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ANIMAL PRE-CLINICAL STUDY OF AN INACTIVATED SARS-CoV-2 VACCINE CANDIDATE (OSVID-19 ®): IMMUNOGENICITY, PROTECTIVE, AND SAFETY ASPECTS

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Abstract

Background: This study aimed to evaluate the outcomes of preclinical studies on the safety and immunogenicity of an inactivated COVID-19 vaccine candidate to warrant further clinical evaluation.

Methods: SARS-CoV-2 positive nasopharyngeal swab specimens were confirmed by real-time polymerase chain reaction and next-generation sequencing. The safety and immunogenicity tests of the COVID-19 vaccine were carried out in rats and Rhesus monkeys, and *Balb/C* mice and Rhesus monkeys, respectively.

Results: The candidate vaccine was well tolerated and induced promising levels of SARS-CoV-2–specific IgG1, IgG2a, and Granzyme B in *Balb/C* mice, and anti-SARSCoV-2 spike IgG and neutralizing antibodies in Rhesus monkeys. Based on cVNT results, the inactivated vaccine in 0.5 and 1 µg/100 µL doses was able to induce a neutralizing effect against the SARS-CoV-2 virus up to a dilution of 1:512 and 1:1000. The protective efficacy of the vaccine candidate was challenged with 2 $\times 10^8$ PFU of live viruses and confirmed by lung CT scan and histopathological evaluations compared to the control group. Repeated intramuscular injection of the candidate vaccine was generally well-tolerated in Rats and Rhesuses. No significant side effects were observed in rats injected with ten full human doses and in the Rhesus monkeys with three full human doses.

Conclusion: Based on the findings presented in this study, it is recommended that this vaccine be moved into human testing commencing with a phase I clinical trial.

Keywords: immunogenicity, toxicology, vaccine

1. Background

A new coronavirus which calls severe acute respiratory syndrome coronavirus 2, initiated the outbreak of coronavirus disease(Covid-19) in China, in December 2019, has now spread worldwide and become a pandemic with a high number of deaths more than 6 million people(1)(2) . SARS-CoV-2 is a member of the genus *Betacoronavirus* in the *Coronaviridae* family(3) (4). SARS-CoV-2 is an RNA-enveloped single-stranded virus which its entire genome encodes various amino acids, structured, and nonstructural proteins. The structural proteins are encoded by the surface or spike (S), envelope(E), membrane(M), and nucleocapsid(N) proteins genes (3) (5).

Three S protein monomers form a homotrimer, which is a major target for vaccine development, able to neutralize antibodies. To deliver the S or truncated S protein to interact with the host cells specific receptors, multiple SARS-CoV-2 vaccine platforms have been developed, including inactivated vaccines, recombinant-subunits proteins, and mRNA/DNA vaccines (6)(7).

As the virus is highly infectious and transmissible, the WHO announced the urgent need for booster vaccination followed general immunization. Since the outbreak commenced, researchers around the world claim to have made more than 350 COVID-19 vaccine candidates at different developmental stages. By Jan 01, 2023 (8)(9), more than 199 vaccines are in the preclinical stage and 176 in clinical trials (10)(11).

Inactivated vaccines have been used for decades due to their advantages such as higher safety profile, lower reactogenicity, and the ability to deliver multiple viral proteins for immune system motivation(12). Additionally, immunocompromised persons can be immunized by this vaccine platform with very few side effects(13)(12). Moreover, the development platforms of such vaccines are well-established, enabling mass production with fewer regulatory barriers to licensing. However, inactivated COVID-19 vaccines licensed for emergency use have shown lower immunogenicity, necessitating repeated boosters compared to the other platforms(14). Additionally, the immunocompromised, children and aged persons can be immunized by this vaccine platform because of its low side effects(15)(16).

Additionally, these vaccines are technically well-developed and could be produced with fewer regulatory barriers to licensing. The inactivated vaccines have been confirmed to be safe and protective in the prevention of infectious diseases like rabies, polio, and influenza. Globally, 14% of all vaccines developed for COVID-19 are inactivated form (17)(18)(19)(20).

