated into two groups (n = 5): G1 received NTs (30 µg) and G2 received RVNTs (30 µg) on day 0. In Experiment 2 (three-dose scheme), 15 rabbits were assigned to three groups (n = 5): G3 received NTs, G4 received RVNTs, and G5 received heat-treated NTs on

days 0, 7, and 14, at doses of 30 µg (prime), 75 µg (first booster), and 75 µg (second booster). Control groups included: G6 (positive control) receiving a commercial tetravalent enterotoxemia vaccine (RVSRI, Iran; n = 5) and G7 (negative control) receiving PBS (n = 5). All groups were monitored for 150 days. Blood samples (3 mL) were collected from the median ear artery before each immunization (days 1, 6, 13) and on days 21, 28, 60, 90, 120, and 150. Samples were allowed to clot at 37°C for 1 hour, centrifuged at 10,000 g for 5 minutes, and sera were stored at -20°C until analysis [18].

In vivo immunization protocol and sampling schedule for immunogenicity assessment

The immunogenicity of injected nanoparticles was assessed using an indirect Enzyme-Linked Immunosorbent Assay (iELISA) to measure antibody titers against ETX [18]. The assay was optimized through a checkerboard method based on prior protocols with modifications [18,19]. Key components such as *C. perfringens* epsilon toxoid (CWEPSILTD), epsilon antitoxin (2CPEPAT), HRP-conjugates, and serum dilutions were titrated to determine optimal concentrations. Briefly, microplates were coated with epsilon toxoid at concentrations ranging from 0.1 to 8 µg/mL. Standard positive and negative sera were serially diluted (1:10 to 1:320), and HRP-conjugates were tested at dilutions from 1:1,000 to 1:8,000. All samples were tested in duplicate and read at 450 nm. The condition yielding the highest OD_{450} positive/negative (P/N) ratio was considered optimal. Further optimization involved parallel microplate setups comparing coating buffers (CBS vs. PBS), incubation times (37°C for 1 - 2 hours vs. 4°C for 6 - 24 hours), blocking agents (1 - 5% BSA, 5% skim milk), blocking durations, and antibody incubation times (30 - 60 minutes). Final assay conditions and serum dilutions (1:5 to 1:80) were selected based on performance.

Determination of iELISA cutoff value

Serum samples from forty rabbits that had not been vaccinated were used as negative controls. Each sample underwent testing in triplicate, and the cutoff value was determined by adding three standard deviations (SDs) to the mean OD_{450} nm of all negative samples. This approach ensures, with 99% confidence, that all negative results lie within the established range [18].

Evaluation of iELISA specificity

The cross-reactivity of the iELISA was evaluated using *C. perfringens* type D (CN_{409}) epsilon toxin, *C. perfringens* type C (CN301) beta toxin, *C. novyi* (CN901) alpha toxin, *C. chauvoi* (Iranian strain), and *C. septicum* (CN913) alpha toxin [20].

Reproducibility of epsilon toxoid and antibody reactions

The reproducibility of epsilon toxoid and antibody reactions was evaluated using different serum samples (at high and low concentrations), as well as negative controls [21]. Each sample was tested in triplicate on three consecutive days. The inter- and intra-assay coefficient of variance (CV) was calculated using the following formula:

$$\text{Mean} = \Sigma xi/n$$

$$SD = \sqrt{\Sigma(ODsample - ODmean)^2}/N$$

$$CV = (SD)/\text{mean} \times 100\%.$$

iELISA procedure for longitudinal antibody detection and titration of neutralizing antibodies

