Potency, toxicity and protection evaluation of PastoCoAd candidate vaccines: Novel preclinical mix and match rAd5 S, rAd5 RBD-N and SOBERANA dimeric-RBD protein

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ABSTRACT

Despite substantial efforts, no effective treatment has been discovered for severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) infection. Therefore, vaccination to reach herd immunity is the ultimate solution to control the coronavirus disease 2019 (COVID-19) pandemic. This study aimed to evaluate the potency, toxicity, and protection of candidate PastoCoAd vaccines as novel mix and match of recombinant adenovirus type 5 (rAd5) containing the full-length spike protein (rAd5-S), rAd5 containing the receptor-binding domain of S protein and nucleoprotein (rAd5 RBD-N), and SOBERANA dimeric RBD protein of SARS-CoV-2. Three vaccine candidates were developed against SARS-CoV-2 using adenoviral vectors, including the prime-boost (rAd5-S/rAd5 RBD-N), heterologous prime-boost (rAd5-S/ SOBERANA vaccine), and prime only (mixture of rAd5-S and rAd5 RBD-N). The rAd5-S and rAd5 RBD-N were produced with a *Cytomegalovirus* promoter and the human tissue plasminogen activator (tPA) leader sequence. The immunogenicity of vaccine candidates was also evaluated in mouse, rabbit, and hamster models and protection was evaluated in a hamster model. Following the injection of vaccine candidates, no significant toxicity was observed in the tissues of animal models. The immunogenicity studies of mice, rabbits, and hamsters showed that responses of total IgG antibodies were significantly higher with the prime-only and heterologous prime-boost vaccines as compared to the other groups (P < 0.009). Virus neutralizing antibodies were detected, and the level of cytokines related to humoral and cellular immunity increased significantly in all vaccinated models. A high cellular immunity response was found in the vaccinated groups compared to the controls. On the other hand, the vaccine challenge test showed that the virus titers significantly decreased in the pharynx and lung tissues of vaccinated hamsters compared to the control group. These successful findings suggest the safety and protection produced by the heterologous prime-boost vaccine (adenovector/ SOBERANA RBD), as well as a single dose of adenovector vaccine in animal models.

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1. Introduction

Since the emergence of severe acute respiratory syndrome-coronavirus 2 (SASR-CoV-2) in early December 2019 in China, the world has been unable to curb this disease successfully. As of July 1, 2021, coronavirus disease 2019 (COVID-19) has claimed 3,945,832 lives around the world, with 181,930,736 cases documented by the World Health Organization (WHO) [1]. The COVID-19 pandemic has not only imposed a significant burden on the healthcare systems globally, but has also affected nearly every aspect of human's life.

Despite significant global efforts, there is no effective treatment for SARS-CoV-2. Therefore, vaccination to reach herd immunity is the ultimate solution to control the COVID-19 pandemic [2]. Various platform technologies have been employed to develop effective COVID-19 vaccines, including mRNA-based vaccines, vector-based vaccines, inactivated whole virus vaccines, and recombinant vaccines. Some of these vaccines have been authorized for emergency use by the WHO, based on the successful reports of clinical trials [3].

Replication-deficient adenoviral vectors are safe platforms, which can stimulate both cellular and humoral immunity. More importantly, these vectors can be produced at a large scale [4,5]. These features make them promising candidates for developing COVID-19 vaccines. So far, three replication-deficient adenovirus-based COVID-19 vaccines, including Ad26.COV2.S by Johnson & Johnson (Janssen), ChAdOx1 nCoV-19 by AstraZeneca (Cambridge, UK), and Sputnik V by Gamaleya (Moscow, Russia), have been approved by the WHO and administered in different countries [6].

Among viral proteins, spike protein (S) plays a key role in the binding and entry of SARS-CoV-2 into the host cell. It is considered as the main target in the design of vaccines against coronaviruses due to the presence of immunodominant epitopes and target antibodies in this protein [7]. Also, nucleocapsid (N) protein contains immunodominant epitopes, which stimulate both humoral and cellular immune systems [8].

The present study aimed to examine the ability of two replication-defective human adenovirus type 5 (Ad5) constructs, containing the full-length S protein or the receptor-binding domains (RBDs) of SARS-CoV-2 S and N proteins, to stimulate both humoral and cellular immune responses in different animal models and provide protection against the virus.

2. Methods

2.1. Ethical statement

The animal experiments were performed on mice, hamsters, rabbits, and rats. This study was approved by the Ethics Committee of Pasteur Institute of Iran (ethics code: IR.PII.REC.1399.057). All animal experiments were performed according to the European Union (EU) Directive on animal testing (86/609/EEC).

2.2. Cells and adenovirus constructs

The human embryonic kidney-adenovirus (HEK-AD) cells were used for the production and packaging of recombinant adenoviral vectors. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Thermo Fisher Scientific, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, USA) at 37 °C in a 5% CO_2 atmosphere. The full-length S gene and RBDs of S and N gene sequences were selected from the GenBank sequence reference for SARS-CoV-2 (Wuhan Hu1; GenBank accession number: MN908947), codon-optimized for human cells, chemically synthesized, and used for the production

of two replication-defective adenoviruses, expressing S protein (rAd5-S) and RBD/N protein (rAd5 RBD-N).

In this study, the Ad5 vector deleted for E1/E3 (provided by S.Y. Hosseini from Shiraz University of Medical Sciences, Shiraz, Iran) was used. Besides, an Ad5-expressing green fluorescent protein (rAd5-GFP) was constructed and used as the negative control. Titration of recombinant Ad5 vectors was carried out, using the QuickTiter™ Adenovirus Titer ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA). The constructs were confirmed by polymerase chain reaction (PCR) and subsequently, by Sanger sequencing. The expression of SARS-CoV-2 proteins was also confirmed by Western blotting on the Ad5 recombinant-transfected HEK-AD cell lysate.

2.3. SARS-CoV-2 cultivation and titration

The SARS-CoV-2 utilized in this study is described elsewhere [9]. Virus cultivation and active virus handling were performed in a biosafety level 3 (BSL-3) laboratory. Briefly, the upper respiratory specimen (a nasopharyngeal swab) from a confirmed COVID-19 patient was inoculated into Vero cells, maintained in DMEM (Gibco, Thermo Fisher Scientific, USA), supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, USA) at 37 °C in a 5% CO_2 atmosphere. The cytopathic effect (CPE) was observed at 72 h after inoculation, and SARS-CoV-2 infection was confirmed by real-time PCR assay. Next, the supernatant of the infected flask was removed, and the virus was concentrated and purified. The median tissue culture infectious dose ($TCID_{50}$) per millimeter was calculated, according to the Spearman-Kärber method [10].
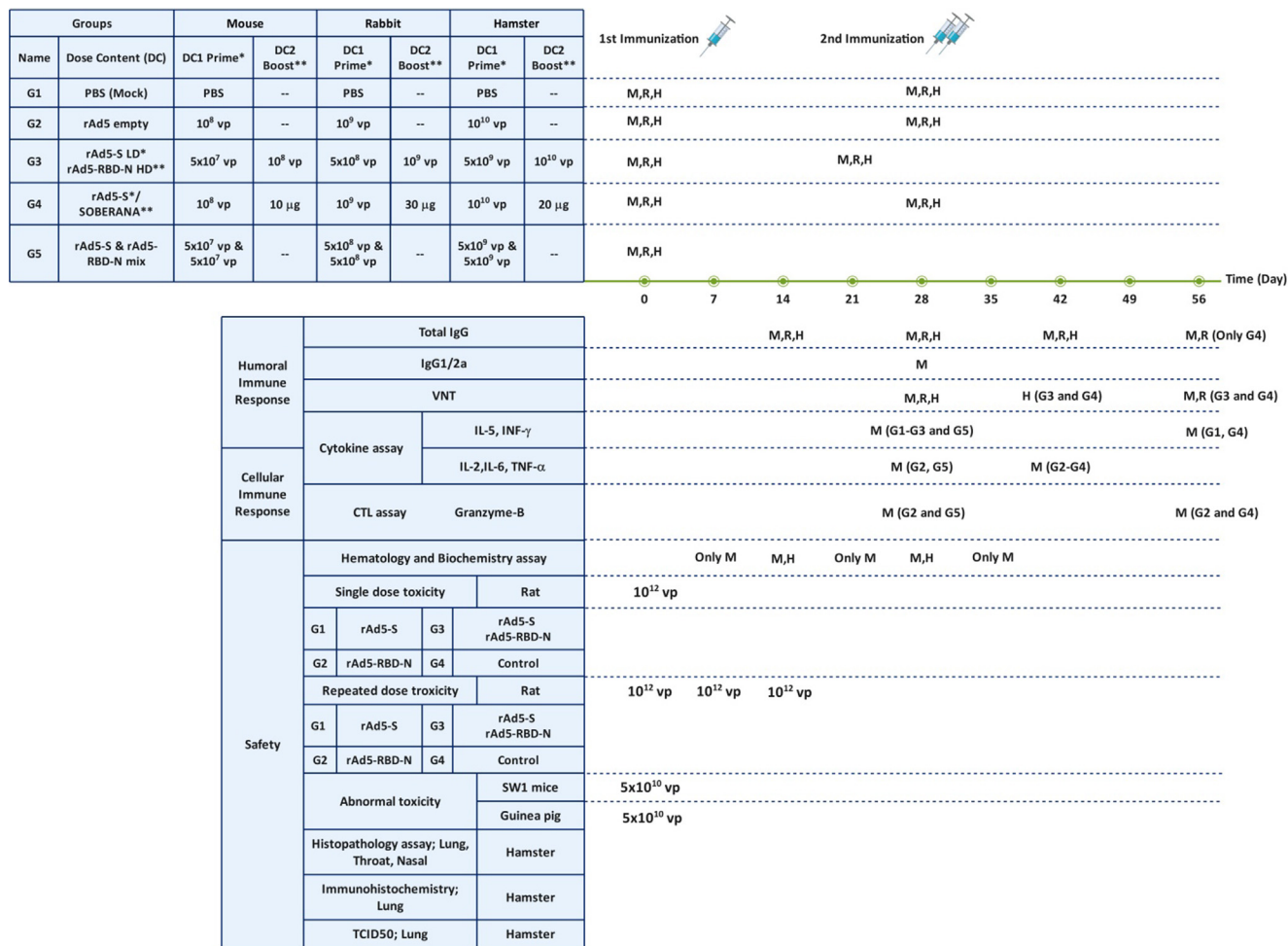
2.4. Toxicology

