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Study of the effectiveness of combined rabies-tetanus vaccine as compared to individual vaccines

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Purpose: Effective treatment for animal bites is essential, encompassing immediate measures and protocols for rabies and tetanus vaccination. In this study, we evaluated the effectiveness of the administration of individual and combined rabies-tetanus (RT) vaccines in mice as model animals.

Materials and Methods: Animal groups were injected with either undiluted Toxovac® combined with Rabies vaccine® (RT/Group 1)/Speeda® (ST/Group 3), 2-fold diluted Toxovac® with Rabies vaccine® (RT1/2/Group 2), or purified tetanus toxoid with Speeda® (Spurf/Group 4). Mice were immunized with either 2 intraperitoneal (IP) doses at one-week interval or one subcutaneous (SC) dose for rabies immunogenicity, and with one SC dose for tetanus immunogenicity. The potency of the vaccines was determined through challenge test, while their immunogenicity was examined by measuring the anti-rabies and anti-tetanus immunoglobulin G response.

Results: All tested vaccines were potent except Spurf; tetanus was not potent. Rabies' immunogenicity for all combinations through both routes of administration showed comparable antibody response & non-significant difference ($p \geq 0.05$) at days 14 and 28 compared to single rabies injected by 2 IP doses. Tetanus' immunogenicity in combinations was compared with Toxovac®. RT depicted higher antibody response on both days 14 and 28. Whereas RT1/2 showed a non-significant difference on both days 14 and 28. Therefore, rabies has a synergistic effect on tetanus in combination.

Conclusion: The immune response to rabies in combination vaccine injected as a single SC dose was as effective as 2 IP doses of single vaccine. Our results highlight the potential of RT combination vaccine via SC as a cost-effective means to provide protective immunity.

Keywords: Rabies; Tetanus; Combination vaccines; Potency test; Immunogenicity test

INTRODUCTION

Rabies is a viral zoonotic disease transmitted through bites of rabid animals, affecting the central nervous system (CNS) and causing fatal inflammation of the brain and spinal cord. Annually, it results in tens of thousands of deaths, particularly in Africa and Asia, with 40% being children under 15. Although rabies claims about

70,000 deaths each year globally, it is entirely preventable through vaccination, either pre-exposure prophylaxis (PrEP) or post-exposure prophylaxis (PEP) [1,2]. The average cost of rabies PEP is approximately US$ 108, contributing to a global annual cost of dog-mediated rabies treatment estimated at US$ 8.6 billion [1]. Rabies PEP is crucial for its life-saving impact. Recent guidelines recommend a 2-dose PrEP schedule (days 0 and 7) for protection up to 3 years [3], replacing the previous 3-dose schedule. Four doses on days 0, 3, 7, and 14 are recommended for PEP, with an additional fifth dose on day 28 [3].

Animal bites or wounds contaminated with saliva or dirt may lead to tetanus (lockjaw), caused by *Clostridium tetani* bacterial spores. Tetanus, causing over 34,000 deaths worldwide in 2019, is prevalent in Asia and Africa, with more than 500 deaths reported in Egypt in 2020. Treatment and prevention involve administering the tetanus vaccine [4-6].

The animal bites treatment protocol encompasses both rabies and tetanus vaccination. If the last tetanus dose is uncertain, a shot should be given within 72 hours after injury, especially if the previous shot was not administered within 5–10 years [7-9].

Combination vaccines have become a worldwide expanded strategy because they offer protection against multiple diseases. They minimize the need for numerous shots and visits, ensuring timely and efficient protection, resulting in fewer delays. This approach also enhances patient compliance by reducing pain and discomfort while saving both time and costs. Additionally, it simplifies the storage of vaccine vials and facilitates record-keeping and tracking [10]. Yet, creating and manufacturing combination vaccines poses challenges due to potential interactions among the antigens and other components involved.

This study aimed to investigate the effectiveness of the combined rabies-tetanus (RT) vaccine as compared to individual vaccines, the route of administration, and the effect of the combination on the efficacy and immunogenicity of each component.

MATERIALS AND METHODS

Vaccines, viruses, and toxins

Standard rabies vaccine: The standard lyophilized rabies vaccine used was produced by the National Institute for the Control of Pharmaceutical and Biological Products, China, with a labelled potency of 6.6 IU/mL.

Rabies vaccine (Speeda®): Purified inactivated freeze-dried vaccine for human use, propagated on Vero cells, produced by Liaoning Cheng Da Biotechnology Co., Ltd. China.

Rabies vaccine®: Purified inactivated freeze-dried vaccine for human use, propagated on Vero cells, produced by Changchun Changsheng Life Science, China.

Tetanus toxoid reference vaccine: Home reference with an assigned potency of 140 IU/mL was produced by the Egyptian Company for Production of Vaccines, Sera and Drugs (VACSERA), Egypt.

Purified concentrated tetanus toxoid: Home reference with known assigned titre (4,300 Lf/mL) was produced by VACSERA, Egypt.

Toxovac® vaccine: Tetanus toxoid vaccine adsorbed on alum for human use, was produced by VACSERA, Egypt.

Rabies virus: Challenge virus strain (CVS) is a fixed rabies virus strain derived from the original Pasteur strain; it was propagated and fixed in mice brain. The virus strain was originally obtained from Pasteur Institute, France, in freeze-dried form.

Tetanus toxin: Home reference produced by VACSERA, Egypt. Its assigned LD_{50} value is 100,000 LD_{50}/mL.

RT combined vaccine: The vaccines were prepared by reconstitution of commercial rabies vaccine (Speeda®/Rabies vaccine®) with commercial tetanus vaccine (Toxovac®) or purified tetanus toxoid.