Based on previous experience with the inactivated vaccines with a well-characterized Vero cell manufacturing platform supported researchers in the rapid development of an inactivated vaccine candidate. The inactivation of the virus has been performed using βpropiolactone or formaldehyde as alkylating agents which formaldehyde has long been used for inactivating antigens for vaccine production(21)(22)(23)(24).

The study of the safety, immunogenicity, and protective efficacy of the developed vaccines in appropriate animals is essential before being applied to clinical trials. Given the long history of developing purified inactivated vaccines based on well-established technologies in Iran, this study aimed to evaluate the outcomes of preclinical studies on the immunogenicity and toxicology studies of an inactivated COVID-19 vaccine candidate known as Osvid-19, while warranting further clinical evaluation.

2. Materials and Methods

2.1 Preparation of the Inactivated SARS-CoV-2 Vaccine

COVID-19 vaccine candidate has been developed by Paya Fan Yakhteh Alborz Co Ltd., Iran. The SARS-CoV-2 viruses isolated from a COVID-19 positive nasopharyngeal swab sample and were confirmed by cell culture virus neutralizing test (cVNT), transmission electron microscopy (TEM), and next-generation sequencing (NGS). The obtained sample was diluted with a viral transfer medium and filtered by Millex-GV filter, 0.22 μm A 25 mm diameter sterile syringe filter with a 0.22 μm pore size hydrophilic Polyvinylidene Fluoride (PVDF) membrane (Merck, Germany).

The working seed lot of the virus was propagated in 90% confluent Vero cell E6 culture with MOI 0.01 at 36°C for 4 days. The harvested vial bulk was confirmed, tittered, and purified following inactivation by formaldehyde 37% (1:4000) (v/v) at 32 °C. To confirm the inactivation process, the inactivated SARS-CoV-2 virus was inoculated to a Vero monolayer and incubated at 37°C in a 5% CO2 incubator for 72 h. The next two subcultures were incubated for 5 and 15 days respectively. The inactivated viral bulk was clarified by centrifugation at 5000 rpm at 4°C for 30 minutes. The supernatant was purified and concentrated by tangential flow ultrafiltration (Pellicon® XL 50 Cassette and Labscale® TFF System; Cat No: XX42 13 L) using a low protein-binding, 50-kDa cutoff membrane before and after ion exchange chromatography using AKTA Explorer 100 FPLC, HiPrep DEAE FF 16/10 column to remove unexpected host cell DNA and contamination.
The desired amount of viral antigen per dose was prepared with 1x PBS, and the final formulation contained 1mg/5µg adjuvant aluminum hydroxide gel. SDS-PAGE confirmed the final purified protein composition, and the total protein concentration was determined by the Micro-Bradford assay. A transmission electron microscope (MIRA3, Tescan Co) was utilized to monitor the structural integrity of the inactivated viral particles. The viral particles were applied to a carbon-coated grid, stained with 1% uranyl acetate, and analyzed at 90000x magnification. Inactivated purified vaccine against COVID-19 (Osvid®19) for preclinical studies contains viral antigen (≥5 µg/dose), aluminum (1 mg/dose), and up to 0.5 ml of PBS (phosphate-buffered saline).

2.2 Study design of animal experiments

The study was designed following the recommendations and guidelines issued by WHO and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) to evaluate human vaccines. SD rats of both sexes (aged 6-8 weeks, weighing 185-250 g) and male *Macaca mulatta* (aged 2-4 years) Rhesus monkeys of both sexes were used for toxicology studies.
Balb/C mice (aged 6-8 weeks, weighting 18-21 g) were purchased from Pasteur Institute of Iran (Tehran, Iran) and used for dose-finding and immunological studies. Additionally, adult male Rhesus monkeys (*Macaca mulatta*) were selected for immunological and vaccine protection studies and were housed in an ABSL3 complex. A veterinary specialist evaluated the health conditions of animals before and during the study. The summary of study design for primary pharmacodynamics and toxicology studies is shown in Table 1.