The adenoviral vector toxicity was examined by single and repeated dose toxicity assays. Four groups of six Wistar rats, aged 7–9 weeks, were administered 100 μL of adenovectors intramuscularly (ten times higher than the human dose, i.e., 10^{12} viral particles [VP]). In the single and repeated dose groups, the animals were sacrificed by CO_2 at 48 h after the final injection, and tissues were collected from the brain, heart, lungs, liver, kidneys, and the injection site of muscles in tubes, containing 10% formalin for pathological examinations.

Abnormal toxicity and local tolerance were examined for rAd5-S and rAd5 RBD-N separately. For this purpose, 5×10^{10} VPs of each virus (equal to the human dose) were administered intraperitoneally to seven guinea pigs and five mice. Three guinea pigs and two mice were injected intraperitoneally with phosphate-buffered saline (PBS) in the mock group. Clinical symptoms, mortality rate, and body weight were monitored up to 14 days. The injection sites were also examined histopathologically (e.g., erythema and edema).

2.5. Immunogenicity studies

2.5.1. Immunogenicity in mice

To avoid bias, all personnel responsible for the injections were blinded to the content of injection vials. Female and male mice (age: 6–8 weeks), purchased from the IPI Animal Production Center, were categorized into five groups (14 mice per group). As shown in Fig. 1, 50 μL of vaccine candidates was administered intramuscularly to the mice. Briefly, a two-dose regimen (prime-boost), including the first-dose rAd5-S (L.dose; 5×10^7 VPs) and the second-dose rAd5 RBD-N (H.dose; 10^8 VPs), was administered in an interval of 21 days. Moreover, a two-dose regimen (heterologous prime-boost), with the first dose of rAd5-S (H.dose; 10^8 VPs) vaccine and the second dose of SOBERANA vaccine (recombi-

Groups		Mouse		Rabbit		Hamster	
Name	Dose Content (DC)	DC1 Prime*	DC2 Boost**	DC1 Prime*	DC2 Boost**	DC1 Prime*	DC2 Boost**
G1	PBS (Mock)	PBS	--	PBS	--	PBS	--
G2	rAd5 empty	10^8 vp	--	10^9 vp	--	10^{10} vp	--
G3	rAd5-S LD* rAd5-RBD-N HD**	5×10^7 vp	10^8 vp	5×10^8 vp	10^9 vp	5×10^9 vp	10^{10} vp
G4	rAd5-S*/ SOBERANA**	10^8 vp	10 µg	10^9 vp	30 µg	10^{10} vp	20 µg
G5	rAd5-S & rAd5-RBD-N mix	5×10^7 vp & 5×10^7 vp	--	5×10^8 vp & 5×10^8 vp	--	5×10^9 vp & 5×10^9 vp	--

LD: Low dose, HD: High dose, M: Mouse, R: Rabbit, H: Hamster

Fig. 1. Graphical abstract of animal studies for evaluating the safety and efficacy of PastoCoAd vaccines.

nant dimeric RBD protein) [11], was administered in an interval of 28 days. Besides, a single-dose regimen (prime only), including a combination of rAd5-S and rAd5 RBD-N (10^8 VPs per 50 µL), was administered (Fig. 1).

Blood sampling (300 µL) was performed from the venous sinus on days 0, 14, 21, 28, and 42 for all groups and on day 56, for group 4. Serum isolation was carried out by centrifugation at 1000*g* for five minutes; the separated sera were kept at −20 °C. The immunogenicity of vaccine candidates was examined in the following steps: (1) evaluation of humoral immune responses via total immunoglobulin G (IgG), IgG1, and interleukin-5 (IL-5) measurements, along with the virus neutralization test (VNT); (2) analysis of cellular immunity by measurement of IgG2a, interferon gamma (IFNγ), and IL-2, along with cytotoxic T cell (CTL) assay; and (3) evaluation of inflammatory cytokines by measurement of tumor necrosis factor-α (TNF-α) and IL-6.

2.5.2. Immunogenicity in rabbits

To avoid bias, all vials were anonymous in this study. Four-month-old female rabbits (weight: 2.5–3.5 kg), purchased from the IPI Animal Production Center (Tehran, Iran), were categorized into five groups similar to the mice; nevertheless, six rabbits were included in each group. The animal groups and vaccine candidate regimens are presented in Fig. 1. For immunological assessments, blood collection (from the marginal ear vein) was performed on days 0, 14, 28, 42, and 49 for all groups and also on day 56 for

group 4. The sera were isolated by centrifugation at 1000 g for five minutes and stored at −20 °C.

2.5.3. Immunogenicity and challenge experiment in golden Syrian hamsters

Golden Syrian hamsters were used as a model of SARS-CoV-2 challenge experiment, because angiotensin-converting enzyme 2 (ACE2) from Syrian hamsters is highly homologous to the human ACE2, and the virus can efficiently replicate in the respiratory tract of hamsters [12]. In the present study, five groups, including the vaccine candidate, negative control (Ad5 empty), and healthy control (mock) groups, were examined. Each group consisted of seven female golden Syrian hamsters, aged 9–11 weeks, with an average weight of 101 g (Fig. 1). Blood samples were taken from all groups on days 14 and 28 post-immunization. Moreover, hematological, biochemical, and total IgG tests were performed. The sera, collected on day 28, were subjected to the VNT, as well.

For the challenge experiment, the hamsters were intranasally inoculated with 5×10^5 TCID$_{50}$ of SARS-CoV-2. Virus inoculation was carried out on day 28 for groups 1, 2, and 5 and on day 42 for groups 3 and 4. Oropharyngeal swabs were collected on days 2, 3, 4, 5, and 7 after the challenge, and the animals' body temperature and weight were recorded. On days 4 and 7 after the challenge, three animals were sacrificed, and the lung and nasal tissues were evaluated for SARS-CoV-2 titer, histopathology, and immunohistochemistry.

2.5.4. Humoral immunity

To evaluate humoral immunity, total IgG antibody tittering was performed, using an ELISA assay on days 0, 14, 28, and 42 in all groups, on day 56 in the mice and rabbits of group 4, and on days 14 and 28 in hamsters. Additionally, IgG1 and IgG2a were measured in mice for all groups on day 28. The anti-S and anti-N IgG, IgG1, and IgG2a antibodies were also evaluated separately by ELISA assays. Briefly, 96-well plates were coated with 2.5 μg/mL of recombinant S and N proteins, purchased from Sina Biotechnology Knowledge-Based Company (Iran), and incubated overnight at 4 °C; then, 5% skim milk (dissolved in PBS, pH = 7.4) was added for one hour at 37 °C in the blocking step. Subsequently, the diluted sera (1:100, 1:500, and 1:1500) in PBS, containing 5% skim milk, were added and incubated for one hour. Next, HRP-conjugated anti-mouse IgG was added, and incubation was performed for one hour at room temperature. After washing with 100 μL of PBS-Tween (PBST), a TMB substrate was added and incubated for ten minutes. Reaction was terminated by adding sulfuric acid (H_2SO_4). The optical density (OD) of all wells was read at 450 and 630 nm by an ELISA reader.