Experimental animals

Male and female Swiss albino mice were purchased from Al-Fahd animal supplier Abu-Rawash, Egypt, weighing 11–15 g and 14–16 g for rabies and tetanus tests, respectively. Animals handling was according to international guidelines [11, 12]. Animals housing was performed with access to water and food and in an environmentally controlled room in compliance with standard laboratory conditions. They were housed in the animal house of the Egyptian Drug Authority, Agouza, Giza (Egypt), and quarantined for one week before use in their specified test. Approval for conducting the study was obtained from the Ethical Committee at the Faculty of Pharmacy, Cairo University (Code: MIC 2–3).

Study protocol

The study protocol experimental design is shown in **Fig. 1**. Potency tests were carried out following the National Institutes of Health (NIH) test [13-15] and challenge assay method protocols [15], and immunogenicity tests were carried out following standard protocol [16,17]. Mice were divided into 4 vaccine combination groups (Groups 1, 2, 3, and 4). In addition to 4 control groups, 2 for rabies tests (Control 1 and 2) and 2 for tetanus tests (Control 3 and 4). Another 2 groups were assigned as diluent control for rabies and tetanus immunogenicity tests (Diluent control 1 and 2

[DC]), as shown in **Table 1**. The 4 combination groups were tested for rabies potency, rabies immunogenicity, tetanus potency, and tetanus immunogenicity.

For rabies potency test, mice were divided into 3 subgroups, each of different dilutions (test vaccine: 4 dilutions of 20 mice each; standard rabies vaccine: 4 dilutions of 20 mice each; working CVS control with 3 back titration dilutions of 10 mice each).

For tetanus potency test, mice were divided into 3 subgroups, each of different dilution (test vaccine: 4 dilutions of 16 mice each; tetanus toxoid reference vaccine: 3 dilutions of 16 mice each; challenge toxin dose as a control: 16 mice each).

For the immunogenicity test, the animals were divided

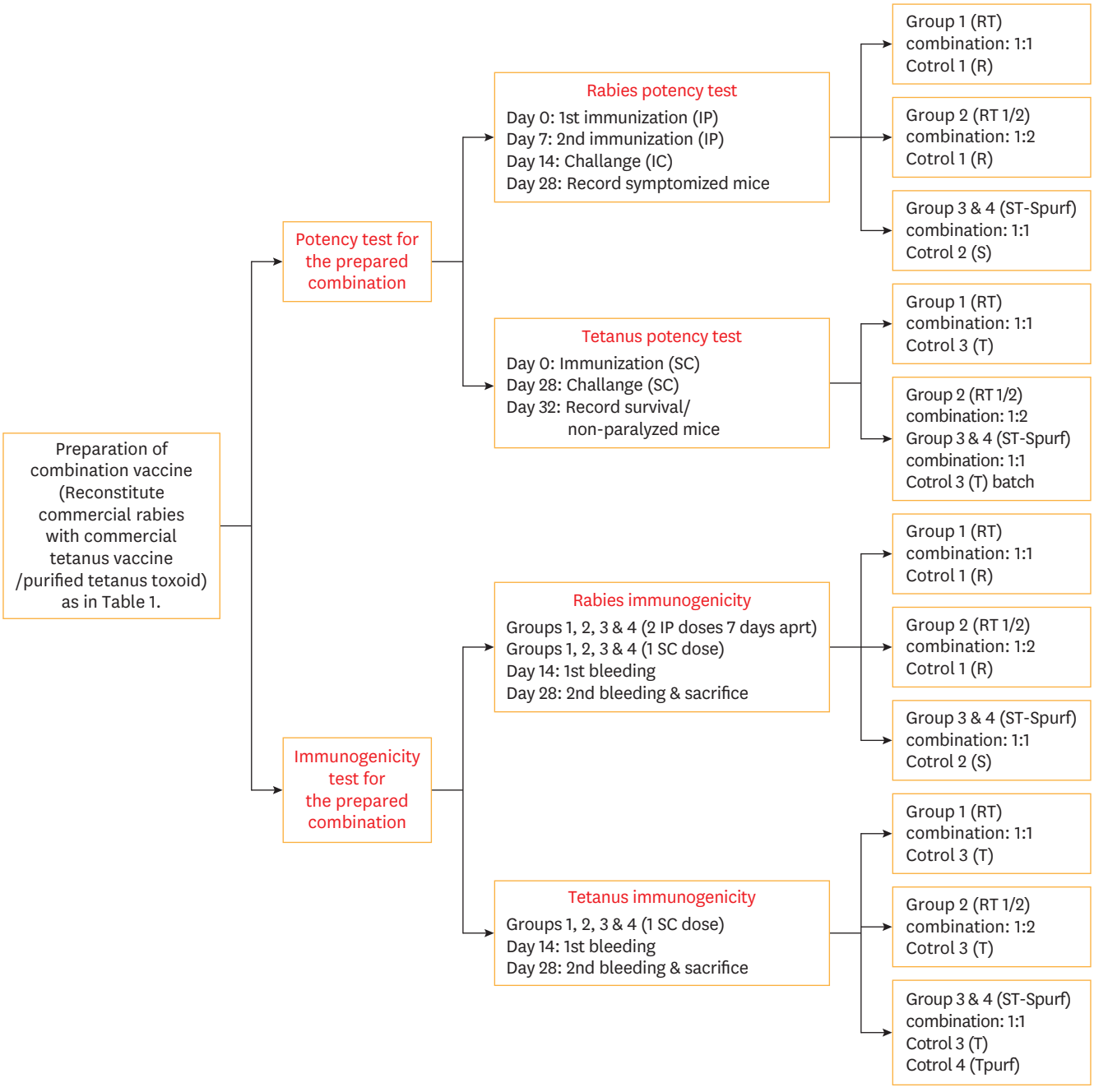

Fig. 1. Study protocol experimental design.
IP, intra-peritoneal; IC, intracerebral; SC, subcutaneous; R, rabies single vaccine (Rabies vaccine®); S, rabies single vaccine (Speeda®); RT, combined rabies-undiluted tetanus vaccine; RT1/2, combined 2-fold diluted tetanus and rabies vaccine; T, tetanus single vaccine (Toxovac®); Tpurf, purified tetanus toxoid; ST, combined rabies (Speeda®)-tetanus vaccine; Spurf, combined rabies (Speeda®)-purified tetanus toxoid.

