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Safety Assessment of Co-Administered SARS-CoV-2 Vaccines in BALB/c Mice

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Abstract:- The onset of Coronavirus disease 2019 (COVID-19) in late 2019 presented a severe worldwide health crisis with widespread morbidity and mortality. Various vaccine platforms have been rapidly developed and approved for broad use in a swift and urgent response to prevent the transmission of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection. However, these vaccines differ significantly in terms of safety. Heterologous prime-boost vaccination enhances vaccine safety compared to homologous vaccination, although it could lead to a higher cumulative number of transient adverse events reported at each visit. Therefore, additional strategies are necessary to improve SARS-CoV-2 vaccine safety. Anecdotal options suggest that vaccine co-administration can significantly reduce these adverse effects and consequently, avert the need for frequent booster doses. This study reports the immunization outcomes against the SARS-CoV-2 virus by assessing the safety profiles of different SARS-CoV-2 vaccines co-administered in BALB/c mice. Vaccine combinations comprising mRNA/adenovirus26-vector, mRNA/inactivated, adenovirus26-vector/inactivated, and mRNA/adenovirus26-vector/inactivated vaccines were prepared in optimized doses, and their activities upon immunization evaluated in comparison with individual mRNA, adenovirus26-vectored and inactivated vaccines. Twenty-eight days post-immunization, safety profiles of the various treatments were evaluated through hematological and biochemical examination. Importantly, the co-administration regimens proved safe and were well-tolerated in mice, as evidenced by the normal hematological and biochemical values.

Keywords:- *mRNA Vaccine; Adenovirus26 Vector Vaccine; Inactivated Vaccine; SARS-Cov-2; Co-Administration; Safety; BALB/C Mice.*

I. INTRODUCTION

The emergence of the coronavirus disease 2019 (COVID-19) in late 2019 has presented a significant global health challenge [1] and resulted in substantial rates of illness and death [2], with far-reaching effects on the worldwide economy [3]. According to data from the World Health Organization (WHO) as of November 4, 2023, there have been 771,820,937 reported cases of COVID-19 worldwide, with 6,978,175 reported deaths [4]. The SARS-CoV-2 virus, characterized by its round shape and protruding surface spikes, is classified as a β-coronavirus and possesses a single-stranded positive-sense RNA genome. Various strategies including vaccination have been employed to combat COVID-19 infection. Consequently, different types of vaccines including mRNA, adenovirus vector, inactivated, protein subunit, DNA vaccines, and others have been developed and approved by the WHO [5].

While numerous prominent COVID-19 vaccines have proven effective in protecting against infection, these vaccine platforms exhibit significant variations in their immunogenicity and safety profiles [6]. After homologous vaccination, studies showed a risk of myocarditis following the second dose of mRNA-based COVID-19 vaccines like BNT162b2, particularly in young males [7], [8]. Additionally, Guillain-Barré syndrome (GBS) and cases of thrombosis and thrombocytopenia were reported following vaccination with vector-based COVID-19 vaccines such as Ad.26.COV2.S and ChAdOx1[9], [10].

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However, compared to homologous vaccination, using heterologous vaccines in the prime-boost immunization strategy has been successful in improving vaccine safety. Many countries have established a heterologous primer-boost vaccination approach against the SARS-CoV-2 virus, finding it to be safe [11], [12], [13]. Despite this, administering vaccines separately could result in a higher cumulative number of transient adverse events reported at each visit [14]. Therefore, there is a continued need for additional vaccination strategies to improve the safety of the existing SARS-CoV-2 vaccines. Moreover, the effective optimization of booster programs remains an ongoing challenge requiring real-time management [15]. To address this, co-administration of vaccines not only significantly reduces adverse effects but also provides convenience for both patients and healthcare providers [14], thereby averting frequent booster vaccinations.