The IL-6 and TNF-α levels were measured in mice using an ELISA kit (Karmania Pars Gene, Iran), according to the manufacturer's instructions. First, 50 μL of samples and standards was added to the precoated plates and incubated for 16–20 h at 4 °C, followed by washing three times. Next, 50 μL of conjugated antibody was added to the plates and incubated at room temperature for one hour, followed by a washing step (four times). Then, 50 μL of HRP-Avidin was added to the plates and incubated in a shaker incubator at room temperature for 30 min at 200 rpm. Finally, after washing four times, 90 μL of the substrate was added and incubated at room temperature for 15 min. The reaction was terminated, and the OD was measured at 450 nm.

Moreover, the IL-5 levels were assessed in the mouse splenocytes by the ELISpot kit (Mabtech, Sweden), according to the manufacturer's instructions. Briefly, 10^5 splenocytes were isolated by density gradient sedimentation and incubated in a precoated anti-mouse IL-5 ELISpot plate (5 μg/mL) with S peptide pools (2 μg/mL) for 16 h at 37 °C in a 5% CO_2 atmosphere. One day after stimulation, the plates were washed five times with PBST and incubated for one hour at room temperature with biotin-conjugated anti-IL-5 antibodies. Subsequently, the plates were washed and incubated for one hour with HRP-Streptavidin. The substrate was added after the washing step, and the reaction was terminated by rinsing with distilled water. The plates were then dried, and spots were counted under a loop microscope in a dark room. The results were reported as the number of specific spots in one million cells.

2.5.5. Cellular immunity

The IFNγ and IL-2 levels were determined by an ELISpot kit (Mabtech, Sweden) and an ELISA kit (Karmania Pars Gene, Iran), respectively, according to the manufacturer's instructions in mice, as described above. Moreover, the response of CD8 T cells was examined in vaccinated mice by the granzyme B activity assay. Briefly, splenocytes were isolated from groups 1, 4, and 5 at two weeks after the final injection. Next, the splenocytes were stimulated with purified S and N antigens (10 μg/mL) in 24-well plates and incubated for 72 h at 37 °C in a 5% CO_2 atmosphere. The granzyme B level was also assessed, using the Granzyme B Mouse ELISA Kit (Invitrogen, USA).

2.5.6. SARS-CoV-2 neutralizing antibody

The levels of neutralizing antibodies were evaluated in the mice, rabbits, and hamsters by the VNT. The sera were heat-inactivated via incubation at 56 °C for two hours. The Vero cells were cultured at 1×10^5 cells/mL in 96-well flat-bottom plates.

Nine dilutions (two-fold) of the sera were prepared in DMEM, containing 1% FBS and 1% Antibiotic-Antimycotic (Gibco, USA). Next, 50 μL of each serum dilution was mixed with 50 μL of 100 $TCID_{50}$ of SARS-CoV-2 and incubated at room temperature for one hour. Subsequently, the mixture was added to the wells, containing monolayers of Vero cells following incubation for 60 min at 37 °C.

All experiments were performed in quadruplicate. One well without the serum/virus mixture, one well without the serum, and one well without the cells were included as the controls (Ad5 empty and mock). After incubation for 60 min at 37 °C, the supernatant was removed, and the cells were washed with DMEM; they were maintained in DMEM, containing 10% FBS and antibiotics, for 72 h at 37 °C in a CO_2 atmosphere. The CPE was examined under an inverted microscope, and the titer of neutralizing antibodies was determined as the reciprocal of the highest serum dilution at which wild-type virus is neutralized in 50% of the wells.

2.5.7. Hematological and biochemical tests

Blood samples were collected from the retro-orbital vein of all groups of mice and hamsters on days 7, 14, 21, 28, and 35 and on days 14 and 28, respectively. Also, blood sampling was performed on days 4 and 7 after the virus challenge experiment for the hamsters. Hematological parameters, including the white blood cell (WBC), red blood cell (RBC), neutrophil, lymphocyte, monocyte, eosinophil, and platelet counts, along with the level of hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW), were measured. Moreover, biochemical parameters, including urea, albumin, C-reactive protein (CRP), fasting blood sugar (FBS), creatinine (Cr), alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH), were evaluated.

2.5.8. SARS-CoV-2 viral load after the challenge in golden Syrian hamsters

On days 2, 3, 4, 5, and 7 after the viral challenge experiment, throat swabs were taken from all groups and subjected to viral titer measurements by $TCID_{50}$. Besides, the lung tissues of the sacrificed animals were subjected to viral titer assessments.

2.5.9. Histopathology of golden Syrian hamsters

On days 4 and 7 after the viral challenge, three hamsters from each group were sacrificed by the intraperitoneal injection of 300 mg/kg of ketamine and 15 mg/kg of xylazine. The lung, tracheal, and nose tissues were removed for histopathological, immunohistochemical, and virus titer (TCID50%) measurements. The tissues were fixed with 10% formalin, and then, paraffin-embedded tissue blocks were prepared. Microsections with a diameter of 3 μm were prepared on a glass slide, and hematoxylin and eosin (H&E) staining was performed for histopathological examinations. The stained sections were examined by light microscopy for scoring the lesions. The intensity of inflammatory cell infiltration into the tissue was scored as follows: 0 = without inflammatory cells, 1 = low number of inflammatory cells, 2 = moderate number of inflammatory cells, and 3 = abundant inflammatory cells.

2.5.10. Immunohistochemistry

The lung and tracheal microsections from paraffin-embedded tissue blocks were incubated with a SARS-CoV-2 S1 antibody (Rabbit Mab 40150-R007, Sino Biological, China) overnight at 4 °C and then incubated in a peroxidase blocking solution for ten minutes at room temperature. Next, secondary anti-polyvalent HRP (EURMAb, Italia) was added and incubated for 30 min at room temperature. The sections were incubated in DAB solution for 3–5 min, and

the cell nucleus was stained with hematoxylin. Finally, the slides were assessed under an optical microscope (Olympus CX33, Japan).

2.5.11. Statistical analysis

GraphPad Prism 8.12 (GraphPad Software Inc., San Diego, USA) was used for statistical analysis and plotting. One-way ANOVA or Mann-Whitney test was used to compare the follow-up criteria between the animal groups. The neutralizing antibody data were analyzed by non-parametric Spearman's correlation test. P-values<0.05 were considered statistically significant at a 95% confidence interval (95% CI).

3. Results

3.1. Toxicology

In this study, single and repeated-dose toxicity (Fig. 2) were measured in rats; abnormal toxicity was examined in mice and guinea pigs; and local tolerance was tested in guinea pigs. The results showed no significant toxicity in the brain, lung, heart, and liver tissues or muscles at the injection site in none of the four groups. In the groups receiving the vaccine candidates (rAd5-S and rAd5 RBD-N), the animals' general condition and weight were in the normal range. Besides, the histological findings revealed no obvious injection-related lesions in the vaccinated animals compare to mock group. The type and pattern of observed changes in submitted specimens of each group were relatively similar to each other. Some suspected lesions were usual and repetitive in most specimens. Although inflammation had variable range in different samples, it was dominant lesion in examined specimens. However, despite some background lesions, no toxic changes attributed to toxicity were found in the any specimens.