Over a 150-day period, 315 rabbit serum samples were analyzed for anti-epsilon antibodies using an iELISA [18]. In brief, 96-well plates were coated overnight at 4°C with 100 µL of epsilon toxoid in bicarbonate buffer (pH 9.6). Plates were washed three times with PBS containing 0.05% Tween-20, blocked with 150 µL of PBS containing 3% BSA at 37°C for 1 hour, and then incubated with 100 µL of 1:10 diluted serum for 1 hour at 37°C. Following washing, 150 µL of HRP-conjugated Goat Anti-Rabbit IgG (1:4,000) was added and incubated at 37°C for 1 hour. After further washing, 100 µL of TMB substrate was added for 30 minutes at room temperature, and reactions were stopped with 50 µL of 2 M H_2SO_4. Absorbance was read at 450 - 620 nm. All assays were performed in triplicate with standard controls to minimize intraplate variation, and results were expressed as mean ± SD. Serum titers against epsilon toxin were also measured by the mouse neutralization test, following European Pharmacopoeia guidelines [16]. Serum dilutions were mixed with standard epsilon toxin and incubated at 37°C for 30 minutes before 0.2 mL was intravenously injected into two NMRI mice (18 - 22 g). Animals were monitored for 72 hours, with mortality recorded at 24-hour intervals. The neutralization titers were determined and reported in international units per milliliter (IU/mL) following the observation period [22].

Challenge test and determination of protective efficacy

On day 21 of the immunization schedule, rabbits were challenged subcutaneously with activated epsilon toxin, prepared from an overnight culture of *C. perfringens* type D strain (CN409, RVSRI, Iran), at a dose of 100 minimum lethal doses (MLD). Each group contained seven rabbits. The MLD was determined following the RVSRI standard protocol (ANB.0024.SOP). In brief, ETX was trypsin-activated, and the culture supernatant was serially diluted (1:1 to 1:200) in PBS. A 0.5 mL volume of each dilution was injected intravenously into two NMRI mice (18 - 22 g). The assay was repeated, and the combined results were used to calculate the MLD [23].

Statistical analysis

Pearson's chi-squared test ($p \leq 0.05$) was applied in SPSS 29.0 (IBM SPSS Statistics for Windows; IBM Corp., Armonk, NY, USA) to evaluate statistical differences in iELISA results among groups. GraphPad Prism

10.1.1 (GraphPad Software, San Diego, CA, USA) was used to visualize optimization data.

RESULTS

Quality control analysis of coated and uncoated nanoparticles

Sterility testing revealed no microbial growth in Thioglycolate and Sabouraud Dextrose broth media (Merck, Germany) following inoculation with both coated and uncoated nanoparticles, confirming the absence of bacterial and fungal contamination in the nanoformulations. In the residual toxicity assessment, no pathological changes were observed in the behavior or injection sites of the tested mice, indicating non-toxicity of the particles. Similarly, safety evaluations in sheep showed no alterations in temperature, appetite, mobility, or other general health indicators. No local adverse effects such as redness, edema, or necrosis were noted at the injection sites. Potency testing demonstrated that pooled sera from immunized rabbits contained an average of 5 IU/mL of epsilon antitoxin, confirming the immunogenic potential of the formulation.

Checkerboard analysis for maximizing sensitivity and specificity

Through checkerboard titration, the optimal ELISA conditions were established, achieving a maximum P/N ratio of 17.603 with 0.25 µg/well ETX and a 1:160 serum dilution (Table 1). The final parameters included a 1:10 serum dilution, a 1:4,000 secondary antibody dilution, CBS as the coating buffer, 3% BSA blocking at 37°C for 1 hour, incubation at 4°C for 24 hours, a 1-hour reaction time, and TMB termination after 20 minutes in the dark (Figure 1).

Cutoff value, specificity, and reproducibility assessment of iELISA

The mean OD_{450} for 40 negative sera was 0.098 with an SD of 0.253, yielding a cutoff of 0.451. No cross-reactivity with antibodies to other clostridial toxins was observed. Intra-assay CVs were < 5% and inter-assay CVs < 10%, confirming the reliability of the repeatedly validated iELISA setup (Table 2).

Evaluation of humoral immune response induced by NTs and RVNTs

Neutralizing antibody titers varied by formulation, with all groups showing similarly low baseline levels on day -1. As shown in Figure 2, single-dose regimens (NTs-exp1, RVNTs-exp1) peaked early (day 6), while three-dose regimens (NTs-exp2, RVNTs-exp2, Heated NTs-exp2) reached a delayed but higher peak on day 21, maintaining elevated titers over 150 days, unlike the decline seen with the commercial vaccine. RVNTs consistently outperformed NTs (Figures 3a - 3c), with RVNTs-exp2 producing the highest titers, surpassing both the positive control and RVNTs-exp1. Heated NTs-exp2 generated higher titers than the commercial vaccine and NTs-exp2, indicating that 60 minutes of heat treatment did not impair antigen release; however, non-heated NTs-exp2 induced the strongest overall response. No immune response was detected in the negative control (PBS), and anti-ETX titers were significantly higher in the three-dose groups compared to single-dose counterparts (Figure 3d).