More interestingly, the studies underscore the growing significance of safely co-administering vaccines to enhance global immunization efforts and actively promote the integration of new vaccines into immunization programs [14]. Recent research has shown improved immune responses and safety when BCG and H107 subunit vaccines were co-administered against *Mycobacterium tuberculosis* [16]. As COVID-19 vaccines seek to reduce the morbidity and mortality linked to the infection, concerns regarding the safety of these vaccines and the possibility of adverse effects following vaccination have led to increased hesitancy [17]. Consequently, it is imperative to evaluate the effect of co-administering COVID-19 vaccines to better understand the feasibility of the immunization strategy. So far, there is little knowledge of how different COVID-19 vaccine platforms may interact and improve their safety when co-administered. In this study, we, therefore, evaluated the hematological and biochemical parameters to investigate the safety profiles of mRNA-based, adenovirus vector-based, and inactivated SARS-CoV-2 vaccines, along with co-administration regimens in a BALB/c mouse model.

II. MATERIALS AND METHODS

A. SARS-CoV-2 Vaccines Used in the Experiment

The COMIRNATY mRNA COVID-19 (Pfizer-BioNTech) vaccine with Lot Number GN6343, the Janssen Ad26.COV2.S (recombinant) vaccine with Lot Number ACB6959, and the SARS-CoV-2 vaccine (Vero Cell), Inactivated (Sinopharm) with Product Code 2021071947 were provided by Kenya Medical Research Institute (KEMRI) and solely utilized for research purposes.

B. Animal Model and Immunization Protocol

Thirty-two female BALB/c mice aged 6-8 weeks old were procured from the Institute of Primate Research (IPR) in Kenya and allowed a 14-day acclimatization period at the KEMRI animal facility under standard conditions of temperature (23 ± 2 °C), humidity (40–70 %), and a 12-hour light/dark cycle. They were divided into 7 treatment groups and one control group, with each group comprising 4 mice. Each mouse in the group received the respective inoculation via intramuscular (IM) injection into either the left or right thigh muscle, or both. Mice in Groups 1(mRNA), 2(Vector), and 3(Inactivated) were immunized with individual mRNA (5µg of Pfizer), adenovirus26-vectored (4×10^9 Viral Particles (VP) of Janssen), and inactivated (0.8 µg of Sinopharm) SARS-CoV-2 vaccines, respectively. On day 14, Groups 1 and 3 received booster doses. Their safety profile was evaluated in comparison with SARS-CoV-2 vaccine combinations, prepared in optimized doses. Group 4 (mRNA/Vector) received the co-administration of mRNA/adenovirus26-vector (5µg of Pfizer and 4×10^9 VP of Janssen) vaccines, Group 5 (mRNA/Inactivated) was immunized with mRNA/inactivated (5µg of Pfizer and 0.8 µg of Sinopharm) vaccines, Group 6 (Vector /Inactivated) received the co-administration of adenovirus26-vector/inactivated (4×10^9 VP of Janssen and 0.8 µg of Sinopharm) vaccines, and Group 7 (mRNA/Vector/Inactivated) was inoculated with mRNA/adenovirus26-vector/inactivated (5µg of Pfizer, 4×10^9 VP of Janssen and 0.8µg of Sinopharm) vaccines. Co-administration groups did not receive booster doses. Group 8 (control) received 50µL of 1× phosphate-buffered saline (PBS).

C. Blood Collection

On day 28 post-immunization, 300µL of whole blood was obtained from mice via the cardiac puncture method [18] after being euthanized by CO_2 asphyxiation. A portion of the collected blood samples was placed in 0.5mL EDTA-containing tubes for hematological analysis, while another portion was placed in non-anticoagulant tubes, centrifuged, and the resulting sera were utilized for biochemical analysis.

D. Hematological Analysis

The whole blood samples collected in EDTA-containing tubes were mixed manually and gently. The complete blood count was conducted utilizing a HumaCount 30^{TS} hematology machine. Hematological parameters including White blood cells (WBC), Neutrophils (NEU), Lymphocytes (LYM), Monocytes (MON), Eosinophils (EOS), Basophils (BAS), Red blood cells (RBC), Hemoglobin (HGB), Hematocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC) and Platelets (PLT) were analyzed.