3.2. Immunogenicity in mice

Vaccine-administered mice, receiving the prime-boost (group 3 receiving the first dose of rAd5-S, followed by the second dose of rAd5 RBD-N) or heterologous prime-boost (group 4 receiving the first dose of rAd5-S and the second dose of RBD SOBERANA) and prime-only (group 5 receiving only a mixture of rAd5-S prime
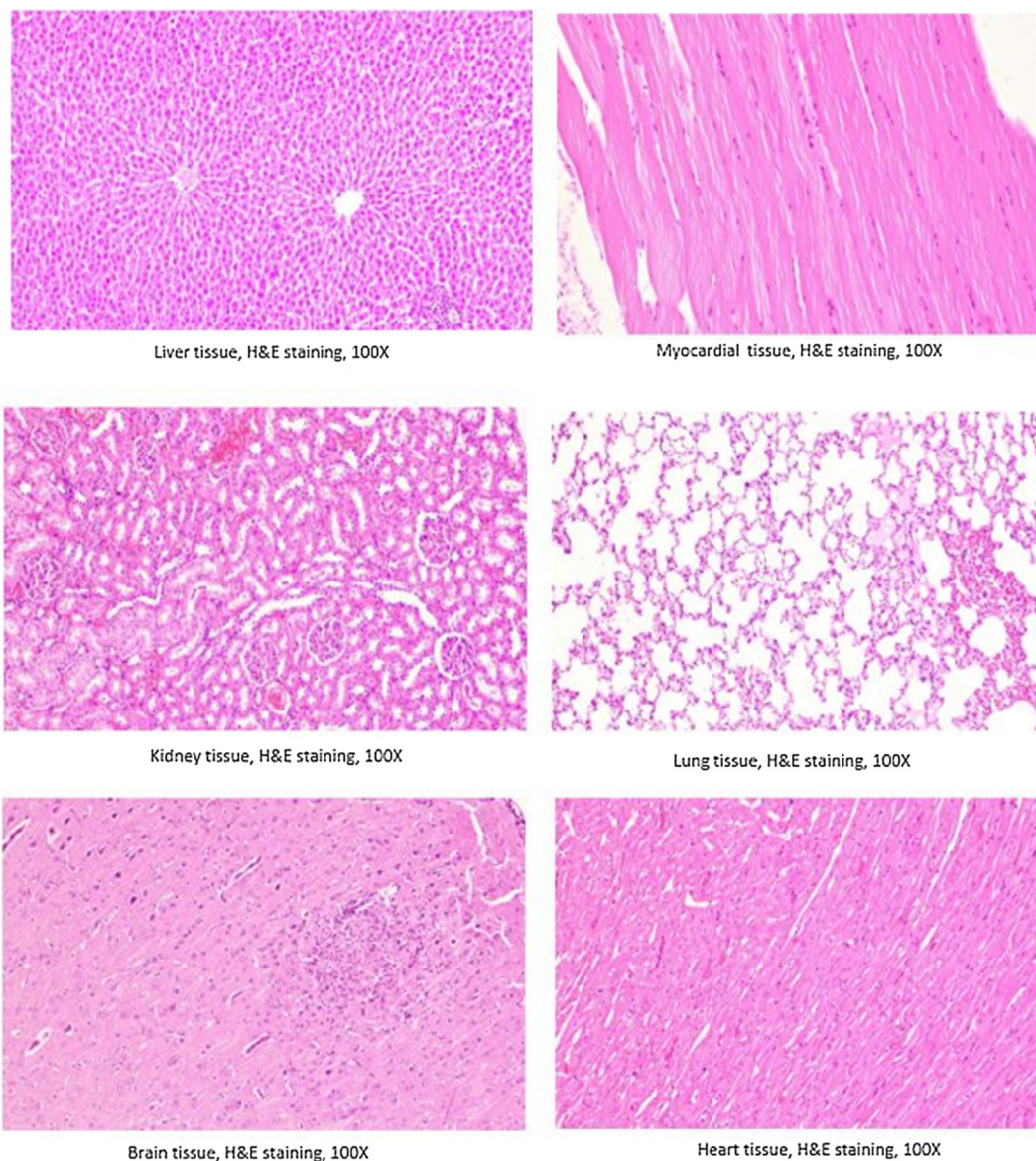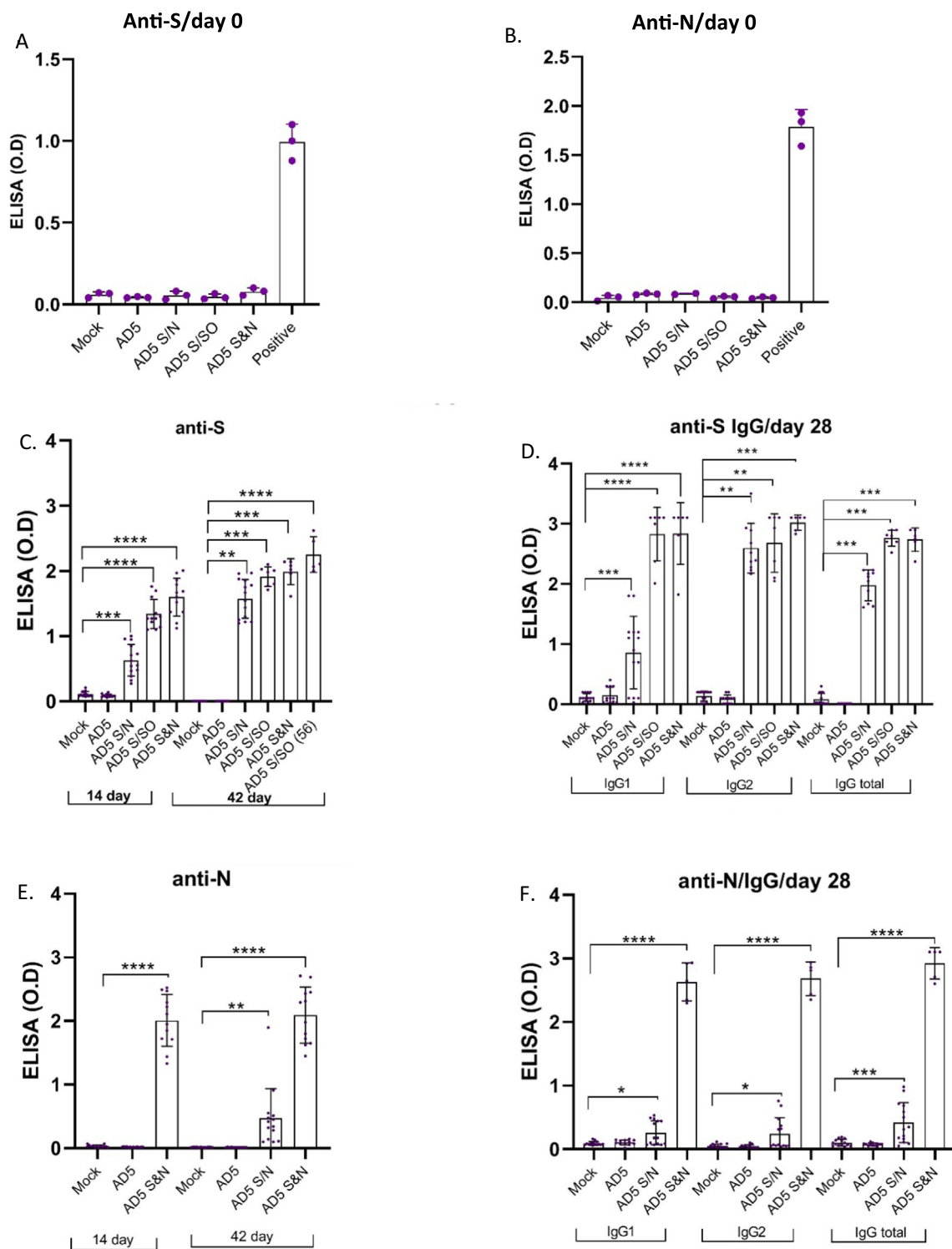

Liver tissue, H&E staining, 100X

Myocardial tissue, H&E staining, 100X

Kidney tissue, H&E staining, 100X

Lung tissue, H&E staining, 100X

Brain tissue, H&E staining, 100X

Heart tissue, H&E staining, 100X

Fig. 2. Repeated dose toxicity. The results showed no significant toxicity in the liver, muscles, kidney, lung, brain and heart tissues.

Mice

Fig. 3. Humoral immune responses in vaccinated BALB/C mice. Serum samples were tested by ELISA assay at 0 (A, B), 14 (C, E), 28 (D, F), 42 (C, E), and 56 (C) days after the inoculation of rAd5-S or RBD-N, along with SOBERANA as a booster dose. The S-specific antibody and N-total antibody levels were also assessed, and the subclasses of IgG1 and IgG2 antibodies were measured. Each dot in the graphs represents replicate samples in the mouse group (P < 0.05, one-way ANOVA test). A 95% CI was considered for all tests. (* *p < 0.05;* ** *p < 0.01;* *** *p < 0.001;* **** *p < 0.0001).*AD5 (rAd5 empty), AD5 S/N (rAd5-S/rAd5 RBD-N), AD5 S/SO (rAd5-S/SOBERANA), AD5 S&N (rAd5-S & rAd5 RBD-N mix).

and rAd-5 RBD-N) vaccines, were investigated for both humoral and cellular immune responses. The humoral immune response was examined on days 0, 14, 28, and 42 in all groups. In the rAd5-S/RBD SOBERANA group, evaluations were performed on day 56, as well (Fig. 3).

The anti-spike total IgG and anti-N total IgG were examined with ELISA assays on days 14, 28, and 42. A significant increase was found in these antibodies in the vaccinated groups, compared to the mock (P < 0.0001). The IgG response was significantly stronger in the prime-only (rAd5-S and rAd5 RBD-N (and heterologous prime-boost (rAd5-S/RBD SOBERANA-vaccinated) mice, compared to the rAd5-S/rAd5 RBD-N vaccinated mice (P < 0.009) (Fig. 3). Also, the levels of IgG1 and IgG2a isotypes were significantly higher in the prime-only group (rAd5-S and rAd5 RBD-N) compared to the other groups (Fig. 3).