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Table 1. Composition of vaccines for different mice groups

Mice group	Composition of test vaccine
Group 1 (RT)	Reconstituted Rabies vaccine® in 0.5 mL Toxovac®.
Group 2 (RT1/2)	Reconstituted Rabies vaccine® in 0.25 mL WFI and 0.25 mL Toxovac® vaccine.
Control 1 (R) (for rabies potency/immunogenicity; Batch 1: control for group1, Batch 2: control for group 2)	Reconstituted commercial Rabies vaccine® in 0.5 mL WFI.
Group 3 (ST)	Reconstituted Speeda® vaccine in 0.5 mL Toxovac®.
Group 4 (Spurf)	Reconstituted Speeda® vaccine in 0.5 mL purified tetanus toxoid 50 LF/mL.
Control 2 (S) (for rabies potency/immunogenicity; as control for groups 3 and 4)	Reconstituted Speeda® vaccine in 0.5 mL WFI.
Control 3 (T) (for tetanus potency/immunogenicity; Batch 1: control for group 1, Batch 2: control for groups 2, 3, and 4)	Commercial adsorbed tetanus toxoid vaccine (Toxovac® vaccine).
Control 4 (Tpurf) (for tetanus immunogenicity; as control for group 4)	Purified tetanus toxoid 50 Lf/mL.
Diluent control 1 (DC; for rabies immunogenicity)	0.9% saline + 2% foetal bovine serum.
Diluent control 2 (DC; for tetanus immunogenicity)	0.9% saline.

RT, combined rabies-undiluted tetanus vaccine; RT1/2, combined 2-fold diluted tetanus and rabies vaccine; R, rabies single vaccine (Rabies vaccine®); ST, combined rabies (Speeda®)-tetanus vaccine; Spurf, combined rabies (Speeda®)-purified tetanus toxoid; S, rabies single vaccine (Speeda®); T, tetanus single vaccine (Toxovac®); Tpurf, purified tetanus toxoid; DC, diluent control; WFI, water for injection.

into 10 groups of 10 mice each. Four mice groups received combination vaccines, and 4 groups served as control and received single vaccine preparation according to the scheme shown in **Table 1**. The control groups for rabies and tetanus received a single vaccine preparation formulated from the same batch used for the preparation of combination vaccines. In addition, 2 groups received DC.

Assessment of potency

Potency of rabies by NIH test method

The degree of protection offered by rabies vaccine (either single or combination vaccine) was tested against reference rabies vaccine in immunized mice according to standard procedure [13-15]. Animal groups were immunized twice at day 0 and day 7 intraperitoneally (IP) with two 0.5 mL doses. Then, they were challenged intracerebrally with a CVS mouse brain strain of fixed rabies virus diluted to 50 LD_{50}/0.03 mL on the 14th day. Mice were observed for 14 days after challenge, and the number of deaths after the first 5 days was recorded, including mice showing signs of rabies virus (paralysis, convulsions) on the 28th day. The median effective dose (ED_{50}) for both reference and test vaccines were calculated based on number of survived mice. The relative potency was calculated using Spearman-Karber formula through comparison of test vaccine ED_{50} with that of the reference vaccine.

Potency of tetanus of the prepared combined vaccines by challenge method

The degree of protection of tetanus vaccine (single and combined preparations) was tested against reference vaccine in immunized mice according to standard procedure [16,19]. On day 28th following subcutaneous (SC) immunization, mice were challenged by tetanus toxin diluted to LD_{50}/dose in 0.5 mL at the lumbar region of the spine so that the distinctive hind limb paralysis could be recognized at early stages. The mice were examined over the next 96 hours for either paralysis or death. ED_{50} of the test vaccine relative to the ED_{50} of the reference vaccine was calculated based on the proportion of challenged animals that did not develop paralysis or survived in each group of vaccinated animals to calculate the potency of the test vaccine. Calculation of the potency was performed using a probit program for parallel line assay (Combistats® software; CombiStats, Strasbourg Cedex, France) [16,19].

Assessment of immunogenicity for the prepared combined vaccines

Immunogenicity of combined RT vaccine against single vaccine (as a control) was evaluated by induction of antibodies in mice, then assessment of anti-rabies/anti-tetanus immunoglobulin G (IgG) in mice sera through measuring optical density (OD) by enzyme linked immunosorbent assay (ELISA).

Induction of antibodies in mice

Groups of 10 mice were inoculated with one of the following: two 0.5 mL doses of combination vaccine/single rabies vaccine IP on days 0 and 7 (for rabies immunogenicity test) or one 0.5 mL dose of combination vaccine/tetanus vaccine/purified tetanus toxoid vaccine SC (for tetanus immunogenicity test). DC groups were inoculated with either two 0.5 mL diluent IP on day 0 and 7, or one 0.5 mL diluent dose SC. Mice sera were collected at day 14 and day 28 post-vaccination for detection of anti-rabies/anti-tetanus IgG antibodies by ELISA technique.