Protective efficacy of nanoparticle formulations in the ETX challenge test

Following subcutaneous challenge with 100 MLD of ETX on day 21, the PBS group (negative control) exhibited 100% mortality within 2 hours. NTs-immunized rabbits showed dose-dependent improvement in survival, increasing from 60% (NTs-exp1) to 70% (NTs-exp2). RVNTs conferred superior protection, achieving 90% survival without boosters (RVNTs-exp1) and complete protection with boosters (RVNTs-exp2). Heated and non-heated NTs in exp2 demonstrated comparable protection, whereas the commercial vaccine failed to protect beyond 24 hours. No deaths occurred in any group after 72 hours post-challenge. Survival patterns closely mirrored antibody titers, with RVNT-treated groups showing the most robust and sustained immune responses. Statistical analysis confirmed significant differences between treated and control groups ($p < 0.0001$). Mortality was assessed at hours 6, 12, 24, 36, and 48, and daily until day 10 post-challenge (n = 10 per group).

DISCUSSION

Developing safe and effective vaccines against bacterial toxins like epsilon toxin remains a challenge, as conventional inactivation methods may compromise antigenicity [8]. Nanovaccines, incorporating targeted antigens and nanocarriers, offer a promising alternative to induce robust immune responses. Here, we evaluate the humoral immune response induced by cell membrane-coated PLGA nanoparticles loaded with *C. perfringens* epsilon toxin in rabbit models. In this study, an iELISA was developed and optimized to accurately determine neutralizing antibody titers, providing a reliable tool for evaluating vaccine-induced immune responses. Comparable approaches have been reported by Wang et al., Peng et al., and Nham et al., who employed optimized ELISA platforms to assess immunogenicity, quantify antibody levels, and fine-tune vaccine formulations for enhanced protective efficacy. These parallels underscore the pivotal role of assay standardization and optimization in generating reproducible and comparable immunological data across studies [18,24]. Our iELISA assay demonstrated high sensitivity, specificity, and reproducibility for detecting anti-epsilon antibodies in rabbits, effectively identifying vaccine-induced immunity. Subsequent immunization studies focused on evaluating the nanotoxoid's ability to induce neutralizing an-

Table 1. Highest P/N ratios identifying the optimal epsilon toxoid coating concentration and standard serum dilutions (P: OD_{450} value of positive samples, N: OD_{450} value of negative samples).

Positive and negative standard sera dilutions	Antigen dilution (µg/mL)						
	0.1	0.25	0.5	1	2	4	8
1:10	9.764	17.424	10.103	9.773	9.018	5.909	5.53
1:20	7.33	16.056	9.983	7.451	5.818	4.637	4.55
1:40	6.371	12.304	9.638	6.44	6.254	5.849	4.100
1:80	6.557	10.580	8.365	6.352	6.297	4.964	2.987
1:160 *	5.894	17.603 *	7.237	5.933	3.957	2.761	1.924
1:320	5.688	9.980	6.931	5.642	5.344	2.228	0.862

*** Optimal values (highest P/N ratio).**

Table 2. Repeatability analysis of positive and negative samples in iELISA: intra- and inter-assay variability based on OD_{450} values.

		Intra-assay		Inter-assay	
		$\bar{x} \pm SD$	CV (%)	$\bar{x} \pm SD$	CV (%)
Positive samples	1	2.493 ± 0.033	1.361	2.751 ± 0.084	3.058
	2	2.622 ± 0.065	2.482	2.766 ± 0.072	2.630
	3	2.668 ± 0.074	2.809	2.282 ± 0.088	3.872
	4	2.746 ± 0.054	1.994	2.639 ± 0.090	3.438
	5	2.502 ± 0.049	1.978	2.506 ± 0.150	5.999
Negative samples	1	0.159 ± 0.006	3.967	0.153 ± 0.013	8.553
	2	0.114 ± 0.009	8.134	0.179 ± 0.005	3.090
	3	0.213 ± 0.008	3.815	0.169 ± 0.007	4.246
	4	0.224 ± 0.004	2.150	0.178 ± 0.007	4.315
	5	0.200 ± 0.008	4.330	0.137 ± 0.008	6.136