The VNT results revealed that the sera from the control groups could not neutralize the virus, and CPE was observed in all serum dilutions. In contrast, virus neutralization was reported in both prime-only and prime-boost groups. In the groups receiving rAd5 S/rAd5 RBD-N, rAd5 S/RBD SOBERANA, and mixture of rAd5-S/ rAd5 RBD-N, the serum dilutions of 1:256, 1:256, and 1:128 neutralized the virus, respectively (Fig. 4).

The cytokine assay results revealed that the levels of inflammatory and non-inflammatory cytokines related to humoral and cellular immunity were markedly elevated in the vaccinated groups compared to the mock. However, no significant difference was found between the prime-only and prime-boost groups (Fig. 5). According to the granzyme B assay results, there was a significant difference in the cellular immunity responses (CD8 T cells) between the vaccinated and mock. However, the difference was not significant between the vaccinated groups (Fig. 5).

The hematological and biochemical factors were normal in both vaccinated and control groups, indicating the non-toxicity of vaccine candidates for the blood cells, kidneys, liver, and other organs. The histopathological results showed that the vaccine candidates caused no toxicity in the brain, lung, heart, kidneys, liver, and injection site tissues.

3.2.1. Immunogenicity in rabbits

In a large animal model, different vaccine candidates were administered to New Zealand white rabbits. The rabbits were vaccinated with the prime-only and prime-boost regimens, similar to the mouse and hamster groups (Fig. 1). The humoral immune response was examined on days 0, 14, 28, and 42 for different rabbit groups. In the rAd5-S/RBD SOBERANA group, evaluations were
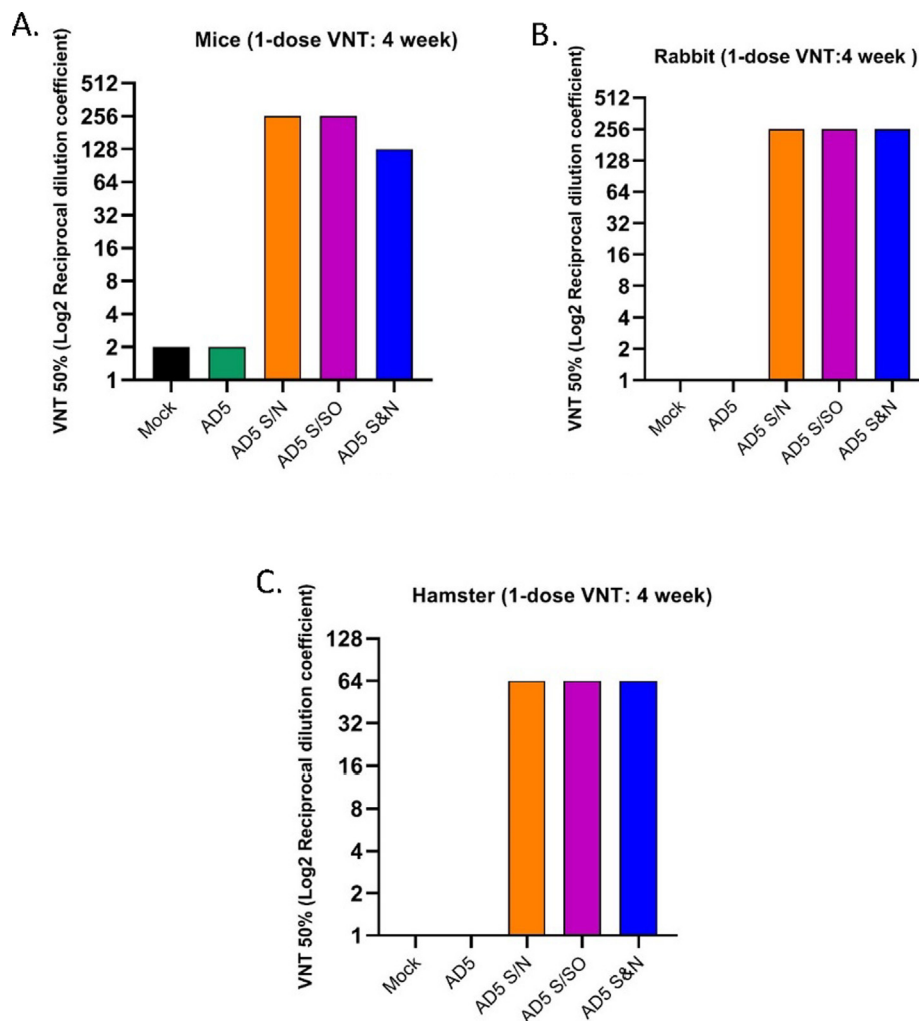