Assessment of anti-rabies antibodies

Anti-rabies IgG response in mice sera was determined as

per the method described by Vorndam and Beltran [17], as follows: ELISA plates (Nunc, Rochester, NY, USA) were coated with 1/50 diluted rabies vaccine and incubated overnight. The next day, plates were washed, blocked, and washed again. Antisera of different groups were 5-fold serially diluted from 1/5 to 1/3,125, dispensed into the plate, and incubated for 2 hours at 37°C. The assay was controlled using DC containing assay diluent. Then plates were washed, and anti-mouse IgG (whole molecule)-peroxidase conjugate (Sigma-Aldrich, St. Louis, MO, USA), diluted at 1:40,000 was added and incubated at 37°C for an hour then chromogen substrate solution (3, 3', 5, 5'-tetramethylbenzidine) (TMB) (Sigma-Aldrich) was added after washing and plates were incubated in dark place. The 0.3 M sulphuric acid stopping solution (Merck KGaA, Darmstadt, Germany) was added and the absorbance was measured by ELISA reader at 450/620 nm. Cut-off value was defined as the average values of DC plus 2 standard deviations (SDs) [17,20-22].

Assessment of anti-tetanus antibodies

Anti-tetanus IgG response in mice sera was assessed according to the method of Yu et al. [18] with some modifications including that ELISA plates (Nunc) were coated by adding 100 µL/well of diluted purified tetanus toxoid to 0.5 Lf/mL in carbonate-bicarbonate buffer (Sigma-Aldrich). Then plates were blocked using 250 µL/well of phosphate-buffered saline (PBS) (Bio Basic, Toronto, Canada) containing 0.05% tween20 (MP Biomedicals, Santa Ana, CA, USA) (PBS-T) and 4% bovine serum albumin (Serva Electrophoresis, Heidelberg, Germany), incubated at 37°C for 90 minutes. Plates were washed with PBS-T then 100 µL/well of 2-fold serially diluted sera in PBS starting from 1/50 to 1/1,600 were added in triplicate and incubated at 37°C for 2 hours. Plates are then washed, and 100 µL/well of 1:40,000 anti-mouse IgG (whole molecule)-peroxidase conjugate (Sigma-Aldrich) diluted in PBS was added, and incubated at 37°C for one hour. Plates were washed and 100 µL/well chromogen substrate solution (TMB) (Sigma-Aldrich) was added, and incubated at room temperature in dark for 30 minutes, then 100 µL/well of KPL stop solution (KPL, Gaithersburg, MD, USA) was added. Absorbance was measured by ELISA reader at 450/620 nm. The cut-off value was determined as the average values of DC plus 2 SD [17].

Statistical analysis

For immunogenicity testing by induction of antibodies in experimental mice, one-way analysis of variance followed by Holm-Sidak's multiple comparison test for determination of significance between single and combination vaccines was used. All data were processed statistically and graphed using GraphPad Prism 8 Software (GraphPad Inc., La Jolla, CA, USA). Differences with p-values less than 0.05 were considered statistically significant.

RESULTS

Assessment of potency

Potency of rabies by NIH test

All combination vaccine preparations elicited protective antibodies sufficient to protect mice against lethal dose of CVS virus in a dose-dependent manner and showed accepted potency values as per World Health Organization (WHO) regulations (≥2.5 IU/dose) [13-15]. The results obtained are shown in **Table 2**.

Potency of tetanus by challenge method

All combination vaccine preparations produced antibodies titer against tetanus toxin sufficient to protect mice against lethal challenge toxin in a dose-dependent manner. They showed accepted potency values as per WHO regulations (≥40 IU/SHD) [19], except Group 4 (Spurf) which received reconstituted rabies vaccine in 0.5 mL purified tetanus toxoid 50 LF/mL. The results obtained are shown in **Table 3**.

Table 2. Rabies potency in single and combination vaccines

Experimental group	Log ED_{50}	Potency (IU/dose)
Control 1 batch 1 (R)	3.033	4.091
Group 1(RT)	2.123	5.319
Control 1 batch 2 (R)	2.273	3.561
Group 2 (RT1/2)	1.925	3.371
Control 2 (S)	2.38	4.555
Group 3 (ST)	2.357	4.317
Group 4 (Spurf)	2.361	4.356

ED_{50}, median effective dose; R, rabies single vaccine (Rabies vaccine ®); RT, combined rabies-undiluted tetanus vaccine; RT1/2, combined 2-fold diluted tetanus and rabies vaccine; S, rabies single vaccine (Speeda®); ST, combined rabies (Speeda®)-tetanus vaccine; Spurf, combined rabies (Speeda®)-purified tetanus toxoid.

Table 3. Tetanus potency in single and combination vaccines

Group	Potency
Control 3 batch 1 (T)	74.88 IU/d
Group 1 (RT)	145.31 IU/d
Control 3 batch 2 (T)	53.74 IU/d
Group 2 (RT1/2)	51.03 IU/d
Group 3 (ST)	126.17 IU/d
Group 4 (Spurf)	<40 IU/SHD

T, tetanus single vaccine (Toxovac®); RT, combined rabies-undiluted tetanus vaccine; RT1/2, combined 2-fold diluted tetanus and rabies vaccine; ST, combined rabies (Speeda®)-tetanus vaccine; Spurf, combined rabies (Speeda®)-purified tetanus toxoid.

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The results showed that the undiluted combination vaccine Group 1 (RT) is roughly twice as potent as the single vaccine of the same batch (Control 3) (T). This was confirmed when the tetanus component in the combination vaccine, diluted 2-fold in Group 2 (RT1/2) demonstrated potency comparable to the single vaccine.