$\bar{x}$: mean OD_{450} of samples, SD: standard deviations, CV: coefficient of variance.

tibodies, a key indicator of antitoxin protection. Neutralizing antibody titers showed a significant dose-dependent increase, suggesting that higher immunization doses can more effectively stimulate humoral immunity and enhance the generation of protective antitoxin responses. In another study, such dose-dependent antibody production has been widely reported in vaccination studies against epsilon toxin [25]. We hypothesized that NTs would elicit a stronger humoral immune response than commercial vaccines, and experimental results confirmed this. Rabbits vaccinated with NTs showed a significant increase in ETX-specific antibody titers by day 21 compared to the commercial vaccine. Antibody levels continued to rise slightly from one to five months ($p < 0.05$) and remained elevated throughout the 150-day study, in both prime-only and prime-boost regimens [7,26]. These findings suggest that the third dose of NTs elicits a stronger and more sustained immune response compared to a single dose. Both our study and prior research indicate that heat treatment of nanoparticles does not significantly enhance antibody titers. Notably, NTs induced antibody persistence for up to five months, significantly longer than the 21-day duration seen with the commercial vaccine [27]. Membrane-coated NTs, such as RVNTs, are expected to have prolonged circulation times compared to uncoated (bare) NTs. To assess this, we compared anti-epsilon antibody titers in RVNT-immunized groups with those receiving bare NTs. RVNTs showed superior responses, likely due to the RBC membrane acting as a barrier to diffusion, a phenomenon consistent with earlier studies [28]. Similarly, Wang et al. reported that PLGA nanoparticles coated with cell membranes exhibited a slightly slower drug release compared to uncoated nanoparticles. The membrane surface acts as a diffusion barrier, enabling more controlled and sustained release, thereby