Fig. 4. The results of viral neutralization test 50% in mouse, rabbit and hamster has been shown. Four week after injection of vaccine, blood samples were collected. VNT has been done in Vero cell lines. In each well 100 TCID50% of the SARS-CoV-2 was added and 4 replicated in each dilution was mentioned. A: mice, B: rabbit and C: hamster. AD5 (rAd5 empty), AD5 S/N (rAd5-S/rAd5 RBD-N), AD5 S/SO (rAd5-S/SOBERANA), AD5 S&N (rAd5-S & rAd5 RBD-N mix).
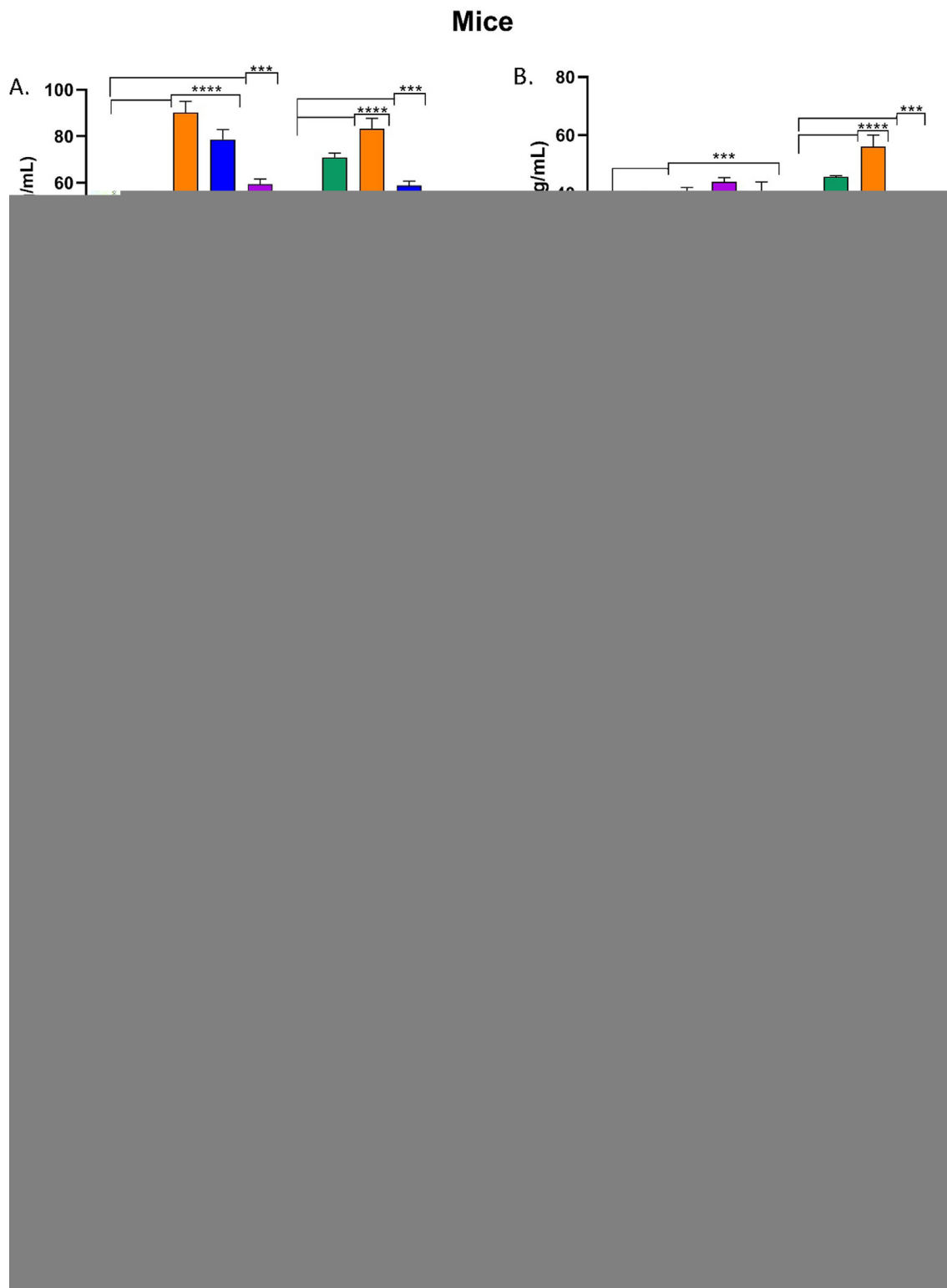
Fig. 5. The cytokine and CTL assays. The levels of IL-6 (A), IL-2 (B), and TNF-α (C) were examined, using the ELISA kit in mice on days 28 and 42. The IL-5 (E) and IFNγ (F) levels were examined by the ELISpot kit on day 28 after immunization. The evaluation of CD8 T cell response (D) in the vaccinated mice was determined by the granzyme B assay for the groups control, heterologous prime-boost (rAd5 S/RBD SOBERANA), and prime only (rAd5-S/rAd5 RBD-N mixture). Each dot in the graphs represents replicate samples in the mouse group (P < 0.05, one-way ANOVA test). A 95% CI was considered for all tests. (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001). AD5 (rAd5 empty), AD5 S/N (rAd5-S/rAd5 RBD-N), AD5 S/SO (rAd5 S/SOBERANA), AD5 S&N (rAd5-S & rAd5 RBD-N mix).
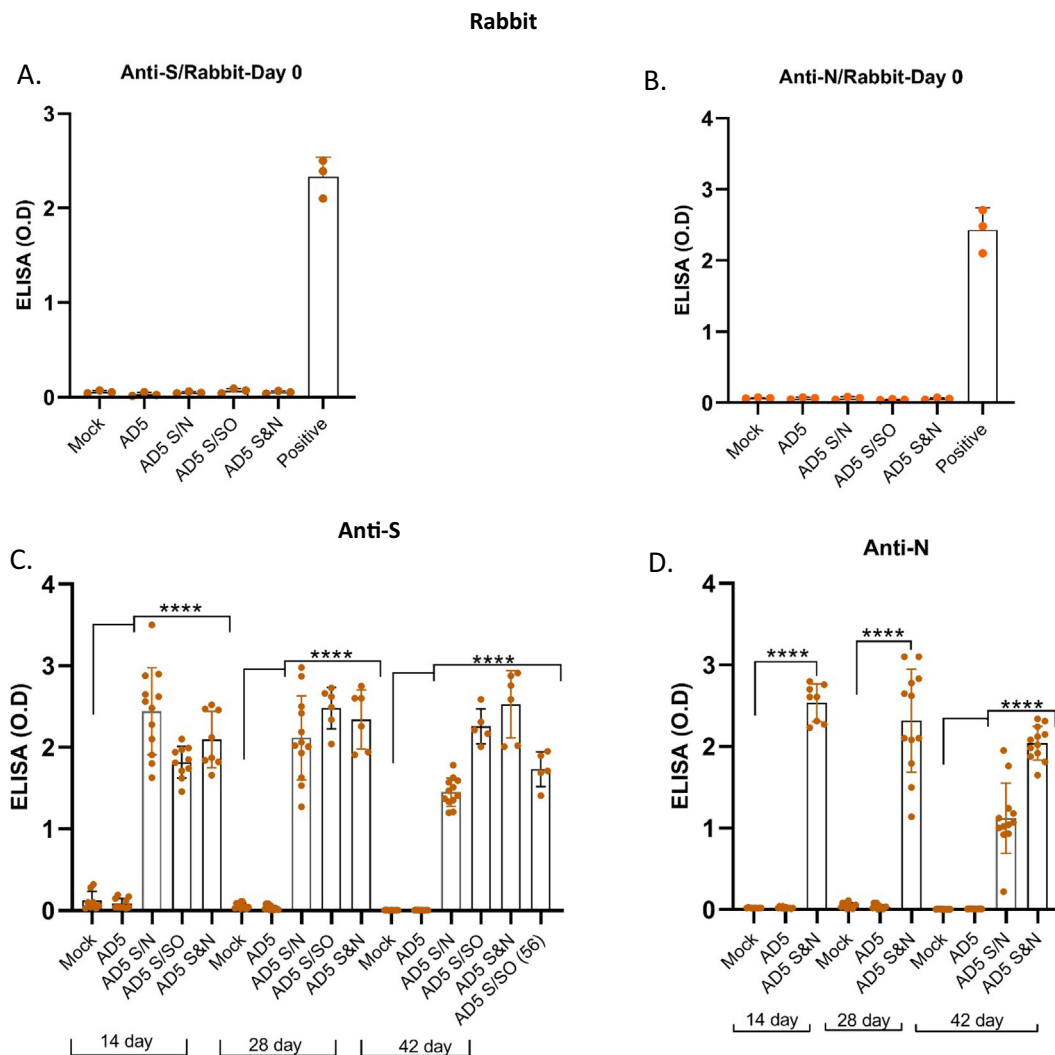
Rabbit

Fig. 6. Humoral immune responses in vaccinated rabbits. Serum samples were examined by ELISA assay on days 0 (A, B), 14, 28, 42 (C, D) and 56 (C) after immunization with rAd5-S or RBD-N, along with SOBERANA as a booster dose. The S-specific antibody and N-total antibody levels were also assessed. Each dot in the graphs represents replicate samples in the rabbit group (P < 0.05, one-way ANOVA test). A 95% CI was considered for all tests. (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001). AD5 (rAd5 empty), AD5 S/N (rAd5-S/rAd5 RBD-N), AD5 S/SO (rAd5 S/SOBERANA), AD5 S&N (rAd5-S & rAd5 RBD-N mix).
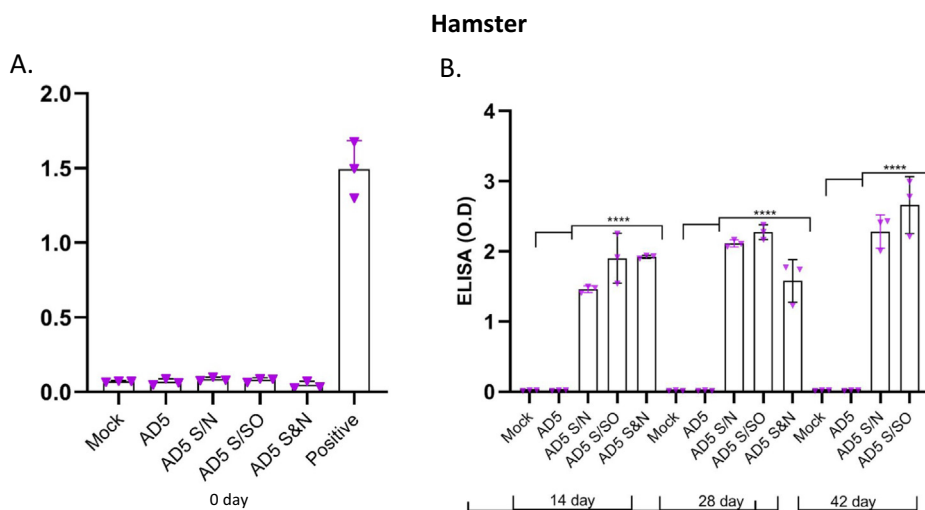
Hamster