Assessment of immunogenicity

Rabies immunogenicity testing

Anti-rabies IgG response in mice sera immunized with single rabies vaccine (R/S) as control injected by 2 IP doses at one-week interval and with combination vaccines (RT, RT1/2, ST and Spurf) administered by 2 routes of administration (traditional 2 IP doses at one-week interval and one SC dose) were evaluated. Sera of the non-vaccinated group injected with diluent were used as DC.

Results shown in **Fig. 2** revealed that the immunogenic response at day 14 for Group 1 (RT) by SC route is comparable to that obtained by IP route and is equivalent to Control 1 (R). Moreover, an elevated level of antibodies was shown for Control 1 (R) than for Group 2 (RT1/2) following SC route or IP route. Unlike the results of day 14, the

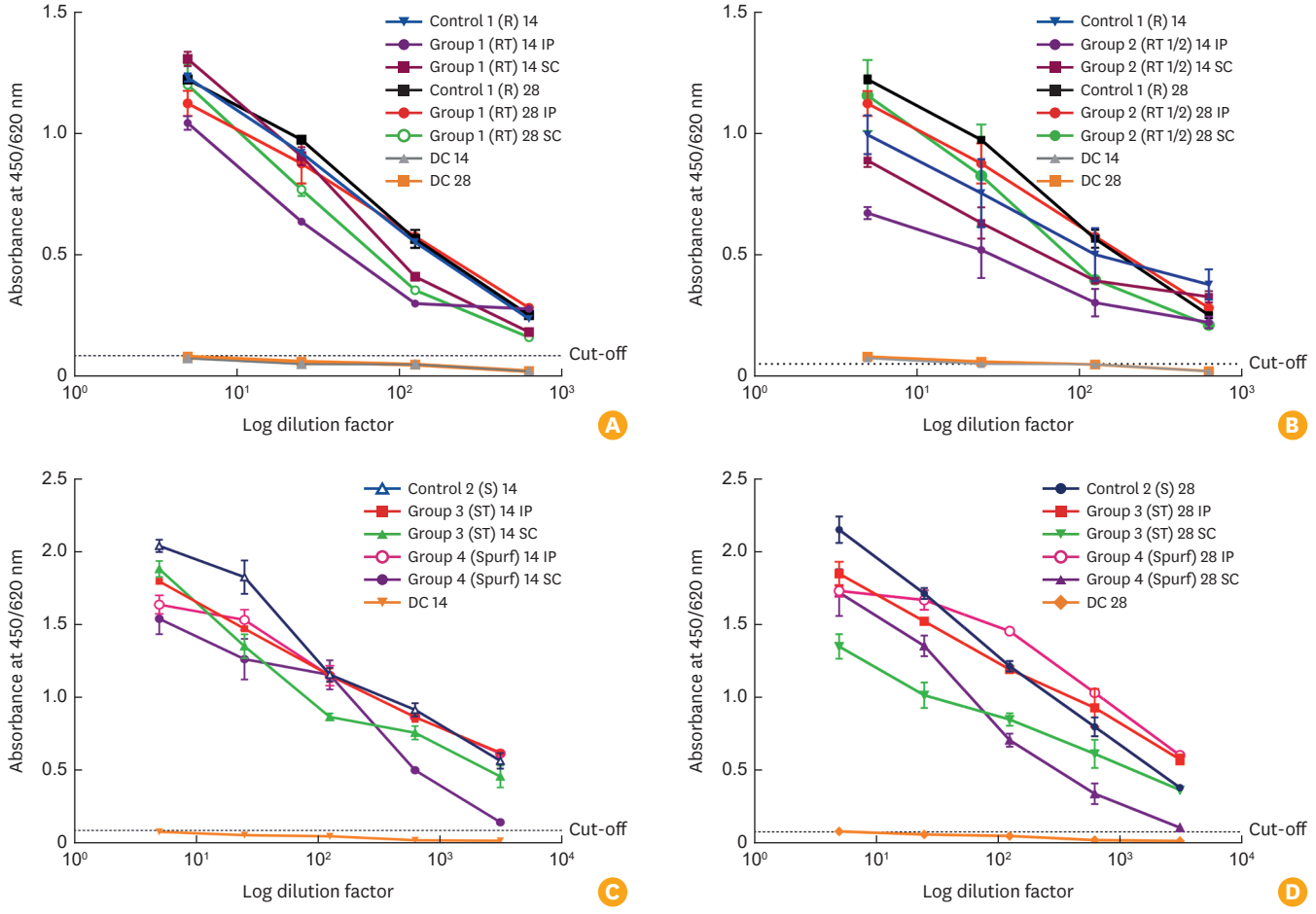

Fig. 2. Anti-rabies immunoglobulin G response to rabies-tetanus combination vaccine in mice' sera determined by enzyme linked immunosorbent assay. Four groups were immunized with combination vaccines (Group 1: RT; Group 2: RT1/2; Group 3: ST; Group 4: Spurf) once by 2 IP doses at one-week interval and another run by one SC dose. Two mice groups were kept as control and were immunized with single rabies vaccine (Controls 1, 2: R/S) as 2 IP doses at one-week interval. Mice sera were collected at day 14 and day 28 post vaccination: (A) Group 1 days 14 and 28; (B) Group 2 days 14 and 28; (C) Groups 3 and 4 day 14; (D) Groups 3 and 4 day 28. Sera of non-vaccinated group injected with diluent were used as DC. Data is the mean absorbance of triplicate wells. Error bars show the standard deviation. Cut-off value is shown as horizontal dashed line. Significance was determined by one-way analysis of variance (Holm-Sidak's multiple comparison test) analysis, no significant difference ($p<0.05$) between combination groups and control groups.