Table 1: List of immunogenicity, protection and toxicology studies performed on animals receiving the purified antigen, candidate vaccine, adjuvant and phosphate-buffered saline.

Study Type	**Test System**	**Antigen**	**Adjuvant**	**Read Out**
Immunogenicity Studies				
Dose range based on Body weight	Balb/c mice	0.5- 1 µg/100µL	Aluminum Hydroxide	Immunity (IgG2a/IgG1, anti- Spike antibody), cytokines, GB, cVNT
Immunogenicity assay	Rhesus	5µg/50 0µL	Aluminum hydroxide	Immunity response, cytokines, sVNT
Protection Studies				
SARS-CoV-2 homologous Immunity response, cytokines challenge with SARS- CoV-2 evaluation, Nasal and Rectal Swab, and CT scan	Rhesus , challenge factors,	Vaccination and 0 clinical signs ,	µL hydroxide BALF	5µg/50Aluminum sVNT, CBC, biochemical
Single-Dose Toxicity				

Toxicology: (single-dose toxicity and local tolerance)	Rat *Sprague dawley*	50 µg	Aluminum hydroxide	CBC, biochemical factors, clinical signs, histopathological features
Toxicology: (single-dose toxicity and local tolerance)	Rhesus monkey	15 µg	Aluminum hydroxide	CBC, biochemical factors, clinical signs, histopathological features
Repeated-Dose Toxicity				
Toxicology: (repeated-dose toxicity)	Rat *Sprague dawley*	5µg Days: 0, 7, 14, 28	Aluminum hydroxide	CBC, biochemical factors, clinical signs, histopathological features
Toxicology: (repeated-dose toxicity	Rhesus monkey	5µg Days: 0, 14, 28	Aluminum hydroxide	CBC, biochemical factors, clinical signs, histopathological features

2.3 Evaluation of two doses of COVID-19 vaccines candidate's Ag in immunized mice; Humoral and Cellular profiles

Immunization studies in animal models were conducted on female *Balb/C* mice (n = 10 / group; six different groups) since they provide valuable "proof of concept" information to support the selection of the appropriate doses, schedules, and routes of administration in clinical trials. The immunization consists of two doses in each group and all factors measurements carried out two weeks and then two months' post-immunization in immunized mice. The mice were kept under standard conditions (Temperature: 23 ± 2 °C, Relative humidity: 45 ± 2%) and provided with standard pellet diet and water. The mice were housed in type III polycarbonate cage and maintained under 12-12 h light/dark cycles. The formulated vaccine, purified inactivated Ag, PBS, and adjuvant with 14 days' interval were administered in six groups, intramuscularly (I.M) as mentioned below:

Group 1 (n=10): Received two doses of inactivated Ag (0.5 µg/100 µL) with a 14-day time interval without adjuvant.
Group 2 (n=10): Received two doses of inactivated Ag (1 µg/100 µL) with a 14-day time interval without adjuvant.
Group 3(n=10): Received two doses of candid vaccine (0.5 µg/100 µL) with a 14-day time interval.
Group 4 (n=10): Received two doses of candid vaccine (1 µg/100 µL) with a 14-day time interval.
Group 5 (n=10): Received two doses of adjuvant (10 µg/100 µL) with a 14-day time interval.
Group 6 (n=10): Control group received two doses of PBS with a 14-day time interval.

The humoral immunity was assessed by evaluation SARS-CoV-2 spike protein-specific immunoglobulin (IgG) subclasses (IgG2a and IgG1) two weeks and then two months' post-immunization in immunized mice. Briefly, an indirect ELISA was performed in order to determine the SARS-Cov2 specific IgG1 and IgG2a antibody concentration in mice serum samples. Briefly, 48-well plates were coated with 1µg/mL of SARS-CoV-2 Ag (virus particle inactivated as purified antigen) for 18 h at 37 °C. Then plates were washed with PBS containing Tween 20, for 60 min at 37 ^{o}C with bovine serum albumin (BSA). The obtained sera were added to each well and incubated for 2 h and then washed. Subsequently, horseradish peroxidase-conjugated goat anti-mouse IgG2a and IgG1 were added and plate incubated for 70 min at 37 °C. Then the wells were washed and TMB substrate, a chromogenic substrate, was added and incubated. Finally, the blocking agent was added and the absorbance was read at 450 nm by ELISA reader.