E. Kidney and Liver Biochemical Analysis

After 28 days following immunization, biochemical markers were determined using a Mindray chemistry analyzer (BS 200) [19]. Biochemical tests analyzed for liver function were Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and Gamma-Glutamyl Transferase (GGT), while Urea and Creatinine were analyzed for kidney function.

F. Data Analysis

The hematological and biochemical results were initially recorded in Microsoft Excel (2016). Statistical analysis was conducted using Graph Pad Prism version 8.0.2 software. The comparison of the experimental groups to the control group was determined by One-way ANOVA followed

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by Tukey's post hoc test. All obtained data were presented in the form of mean ±SD and the bar charts were generated using Graph Pad Prism version 8.0.2 software. P-value <0.05 was considered as statistically significant.

III. RESULTS

A. Hematological Assessment

To evaluate the overall health of female BALB/c mice immunized with the various vaccine regimens, complete blood count (CBC) was analyzed to measure different hematological parameters after 28 days. The findings of the hematological parameters analyzed are depicted in Table I.

The leukogram parameters, including white blood cells (WBC), neutrophils (NEU), eosinophils (EOS), basophils (BAS), and lymphocytes (LYM), were within the normal range across all experimental groups, except for monocytes (MON), which showed elevated values compared to the normal range across all the experimental groups. On the other hand, the erythrogram parameters, such as red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), remained within the reference range in all groups except for the Inactivated group, which showed lower RBC, HGB and HCT counts versus reference range. Regarding platelet (PLT) counts, the experimental groups exhibited normal values, except for the mRNA, Inactivated, and mRNA/Vector/Inactivated groups, which demonstrated reduced PLT counts versus the expected normal range.

Table 1: Complete Blood Count of Immunized Balb/C Mice

Parameter	mRNA	Vector	Inactivated	mRNA/ Vector	mRNA/ Inactivated	Vector/ nactivated	mRNA/ Vector/ Inactivated	Unvaccinated	Reference range
RBC(×10⁶/µL)	8.52± 0.03	8.80± 0.84	5.64± 1.05	7.84± 0.00	7.76± 0.79	8.18± 0.07	8.78± 0.63	8.44± 0.43	7.8–10.3
HGB (g/dL)	14.55± 0.35	15.55± 1.90	11.20± 0.42	14.10± 0.14	14.15± 1.06	14.55± 0.07	15.10± 0.56	14.55± 0.77	11.6–15.9
HCT (%)	45.40± 2.12	49.15± 4.25	29.75± 3.32	40.05± 0.07	41.85± 0.21	44.50± 0.98	46.80± 0.70	47.50± 1.69	35.2–49.9
MCV (FL)	53.20± 2.26	55.70± 1.55	53.05± 2.85	51.05± 0.07	54.15± 3.30	54.35± 1.62	53.35± 3.04	56.20± 0.84	39.2–56.2
MCH (Pg)	17.05± 0.35	17.55± 0.49	20.85± 1.90	16.60± 0.56	18.20± 0.56	17.75± 0.21	17.15± 0.63	17.15± 0.07	12.4–18.4
MCHC(g/dL)	32.00± 0.70	31.60± 0.00	39.10± 0.84	32.15± 3.88	33.75± 2.33	32.70± 0.56	32.20± 0.70	30.60± 0.56	27.2–40.8
WBC(×10³/µL)	7.00± 0.14	8.30± 0.42	4.35± 1.06	10.75± 0.07	8.95± 1.76	2.65± 0.49	9.80± 1.69	10.25± 2.19	1.09–11.3
NEU (%)	15.50± 3.53	11.50± 3.53	8.50± 2.12	9.00± 0.00	23.50± 7.50	9.50± 2.12	23.00± 7.00	21.50± 3.53	11–29
LYM (%)	73.00± 1.41	78.50± 3.53	83.00± 5.65	82.00± 0.00	82.50± 4.95	81.50± 3.53	65.50± 6.36	70.50± 8.50	65–87
MON (%)	10.50± 2.12	9.00± 0.00	9.00± 1.14	8.00± 0.00	8.00± 1.14	8.00± 1.14	10.50± 3.53	11.50± 2.12	0–6
EOS (%)	1.00± 0.00	1.00± 0.00	1.00± 0.00	1.00± 0.00	1.00± 0.00	0.50± 0.70	1.00± 0.00	1.50± 0.70	0–5
BAS (%)	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0–1
PLT (×10³/µL)	164.5± 6.36	579.0± 21.00	232.5± 7.77	515.0± 1.41	522.5± 18.50	487.0± 7.07	149.50± 14.85	379.0± 9.89	322–798