RT, combined rabies-undiluted tetanus vaccine; RT1/2, combined 2-fold diluted tetanus and rabies vaccine; ST, combined rabies (Speeda®)-tetanus vaccine; Spurf, combined rabies (Speeda®)-purified tetanus toxoid; IP, intraperitoneal; SC, subcutaneous; R, rabies single vaccine (Rabies vaccine®); S, rabies single vaccine (Speeda®); DC, diluent control.

immune response at day 28 for all groups was lower than Control 1 (R) either by IP route or SC route. Even though all combination vaccine groups (1, 2, 3 and 4) injected by 2 IP doses showed non-significant difference ($p \geq 0.05$) compared to single rabies vaccine at day 14 (p=0.6578, 0.0658, 0.9238, and 0.9238, respectively), and day 28 (p=0.7926, 0.8711, 0.9890, and 0.9890, respectively). They also showed a non-significant difference when injected as one dose by SC route compared to single rabies vaccine at day 14 (p=0.834, 0.2745, 0.6467, and 0.716, respectively) or day 28 (p=0.7652, 0.8455, 0.1446, and 0.1446, respectively). Both routes of injection (2 IP doses and one SC dose) showed non-significant difference compared to each other across all combination vaccine groups. There was a significant difference between the immunized groups and the unimmunized DC group.

Tetanus immunogenicity

Anti-tetanus IgG response in mice sera immunized with single tetanus vaccine (T), purified tetanus toxoid (Tpurf) as controls and combination vaccines (RT, RT1/2, ST and Spurf) were evaluated. Sera of non-vaccinated group injected with diluent were used as DC.

Results shown in **Fig. 3** revealed that immune response increased significantly in Group 1 (RT) combination vaccine compared to Control 3 (T) at both days 14 and 28 (p=0.0043 and 0.0010), respectively. Whereas upon 2-fold dilution of tetanus component as per Group 2 (RT1/2), the antibody level decreased at both days 14 & 28. However, there was no-significant difference between Group 2 (RT1/2) and Control 3 (T) at both days 14 and 28 (p-values=0.0880 and 0.7903, respectively). The antibody level of both Group 3 (ST) and Control 3 (T) at day 14 is nearly equivalent followed by Control 4 (Tpurf) and Group 4 (Spurf), and they showed non-significant difference compared to Control 3 (T) (p=0.9129, 0.1482, and 0.1126, respectively). While at day 28, Group 3 (ST) had the highest immune response and showed a significant difference against Control 3 (T), Group 4 (Spurf) and Control 4 (Tpurf) also showed significant difference against Control 3 (p=0.0135, 0.0015 and 0.0010, respectively). Moreover, Groups 3 (ST) and 4 (Spurf) showed non-significant difference compared to each other at day 14 (p=0.0979). However significant difference in antibody response was shown at day 28 between Groups 3 and 4 (p-value <0.0001). At day 28, Control 4 (Tpurf) and Group 4 (Spurf), their ODs were below the cut-off value. There was a significant difference between the immunized groups and the unimmunized DC group except for Control 4 (Tpurf) and Group 4 (Spurf) at day 28 (both p-values=0.9811).

DISCUSSION

Rabies virus infects the mammalian CNS, causing progressive fatal inflammation of the brain and spinal cord and, finally death. Rabies prophylaxis is a major priority due to the fatal outcome of the disease. PEP has a very important place due to its life-saving effect [1]. For any rabies vaccination strategy to be effective, it must achieve sufficient coverage in young children, the highest-risk group. Therefore, it is recommended to administer human rabies vaccine in combination with an existing standard childhood vaccine included in the WHO Expanded Programme of Immunization [23].

PEP schedules that use less vaccine spare cost may also be critical to overcome vaccine shortages in settings where vaccine availability is limited, especially where patients can be pooled for injections. Abridged schedules with fewer doses and changing administration routes during a single course have the potential to save costs, increase patient compliance, and thereby improve equitable access to life-saving PEP for at-risk populations [24,25].

Injuries resulting from animal bites or wounds contaminated with dirt or saliva may also lead to tetanus infection (lockjaw) [26,27]. Animal bite treatment guidelines include both rabies vaccination and a tetanus vaccine shot if not administered within 5–10 years [7-9].

WHO has declared 2030 a target year to wipe out dog-mediated human rabies. However, increasing access to vaccines in low-income and middle-income countries is a complex challenge due to limited vaccine supplies, vaccine nationalism in high-income countries, hesitancy to vaccination, and distribution and registration problems. Alternative dose-sparing strategies are needed to alleviate vaccine shortages [28].

One of the applied dose-sparing approaches is the intradermal (ID) vaccination. Research over the past decades encompassing vaccines such as hepatitis, influenza, polio, and rabies has demonstrated that ID immunization produces comparable or enhanced immunogenicity, even with a fractional dose, as compared to intramuscular or SC immunization [29].

In 1948, the development of combination vaccines began by combining diphtheria, tetanus, and pertussis vaccines into a single product (diphtheria-tetanus-pertussis), which continues to be utilized for children's vaccination, serving as a pivotal component in pediatric immunization. Subsequently, numerous other combined vaccines have been developed, such as mumps, measles and rubella, diphtheria, tetanus, acellular pertussis, hepatitis B, *Haemophilus influenzae*, and