Fig. 7. Humoral immune responses in hamsters. Serum samples were examined by ELISA assay on days 0 (A), 14, 28 and 42 (B) after immunization with rAd5-S or RBD-N, along with SOBERANA as a booster dose. The S-specific antibody levels was assessed. Each dot in the graphs represents replicate samples in the hamster group (P < 0.05, one-way ANOVA test). A 95% CI was considered for all tests. (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001). AD5 (rAd5 empty), AD5 S/N (rAd5-S/rAd5 RBD-N), AD5 S/SO (rAd5 S/SOBERANA), AD5 S&N (rAd5-S & rAd5 RBD-N mix).
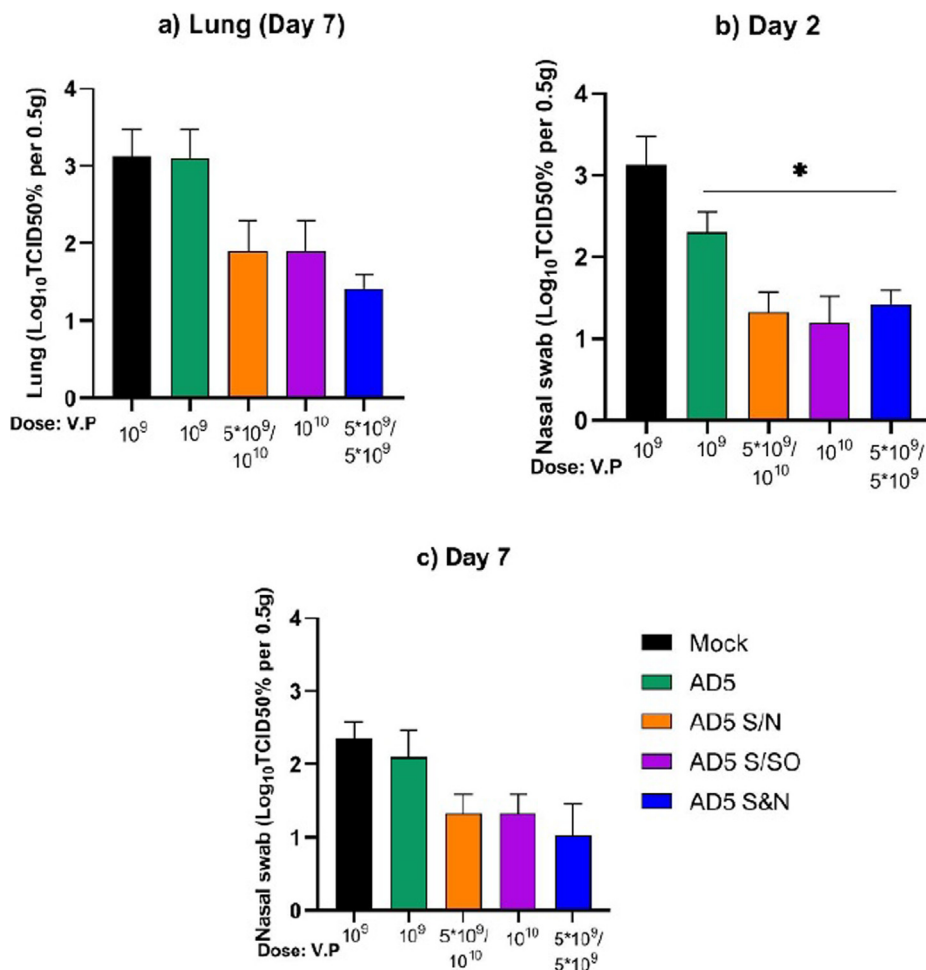
Fig. 8. Protection against SARS-CoV-2 viral replication in hamsters immunized with Ad5-based vaccines. The hamsters were intramuscularly immunized with a one-dose regimen (mixture of rAd5 S and rAd5 RBD-N) and a two-dose regimen (rAd5-S/rAd5 RBD-N and rAd5-S/booster SOBERANA). An empty Ad5 vector was used for the negative control group. The hamsters received an intranasal inoculation with 5×10^5 $TCID_{50}$ of SARS-CoV-2 strain. In the prime-only group, the viral challenge was performed at 28 days after vaccination, while in the two-dose group, the challenge was performed at 42 days after vaccination. Throat swab samples were taken daily after inoculation, and the viral load was calculated as TCID50%/mL. Comparisons were made between the two groups by one-way ANOVA test (P < 0.05). (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). AD5 (rAd5 empty), AD5 S/N (rAd5-S/rAd5 RBD-N), AD5 S/SO (rAd5 S/SOBERANA), AD5 S&N (rAd5-S & rAd5 RBD-N mix).

performed on day 56, as well. The anti-spike total IgG and anti-N total IgG levels were measured using ELISA assays on days 14, 28, and 42. A significant increase was observed in these antibodies in the vaccinated groups compared to the mock (P < 0.0001). The IgG response was significantly stronger in the prime-only (rAd5 S and rAd5 RBD-N (and heterologous prime-boost (rAd5-S/RBD SOBERANA), compared to the prime-boost (rAd5-S/rAd5 RBD-N) group (P < 0.009) (Fig. 6). The VNT results indicated that the sera from the mock could not neutralize the virus, and CPE was observed in all serum dilutions. In contrast, virus neutralization was observed in both prime-only and prime-boost groups up to a dilution of 1:256 (Fig. 4).

### 3.2.2. Immunogenicity in Syrian golden hamsters

The hamsters were vaccinated with the prime-only, prime-boost, and heterologous prime-boost regimens. In the prime-only regimen, a single dose containing a mixture of rAd5-S (5×10^9 VP) and rAd5 RBD-N (5×10^9 VP) was administered. On the other hand, the prime-boost group received the first dose of rAd5-S (5×10^9 VPs), followed by the second dose of rAd5 RBD-N (1×10^{10} VPs) in an interval of 21 days. Also, the heterologous prime-boost group received the first dose of rAd5-S (1×10^{10}

VPs) and the second dose of RBD SOBERANA (20 μg). They were investigated for both humoral and cellular immune responses in an interval of 28 days.

The humoral immunity response was examined in all groups on days 14 and 28 after vaccination. The hematological and biochemical tests were performed for all groups on days 14 and 28 after vaccination and on days 4 and 7 after the viral challenge experience. All factors nearly, were in normal range between all groups except some difference. Platelet counts were normal between groups on days 14 and 28.

The results of total IgG antibody measurements showed a significant difference in the antibody production on days 14, 28, and 42 in the vaccinated groups compared to the mock (Fig. 7).

The VNT results showed that the sera from the control groups could not neutralize the virus, and CPE was observed in all serum dilutions. In contrast, virus neutralization was observed in all vaccinated groups up to a dilution of 1:64 (Fig. 4). The cell blood count showed leukocytosis in the vaccinated group; however, no elevation was seen in the WBC in any of the control groups, indicating the immune system stimulation because of vaccination. No vaccine-related toxicity was observed in the pathological investigation.

Hamster
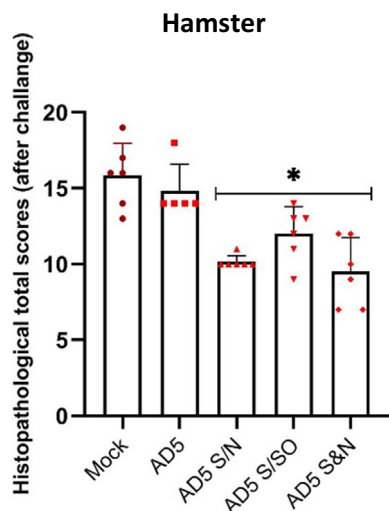
Fig. 9. On days 4 and 7 after the viral challenge, the lung and tracheal tissues were isolated and scored for the presence and severity of alveolitis, alveolar damage, alveolar edema, alveolar hemorrhage, type II pneumocyte hyperplasia, bronchitis, bronchiolitis, and peribronchial or perivascular cuffing. The sum of scores is presented as the sum of lower respiratory tract parameters. A 95% CI was considered for all tests. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). AD5 (rAd5 empty), AD5 S/N (rAd5-S/rAd5 RBD-N), AD5 S/SO (rAd5 S/SOBERANA), AD5 S&N (rAd5-S & rAd5 RBD-N mix).

The viral titer measurements (TCID50%) in the throat swabs revealed a significantly higher titer in the mock two days after the challenge, compared to the vaccinated animals. A similar difference was seen in the viral titer of throat swabs and lung samples on day 7 after the challenge; however, the difference was not statistically significant (Fig. 8). The histopathology induced by SARS-CoV-2 challenge significantly reduced after immunization with the prime-only, prime-boost, and heterologous prime-boost regimens in the lower respiratory tract, compared to the mock (Fig. 9). The immunohistochemical (IHC) staining of SARS-CoV-2 S1 in the vaccinated groups was lower on days 4 and 7 after the challenge compared to the mock, which was consistent with the viral load and histopathology results (Fig. 10). The analysis of body weight loss by measuring the area under the curve in different vaccine groups showed in Fig. 11. The values were compared using one-way ANOVA followed by a Tukey's multiple comparison test. There is significant difference between mock and AD5 S&N group. This group clearly lost less weight than the mock group.

4. Discussion

So far, several vaccine platforms, mostly designed for the SARS-CoV-2 virus spike gene with many B and T cell epitopes, have received use authorization, as they can effectively prevent against the severe form of COVID-19 [13-15]. Adenoviral vectors, due to high gene transfer capacity, safety, and convenience, are extensively used in SARS-CoV-2 vaccine studies [16]. In the present study, the safety and immunogenicity of three adenovirus-based vaccine candidates, including the prime-only, prime-boost, and heterologous prime-boost vaccines, were examined in mice, rabbits, and hamsters. The mouse and rabbit models, as convenient immunogenicity models, along with hamsters, as suitable viral challenge models, have been used in many studies [17,18]. In line with previous research on adenovector-based vaccines, no vaccine-induced side effects were observed in the brain, lung, kidney, or liver tissues of vaccinated animals compared to the mock [18,19].