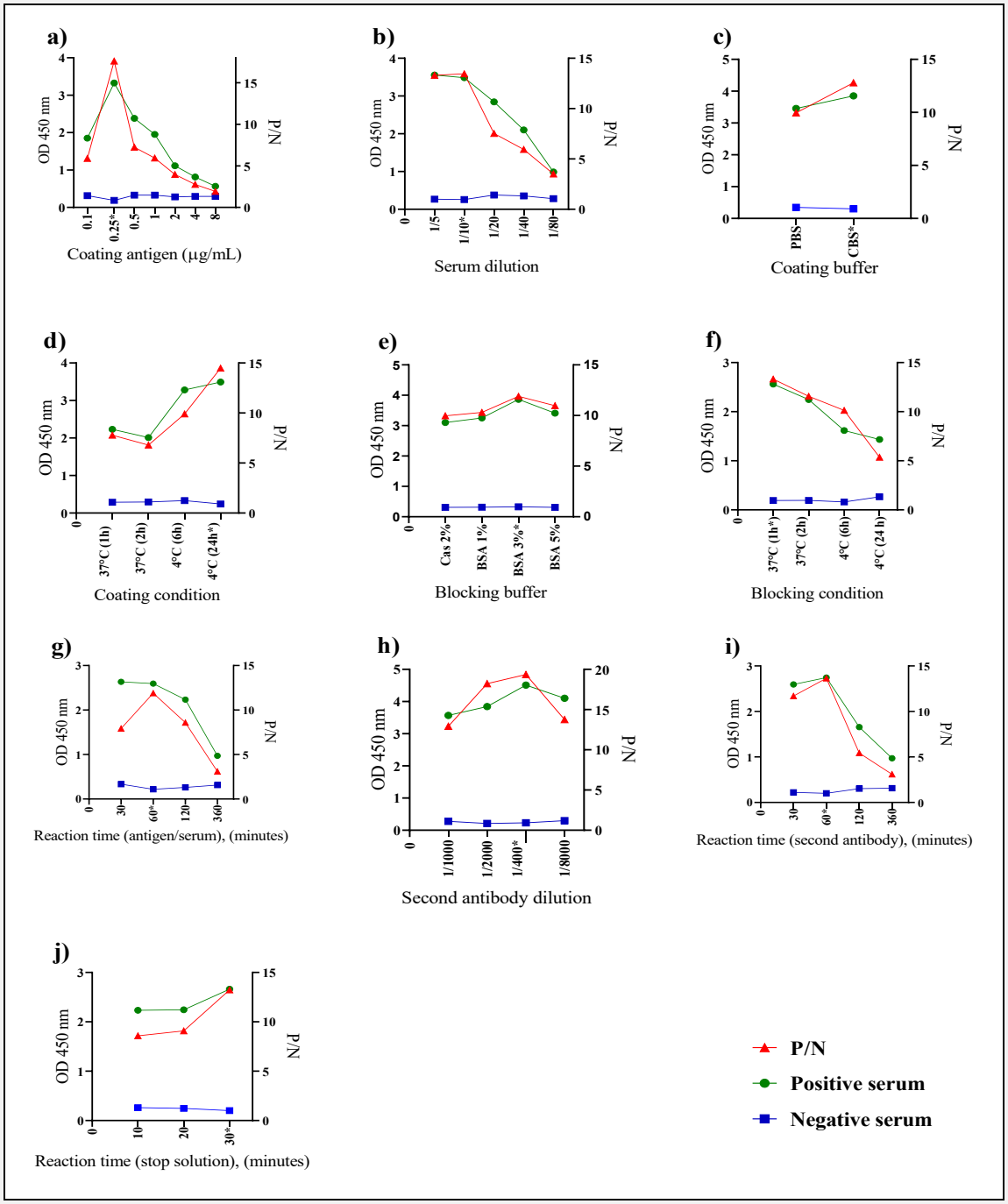


Figure 1. Optimization of iELISA working conditions.

a, b: checkerboard titration to determine optimal epsilon toxoid coating concentration and serum dilution, c: selection of coating buffer, d: coating conditions, e: blocking agent, f: blocking conditions, g: antigen and serum incubation time, h: secondary antibody dilution, i: secondary antibody incubation time, and stop solution incubation time. Asterisks (*) indicate the optimal conditions identified for the iELISA (n = 3). Data are expressed as mean ± SD.

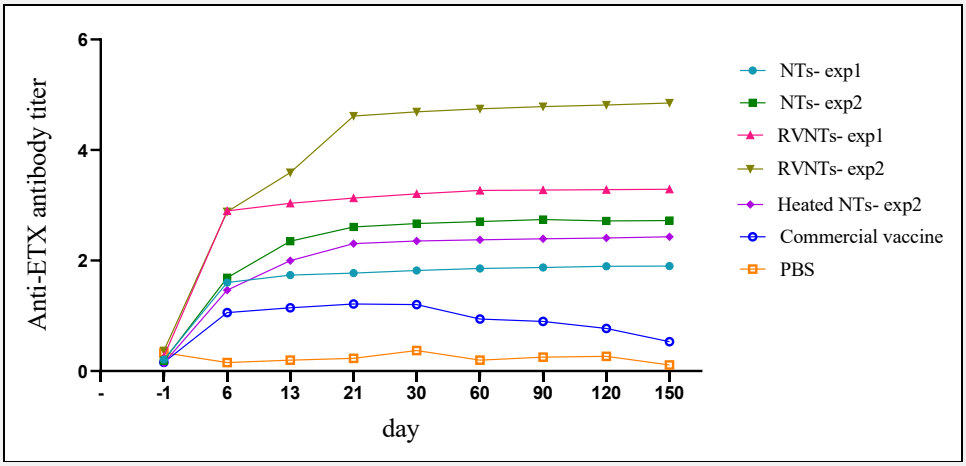


Figure 2. Immunogenicity of nanoparticle formulations over a 150-day period. NTs-exp1 and NTs-exp2 received one and three doses of NTs, respectively.

RVNTs-exp1 and RVNTs-exp2 received one and three doses of RVNTs, respectively. The Heated NTs-exp2 group received three doses of heat-treated NTs. Positive and negative controls were immunized with a commercial tetravalent enterotoxemia vaccine or phosphate-buffered saline (PBS), respectively. Data are shown as mean ± SD.