Additionally, the cell culture Virus Neutralization Test (cVNT) was performed to analyze vaccine protectively. Briefly, two weeks after the final immunization in BALB/c mice, 50 µl blood sample were collected to evaluate vaccines immunogenicity by cVNT. Isolated sera diluted in different concentrations and mixed with 50 µL of 100 TCID50/mL of SARS-CoV2 in DMEM and incubated for 1h at 37°C then inoculated onto monolayers of 2×10^4 Vero cells in 24-well plates for 1h at 37 °C. Subsequently, the supernatant was removed and the cells were washed. The cells transferred to DMEM and incubated for 72 h at 37°C with 5% CO_2 and the cytopathic effect (CPE) of each samples were recorded with microscopes, and the neutralizing titer was calculated.

To evaluate the vaccines effects on T-cell immunity, the percentage of SARS-CoV-2specific $CD8^+$ (Anti-CD8 (Mouse) mAb-PE) and $CD4^+$ (PE anti-mouse CD4 Antibody) T-cell propagation was measured by CFSE assay in spleen cell samples obtained from the immunized mice by using a BD FACSCalibur™ flow cytometer (US, Becton and Dickinson Co).

The level of *TNF-α* (ab208348), *IL-10* (ab100697), *IL-6* (ab46100), and *IL-4* (ab100710), in response to Ag stimulation, was measured by cytokine specific commercial ELISA kits, 21 days following immunization. Briefly, 5×10^6 cells isolated from immunized mouse spleen were incubated with SARS-CoV-2 virus in a humidified 37 °C Co_2 incubator. After 72 h incubation, the cytokine profile was analyzed using the supernatant of the cultures.

Additionally, the vaccine's potential to induce cellular cytotoxicity was evaluated by examining *Granzyme B* activity in splenocytes isolated from the immunized mice 21 days' post-immunization using a commercial mouse *Granzyme B* ELISA kit (MYBIOSOURCE (MBS), United States). Briefly, red blood cells were lysed by RBC lysis buffer for 10 min at room temperature, and the splenocytes were suspended at a density of 1-2 × 106 cells/mL in RPMI 1640, 10% FBS, L-glutamine, Pen-strep (5000 IU/mL, 5 mg/mL). The purified inactive SARS-Cov-2 Ag was added to cells in a 48-well plate and incubated at 37°C with 5% CO2 for 72 h. Then supernatants were collected for measurement of *Granzyme B* activity.

2.4 Immunogenicity, and Protection studies on vaccine candidate in adult Rhesus monkey

Adult female Rhesus macaque monkeys (n = 3 / group) were purchased from Shahid Meysamy Complex Laboratory Animal (Iran) and maintained in stainless steel cages under stable conditions at 22±2 °C, 55% relative humidity in a room with air exchange of 10 times per hour. Clinical reports including body temperature, clinical and behavioral symptoms, weight gaining, biochemical and hematological factors, and immunological parameters were evaluated weekly.

Before the experimental study, C - reactive protein (CRP), inflammatory and proinflammatory cytokines, and serum Vitamin D and Parathyroid hormone (PTH) were evaluated to confirm the healthy condition of monkeys. Also, sterile nasal swab samples were taken from the ends of the monkeys' noses and pharynx and transferred to the laboratory to be evaluated for SARS-CoV-2 RdRP and E genes. The vaccine candidate was administered intramuscularly at a concentration of 5 µg purified inactivated Ag in 500 µL as appropriate dose mentioned for first human use.