Abbreviations: WBC: White Blood Cell Count, NEU: Neutrophils, LYM: Lymphocytes, MON: Monocytes, EOS: Eosinophils, BAS: Basophils, RBC: Red Blood Cell Count, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Hemoglobin, MCHC: Mean Corpuscular Hemoglobin Concentration, PLT: Platelet Count. Values were Presented as Mean ± SD. Mice Reference Range According to [20], [21].

B. Biochemical Assessment

Twenty-eight days post-immunization, renal and liver biochemical tests were conducted to evaluate kidney and liver function. The results of the biochemical parameters analyzed are illustrated in Fig. 1. It was observed that all treated groups did not elicit significant variations in Urea ($p > 0.5523$), Creatinine ($p > 0.5157$), ALT ($p > 0.0719$), and GGT ($p > 0.2498$) levels compared to the unvaccinated group, except for AST parameter. Although One-Way ANOVA revealed a significant difference in AST levels ($p < 0.0329$) among the groups, Tukey's multiple comparisons test demonstrated no significant variations in AST levels between the vaccinated and unvaccinated groups ($p > 0.05$).

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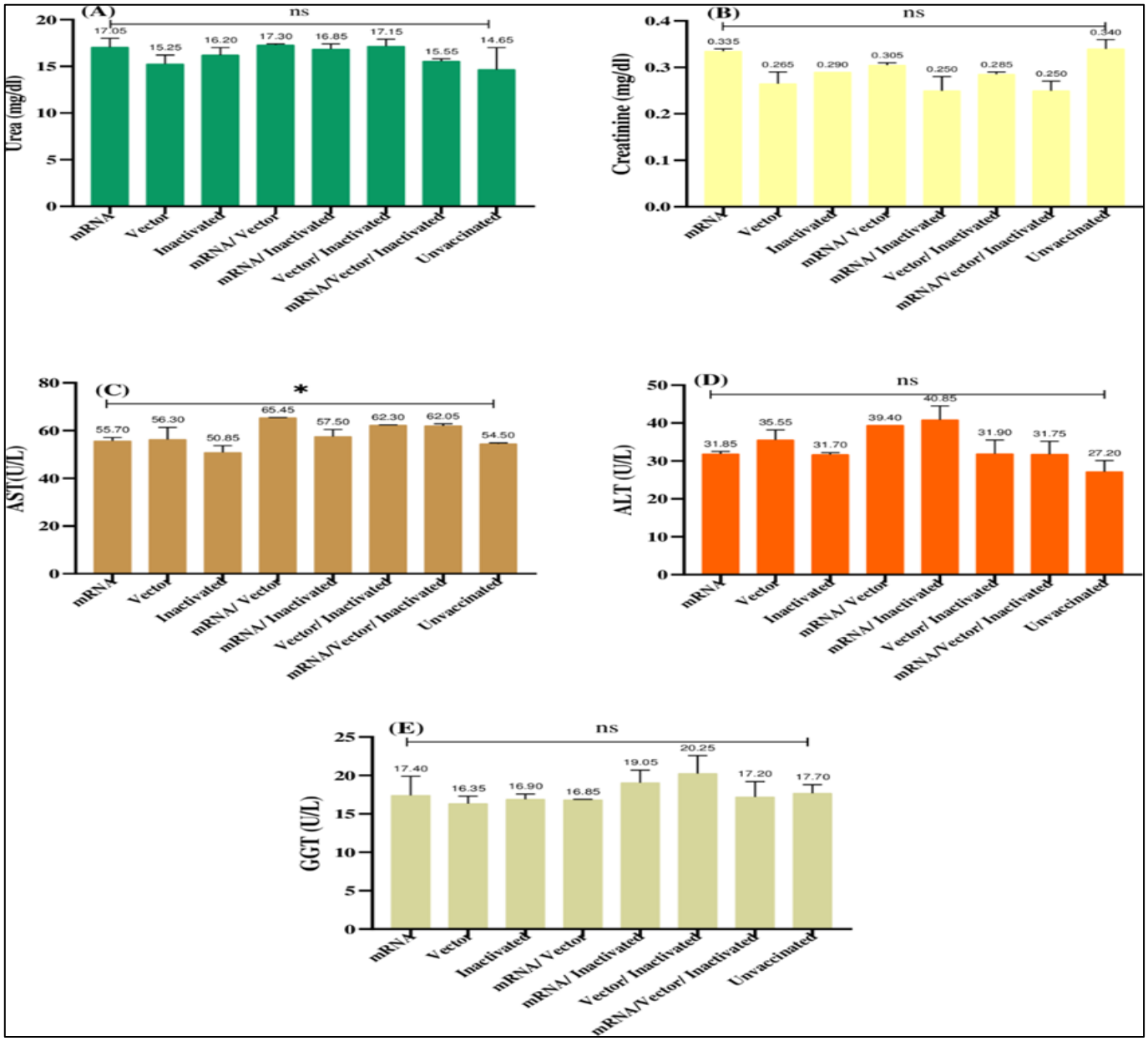

Fig 1: Kidney and Liver Biochemical Analysis at Day 28 Post-Immunization. AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase, GGT: Gamma Glutamyl Transferase. Levels of (A) Urea, (B) Creatinine, (C) AST, (D) ALT, (E) GGT. Statistical Differences were Compared using GraphPad Prism Version 8.0.2. Error Bars Depict Mean ± SD. * Indicates Statistical Significance at $p < 0.01$, while "ns" Denotes $p > 0.05$, Indicating no Significant Difference

IV. DISCUSSION

As COVID-19 vaccines seek to reduce the morbidity and mortality linked to the infection, concerns regarding the safety of these vaccines and the possibility of adverse effects following vaccination have led to increased hesitancy [17]. Consequently, it is imperative to evaluate the effect of co-administering COVID-19 vaccines on hematological and biochemical parameters to better understand the feasibility of the immunization strategy. Notably, no mortality or alarming adverse events were observed in any experimental group throughout this experiment.

When exploring vaccination-related body reactions, it becomes increasingly important to prioritize the investigation of hematological side effects. The present study demonstrated normal leukogram values except for increased levels of monocytes in all experimental groups following 28 days of vaccination. Literature underscores the pivotal role of monocytes in effectively managing and eliminating viral, bacterial, fungal, and protozoal infections, while also implicating in inflammation [22], [23]. Moreover, research has demonstrated an increase in monocyte levels following COVID-19 vaccination [24]. Consequently, our findings suggest that the recruitment of monocytes would potentially facilitate the clearance of the SARS-CoV-2 infection following the COVID-19 vaccination.