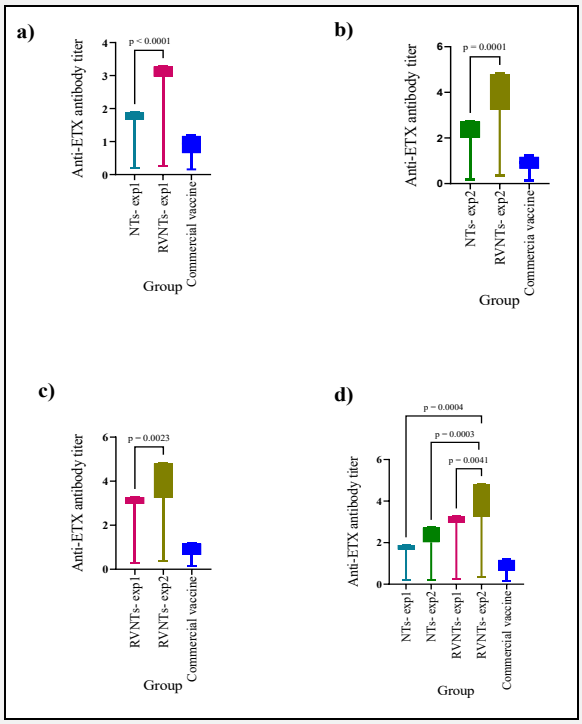


Figure 3. Comparison of anti-ETX antibody titers between RVNTs and bare NTs in a) experiment 1 and b) experiment 2. c) Comparison of anti-ETX titers between RVNT-treated groups from experiments 1 and 2. d) Comparison of anti-ETX titers from all groups relative to the RVNTs-exp2 group.

Data are presented as mean ± SD. Statistical significance was determined using RM one-way ANOVA.

supporting long-term therapeutic delivery [29]. Consistent with the observations reported by Hu et al., the present study confirmed that RBC membrane coating markedly improves nanoparticles' stability and extends their *in vivo* circulation time compared to uncoated counterparts [9]. Hu et al. demonstrated that bare nanoparticles rapidly aggregate in serum and that RBC membrane vesicles are cleared from the bloodstream within minutes, whereas the incorporation of a solid core significantly enhances structural rigidity and particle stability. In agreement, our RVNTs formulation comprising a PLGA core enveloped by RBC membrane exhibited prolonged systemic retention and sustained antigen availability. This outcome highlights the synergistic role of the rigid polymeric core in maintaining structural integrity and the biomimetic membrane in evading immune clearance, collectively contributing to improved pharmacokinetics and potential enhancement of vaccine efficacy. Erythrocyte membranes possess a repertoire of membrane proteins, saccharides, and lipids that collectively contribute to their remarkable longevity in circulation, often exceeding three months [30]. Leveraging this unique biological interface to coat nanoparticles can substantially attenuate their immunogenicity and markedly extend systemic residence time. In conclusion, RVNTs enhanced stability, circulation time, and antigen persistence, while reducing immunogenicity and inducing strong immune responses against *C. perfringens* epsilon toxin [31]. These results highlight RVNTs as a promising nanovaccine platform for clostridial enterotoxemia prevention. The study was restricted to a single animal model, a limited observation period, and lacked mechanistic insight into antigen processing. Scalability for large-scale production was not assessed. Further research should address long-term immunity, cross-protection across toxinotypes, and multi-species efficacy, as well as explore polyvalent RVNTs formulations for broader protection.

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Ethical Considerations:
The Islamic Azad University Ethical Committee, Kerman Branch, approved the project protocol (Ethical code: IR.IAU.Kerman.REC.1400.011). The participants filled out a written informed consent before sampling.

Declaration of Generative AI:
This study, derived from a PhD thesis at the Razi Vaccine and Serum Research Institute (Iran), was conducted and written without the use of any generative AI tools in its design, analysis, interpretation, or writing process.

Declaration of Interest:
This study was conducted without any specific financial support from public, commercial, or nonprofit funding agencies. The authors declare no actual or potential conflicts of interest financial or otherwise that could have influenced the design, execution, or interpretation of the research presented in this manuscript.

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