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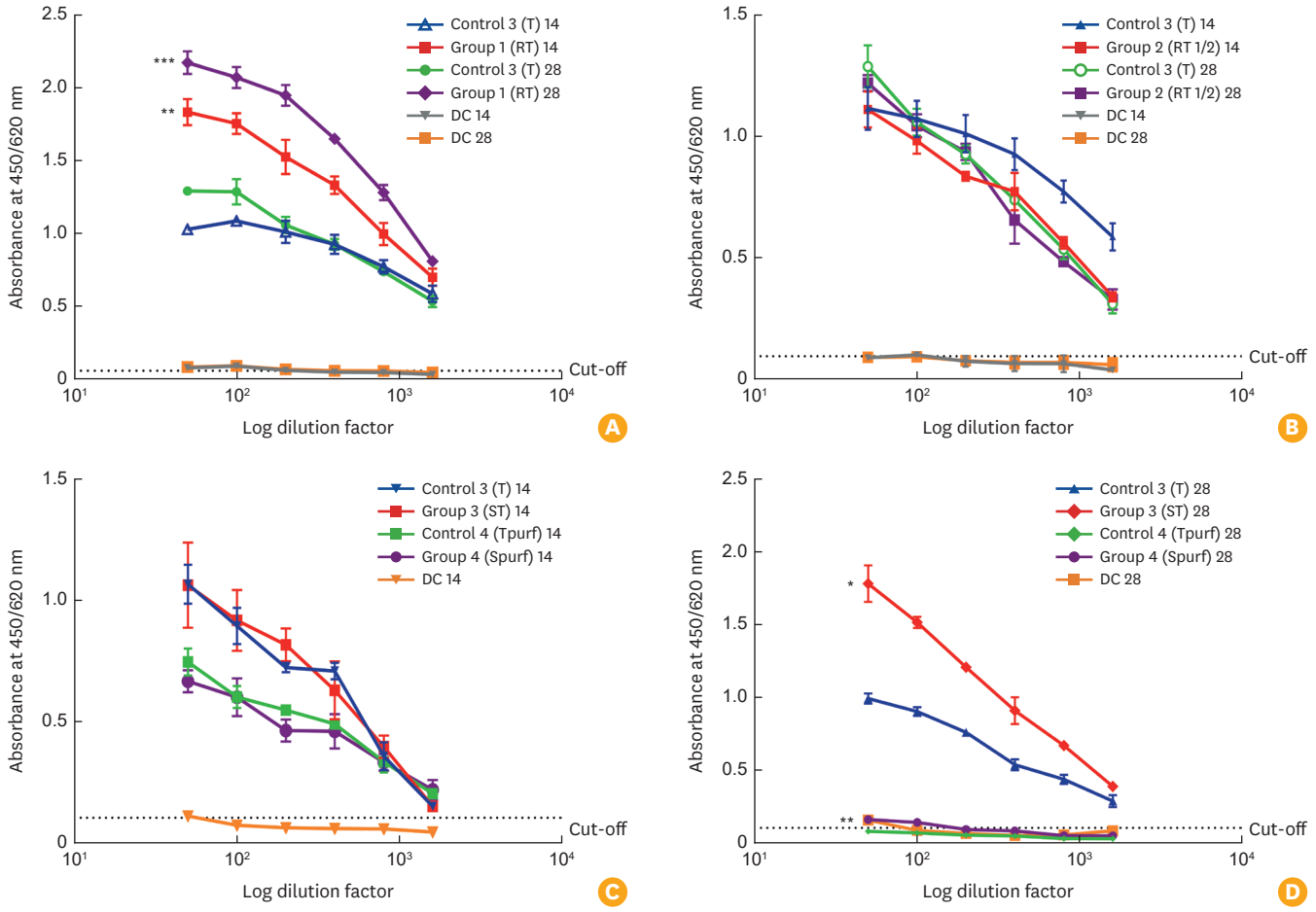

Fig. 3. Anti-tetanus immunoglobulin G response to rabies-tetanus combination vaccine in mice' sera determined by enzyme linked immunosorbent assay. Four mice groups were immunized with combination vaccines (Group 1: RT; Group 2: RT1/2; Group 3: ST; Group 4: Spurf) by one SC dose. Two groups were kept as control and were immunized with T/Tpurf (Controls 3, 4: T/Tpurf) as one SC dose. Mice sera were collected at day 14 and day 28 post vaccination: (A) Group 1 days 14 and 28; (B) Group 2 days 14 and 28; (C) Groups 3 and 4 day 14; (D) Groups 3 and 4 day 28. Sera of non-vaccinated group injected with diluent were used as DC. Data is the mean absorbance of triplicate wells. Error bars show the standard deviation. Cut-off value is shown as horizontal dashed line. Significance was determined by one-way analysis of variance (Holm-Sidak's multiple comparison test) analysis, asterisks indicate statistically significant difference between combination vaccine groups and their relevant control group.

RT, combined rabies-undiluted tetanus vaccine; RT1/2, combined 2-fold diluted tetanus and rabies vaccine; ST, combined rabies (Speeda®)-tetanus vaccine; Spurf, combined rabies (Speeda®)-purified tetanus toxoid; T, tetanus single vaccine (Toxovac®); Tpurf, purified tetanus toxoid; SC, subcutaneous; R, rabies single vaccine (Rabies vaccine®); S, rabies single vaccine (Speeda®); DC, diluent control.

*p<0.05, **p<0.01, ***p<0.001.

inactivated poliovirus vaccines (DTaP-HepB-Hib-IPV). Dose sparing through the combination of multiple vaccines into a single syringe has been documented to yield positive outcomes such as the reduction of trauma to infants, enhanced compliance, and reduction of costs [30].

However, the safety, efficacy, and immunogenicity of a combined vaccine may be affected by interactions, not only between the antigens but also with other components such as adjuvants, stabilizers, and preservatives [31,32].

Limited research exists on the impact of administering tetanus toxoid vaccination simultaneously for rabies and tetanus prophylaxis, along with the most appropriate route of vaccine administration. However, the timely delivery of both vaccines is crucial and has the potential to save lives.

This study aimed to investigate the effectiveness of the administration of individual and combined RT vaccine in mice models via different routes of administration.