The evaluation of anti-S and anti-N total IgG responses showed that the vaccinated groups had significantly higher IgG titers compared to the mock. Additionally, the prime-only and heterologous prime-boost regimens stimulated stronger IgG responses compared to the homologous prime-boost regimen. This finding could be attributed to the high dose of adenovector in the heterologous prime-boost regimen or the greater stimulation of the immune system by both S and N antigens. In this regard, Dangi et al. showed that the N protein of SARS-CoV-2 with conserved and immunogenic T cell regions could be an ideal target for SARS-CoV-2 vaccine design [20]. Accordingly, these viral epitopes, together with the T and B lymphocyte epitopes, the Spike protein region, act as immunodaminant epitopes in the strong stimulation of the immune system against the virus[20,21].

On the other hand, because there are many mutations in the S region, which can reduce the vaccine efficacy, the N gene epitopes can be considered as conserved and synergistic immunopotent regions in vaccine studies [8,20]. An increase in the neutralizing antibody titer on day 28 after vaccination, along with an increase in cytokines and IgG1/IgG2 (elevated levels of IgG1 as a humoral immune response and IgG2a as a marker of cellular immune response), indicated that both arms of the immune system were equally stimulated and activated by our vaccine candidate; this finding is in line with the ability of adenovirus-vector vaccines to trigger humoral and cellular immune responses. The results of the CTL assay showed the strong stimulation of Th1 and CTL in mice receiving the prime-only and heterologous prime-boost vaccine regimens, which can be promising observations in the prevention of SARS-CoV-2 [22,23], as shown for the influenza virus and other respiratory viruses [24,25].

The viral load and IHC staining of SARS-CoV-2 S1 protein were lower in the immunized groups compared to the mock, and no histopathological lesions were found in the hamsters' lung tissues following a viral challenge with a high dose of SARS-CoV-2, which strongly supports the efficacy of vaccine candidates. Hematological and biochemical factors were normal except for the results of increased platelet count in the groups rAd5 S / rAd5 RBD-N and rAd5 S / RBD SOBERANA on day 4 after viral challenge which could be due to inflammatory reactions related to high immune stimulation[26].

In this regard, Doremalen et al. showed that a single-dose ChAdOx1 vaccination in the rhesus macaques induced potent humoral and cellular immune responses; also, in challenged rhesus macaques, a reduced SARS-CoV-2 load was found in the respiratory tract tissue and fluid samples of vaccinated animals compared to the controls [27]. Consistent with the results of these studies, and also the results of immunohistochemistry, toxicology and body weight loss of these groups we found that the single-dose vaccine regimen had adequate efficacy as a vaccine candidate.
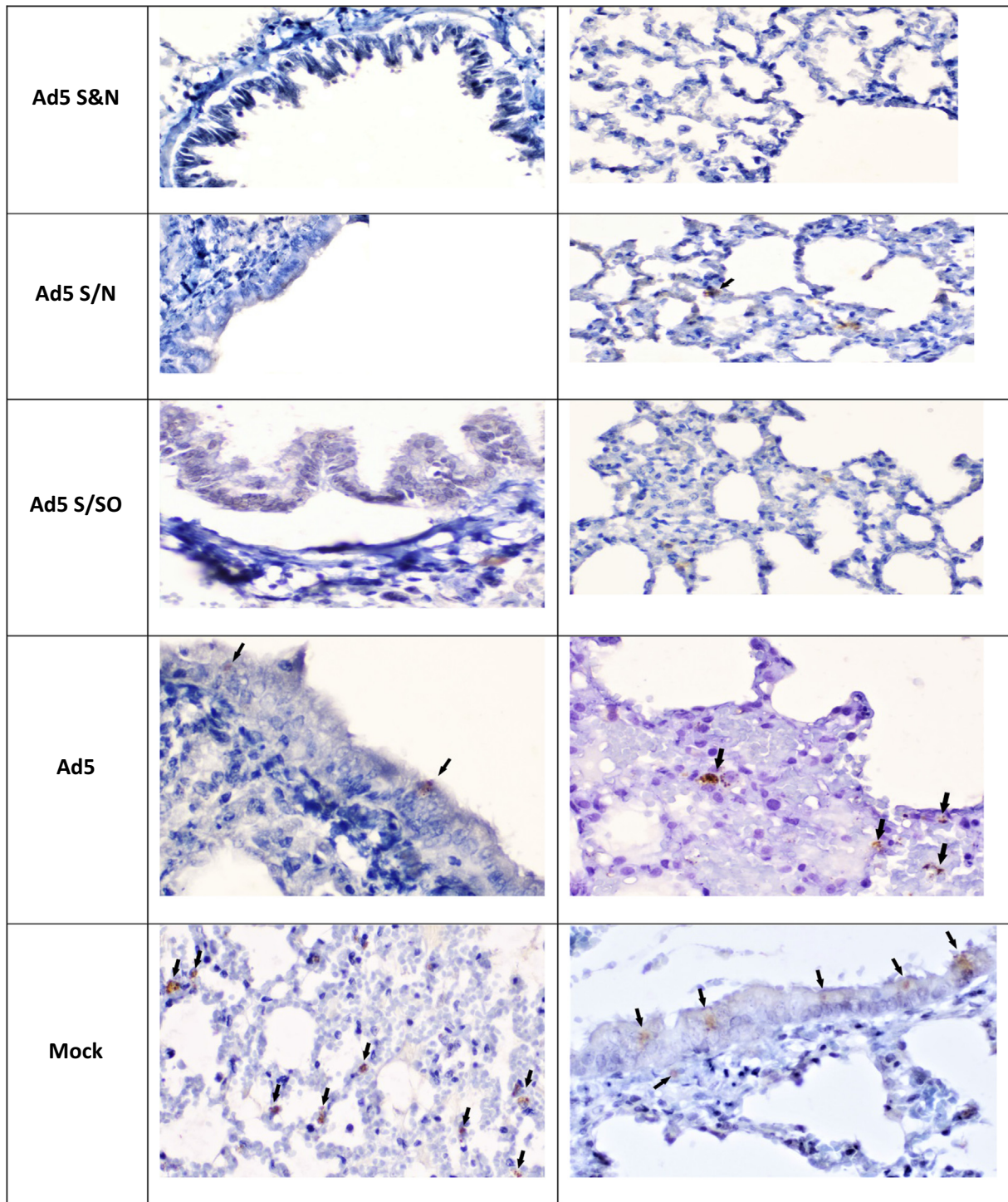
Fig. 10. Immunolocalization of S1 protein in the bronchial epithelium and the lungs of hamsters following the viral challenge. Immunoreactivity and lack of immunoreactivity to S1 protein were observed in the control and vaccinated groups, respectively (100 × ma).

Hamster body weight dynamics
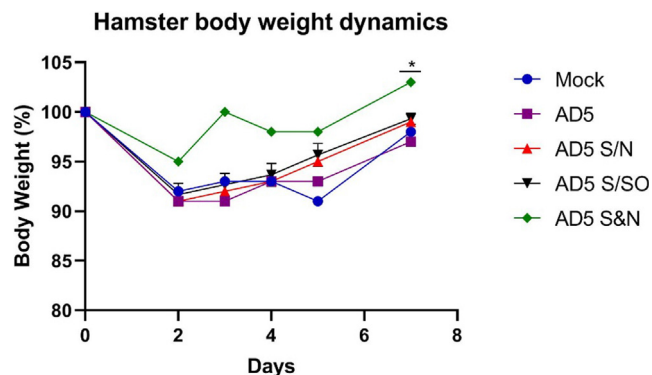
Fig. 11. The picture shows analysis of body weight loss in different vaccine group (normalized to 100% value). The values were compared using one-way ANOVA followed by a Tukey's multiple comparison test. The AD5 S&N group was significant with weak evidence (P-value 0.0259). (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Error bars represent mean ± SD. AD5 (rAd5 empty), AD5 S/N (rAd5-S/rAd5 RBD-N), AD5 S/SO (rAd5 S/SOBERANA), AD5 S&N (rAd5-S & rAd5 RBD-N mix).

5. Conclusion

In conclusion, the results of the present study showed that the three vaccine regimens were immunogenic in the three animal models and provided protection in challenged SARS-CoV-2 hamsters. However, a single dose of rAd5-S and rAd5 RBD-N is suggested as an effective and inexpensive candidate for mass vaccination after successful confirmation of the results in human clinical trials.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval

All procedures performed in studies involving animal participants were in accordance with the ethical standards of the institutional and/or national research committee.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.03.066.

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