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Further, the erythrogram parameters (RBC, HGB, HCT, MCV, MCH, and MCHC) did not exhibit significant differences between the treated and the control groups, except in the case of the inactivated vaccine group, where reduced RBC, HGB, and HCT values were observed. Physiologically, a decrease in RBC, HGB, and HCT levels suggests the presence of anemia. However, it is important to acknowledge that our findings are not conclusively definitive, as we did not investigate other relevant laboratory tests such as reticulocyte count and peripheral blood smear test, commonly employed in the diagnosis of anemia [25].

On the other hand, our study demonstrated decreased platelet counts in mRNA, Inactivated, and mRNA/Vector/Inactivated groups. The vaccines have been demonstrated to elicit thrombocytopenia [26]. Clinical presentations such as thrombotic events, either with or without thrombocytopenia were observed in individuals who received Pfizer, Janssen, or AstraZeneca vaccines. Furthermore, cases of thrombocytopenia (TP), immune thrombocytopenic purpura (ITP), and thrombotic thrombocytopenic purpura (TTP) have been documented following vaccination with Pfizer, Moderna, AstraZeneca, or Janssen vaccines [27]. Studies have shown that mRNA and adenovirus vector vaccines have the potential to induce functional anti-PF4 antibodies. These antibodies can trigger abnormal platelet activation and potentially dangerous blood clot formation by interacting with Fcγ receptor IIA on platelets, resulting in a decrease in platelet count and the development of vaccine-induced thrombotic thrombocytopenia (VITT) [28], [29], [30], [31]. Furthermore, immune thrombocytopenia has also been linked to inactivated vaccines [32]. We speculate that the reduction in platelet count could result from the immune-related reactions in vaccinated BALB/c mice. Since the present study did not assess anti-platelet factor 4 antibodies, the findings remain inconclusive. Moreover, additional tests such as platelet activation, prothrombin time (PT), activated partial thromboplastin time (APTT or PTT), fibrinogen level, and D-Dimer test, which could offer a more comprehensive insight into the occurrence of thrombocytopenia [27], [33], were not included in the analysis. Physiologically, the observed thrombocytopenia could impair the blood clotting ability, potentially leading to an increased risk of excessive bleeding in the vaccinated mice [26]. However, this risk was not identified in the experiment.

Serum biochemical tests play a crucial role in diagnosing and managing renal and liver diseases. Kidney function, assessed through biochemical tests such as Urea and Creatinine tests, showed no statistically significant differences in values between the vaccinated and control groups, indicating no evidence of kidney damage. Additionally, liver function, evaluated using biochemical tests including ALT, AST, and GGT as markers of hepatocellular injury, did not exhibit variations in ALT and GGT levels across the treatment groups compared to the control group. However, a significant increase in AST was observed in the mRNA/Vector, Vector/Inactivated, and mRNA/Vector/Inactivated groups compared to the control group. It is important to note that while an elevation of AST alone does not conclusively determine liver damage, it is suggestive, especially when correlated with the elevation of ALT, which is exclusively found in the liver [34]. In this study, ALT levels were not statistically significant compared to the control group. It is essential to conduct additional tests, including assessment of liver metabolism (total bilirubin) and liver synthetic function (serum albumin and prothrombin time) for a conclusive determination of liver damage [34].

In conclusion, co-administration regimens proved to be safe and well tolerated in BALB/c mice. However, further investigation is needed to observe vaccinated animals for extended periods following vaccination with co-administered vaccines to draw definitive conclusions.

ETHICAL STATEMENT

All mice experiments were conducted in accordance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals at the Kenya Medical Research Institute (KEMRI). The protocols for animal studies were thoroughly reviewed and granted approval by both the KEMRI Animal Care and Use Committee and the Mount Kenya University (MKU) Animal Care and Use Ethics Review Committee, with reference numbers KEMRI ACUC/02.06.2023 and REF: MKU/ISERC/2904 (approval number 1948), respectively.

- *Data Availability*
  All data are included in the manuscript

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- *Conflict of Interest*
  All authors declare no conflicts of interest in this paper.

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