Well-known commercial vaccines with recognized safety and efficacy were used in formulating the candidate combination vaccines. Toxovac® served as the commercial

adsorbed tetanus toxoid vaccine, while 2 commercial rabies vaccines Rabies Vaccine® and Speeda® vaccine, were used.

The efficacy of the candidate combination vaccines was evaluated through testing potency using NIH and challenge assay methods and immunogenicity using ELISA. Different vaccine combinations were prepared, and animal groups were injected with one of the following: RT (undiluted Toxovac® vaccine (T) combined with Rabies Vaccine®), ST (undiluted T combined with Speeda® vaccine), RT1/2 (2-fold diluted T with Rabies Vaccine®) and Tpurf (purified tetanus toxoid (Tpur) with Speeda® vaccine).

Both virus neutralization (e.g., rapid fluorescent focus inhibition test [RFFIT], fluorescent antibody virus neutralization [FAVN] test) and ELISA are suitable for monitoring the antibody response of vaccinated animals in the framework of rabies control. However, RFFIT and FAVN are less easily standardized and require the use of live rabies virus, containment facilities, and skilled professionals and therefore not used by many countries [32,33].

ELISA assays are considered suitable alternative approach for rabies serology, providing consistent results in several laboratories, particularly for quantitation of antibodies titer in PEP and sero prevalence surveys in different wildlife reservoirs. Also, ELISA could be used to quantify human anti-rabies antibodies titers following vaccination. ELISA can be performed in any laboratory with an ELISA reader, the sera samples could be monitored at regular intervals, and only a small volume of sera is required as compared to the RFFIT [34-36].

Several works demonstrated a good correlation between these assays and vector network analyzer tests, based on detecting and measuring anti-G protein antibodies [36,37].

In the present study, the potency of various combination vaccine groups was assessed for both rabies and tetanus components through the determination of Log ED50 by NIH test for rabies and challenge method for tetanus to ensure the antigen content of both met the WHO guidelines. Results shown indicate that the combination vaccines when administered generated antibodies titer against rabies and tetanus sufficient to protect mice against lethal dose of CVS virus and tetanus toxin in a dose-dependent manner. Consequently, they successfully passed both rabies and tetanus potency tests, except for the tetanus component in Group 4 (rabies-purified tetanus toxoid), which did not meet the criteria. This result suggests that purified, un-adjuvanted tetanus toxoid alone couldn't trigger a protective immune response and necessitates an adjuvant to enhance its immunogenicity [38]. The tetanus potency assessment in combination vaccines also revealed that the undiluted combination vaccine is twice as potent as single vaccine from the same batch, suggesting a synergistic effect of rabies. This was affirmed by the 2-fold dilution of tetanus component in combination vaccine in group 2, wherein the potency was found to be comparable to the single vaccine.

Immunogenicity testing of the candidate vaccine combination showed that all combination vaccines proved to be immunogenic. Even though the antibody response to the rabies control group was higher than combination vaccine groups, they showed an insignificant difference in response compared to the rabies control group injected by 2 IP doses at both days 14 and 28 post-immunization.

Furthermore, the route of administration of vaccine combinations was assessed to explore the potential of unifying the route of administration and reducing the number of doses for an economically efficient induction of a protective immune response. The combination vaccines administered through a single SC dose exhibited insignificant difference compared to the rabies control group receiving 2 IP doses on both days 14 and 28.

Presumably, the reason behind the equivalence between one SC dose and 2 IP doses is the presence of dendritic cells SC. These cells facilitate the capture of antigens and contribute to an enhanced immune response [39]. Consequently, fewer doses of the candidate combination vaccines are required to elicit protective immunity, providing the added benefit of vaccine dose sparing in a cost-effective manner.

When examining the anti-tetanus IgG response in different RT vaccine combinations administered through a single SC dose and compared to the tetanus control group, it became evident that all combination vaccines were immunogenic. The antibody response of the 2-fold serially diluted tetanus component in Group 3 exhibited an insignificant difference compared to Control 2 confirming our previous suggestion that rabies has a synergistic effect on tetanus in combination at both days 14 and 28 for Group 1 and at day 28 for Group 3.

In addition, rabies combination with purified tetanus toxoid (un-adsorbed) showed a non-significant difference compared to tetanus control at day 14 but decreased significantly at day 28. This result could be attributed to the short-term immune response elicited by purified toxoid in combination vaccines at day 14 that declines at day 28 revealing the importance of alum adjuvant in tetanus toxoid vaccines.

In the same context, a study conducted by Mawas et al. [40] to investigate immune interaction between Hib vaccine combined with DTaP vaccine as compared to both vaccines administered separately in a rat model showed that immunogenicity of DTaP components was similar or greater in combined vaccine versus separate vaccines. While

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combined administration of Hib vaccine and tetanus toxoid decreased immunogenicity for both. In addition, Hib immunogenicity decreased significantly when given combined with filamentous hemagglutinin (one of pertussis components) and following adsorption to aluminium hydroxide.

Rabies vaccine has a synergistic effect on tetanus in combination. The immune response to rabies in combination vaccine administered as a single SC dose proved as effective as 2 IP doses of a single rabies vaccine.

Achieving vaccine dose sparing is possible by reducing the tetanus content by 2-fold in the combination and decreasing the number of rabies doses ensuring protective immunity.

Our proposed vaccine combination offers advantages such as dose sparing, using one effective route of administration, and reducing cost by decreasing the required doses of rabies and tetanus content.

Further clinical studies are essential to provide a comprehensive understanding of our candidate vaccine.

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Declaration

During the preparation of this work the author(s) used [ChatGPT, December 2023] to improve readability and language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Author Contributions

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