Different experimental groups include treatment groups received two doses of vaccine, adjuvant received group, and the control group received PBS. All the animal groups were immunized twice (days 0 and 21), and the challenge was performed intranasally and intratracheally 14 days after the second immunization at a concentration of 2×10^8 PFU of the isolated and confirmed SARS-CoV-2 virus. After the challenge, anal and nasopharyngeal swab were collected 3, 5, 7, 14, and 28 days to evaluation SARS-CoV-2 RdRP and E genes. Additionally, bronchoalveolar lavage was done before the challenge, two days and two weeks followed under general anesthesia by using sterile normal saline solution.

The clinical signs, biochemical and hematological factors, *IL-1 β*(MBS735135), *IL10*(MBS2501888), *TNF-α*(MBS2513510), *IFN-γ*(MBS764170), and *IL-6* (MBS765559), modified-SARS-Cov2 specific IgG, modified-SARS-COV2 Neutralizing Ab test (Pishtaz Teb Co, Iran), lung CT-Scan, and histopathological features of the organs were evaluated. Flow cytometry evaluation was performed

on peripheral blood samples by Anti-CD4 antibodies (MT310), and PE antibodies to Anti-CD8 antibody (ab28017) in BD FACSCalibur ™ flow cytometer.
Briefly, in order to evaluate specific antibodies against spike protein in monkeys, serums with a dilution of 1.100 were used according to the kit protocol. The diluted sera were added to plates coated with purified S proteins and incubated at 37 ° C for 60 minutes. The plate was washed five times with a washing buffer and then, goat anti-monkey IgG HRP conjugated antibody was added to each well and the plate was incubated for 30 minutes at 37 ° C. After the washing step, colorimetric detection was performed using TMB as substrate for 15 minutes at room temperature. The reaction was stopped by the addition of 50 µl of sodium sulfate and the OD uptake at 450 nm was measured using an ELISA reader.
Thoracic radiography (Ventrodorsal and lateral) and lung computed tomography scan before initialing the study and followed the challenge were performed under general anesthesia using Ketamine 10% (Bremer Pharma GmbH, Germany) + Xylazine 2% (Alfasan®, Netherlands).
Seven days followed the challenge, early sacrifice was done on euthanized monkeys and the vital organs were isolated and fixed in 10% formalin solution for tissue processes. The fixed samples were embedded in paraffin, and serial paraffin sections (a thickness of
4-6 µm) were prepared for hematoxylin and eosin staining. An optical microscope was performed for further histological evaluation of the tissues. Euthanasia in Rhesus was performed intravenously with sodium thiopental 100 mg/kg B.W. according to NRC guidelines on animals.

2.5Design of toxicology study

Single-dose toxicity and local tolerance toxicity studies were performed on *Sprague Dawley* (SD) rats (10 heads/group/ both sexes) by administration 10 full human dose vaccine (10 FHD) as appropriate rodents and as well as on *Rhesus monkeys* (3 FHD) as non-rodent animals (3 heads/group/ both sexes) using purified antigen formulated with aluminum hydroxide as candidate's vaccine. Mortality, clinical signs, injection site local reactions, body weight gaining, food consumption, biochemical, and hematological factors were monitored during the immunization period and up to 28 days afterward. The histopathological evaluation was performed on different organs isolated from the animals under study, including lung, heart, liver, and kidney and injection site muscle.
In addition to the standard n+1 dose approach, the repeated dose toxicity studies were designed specifically to mimic two separate immunization series repeated at a prolonged interval, as might occur in a prime-boost strategy in pandemics or in an annual vaccination strategy. The systemic toxicity of intramuscular administration of repeated dose of vaccine (5 µg Ag) was assessed in *Rats* (0, 7, 14, and 28 days) and *Rhesus monkeys* (0, 14, and 28 days), I.M. All studies were conducted according to good laboratory practice (GLP) requirements. Based on a pre-specified schedule, the process of sacrificing of rats and monkeys was carried out in two stages as followed:

1) *Early sacrifice was performed on females and males per group after fourteen days.*
2) *Late sacrifice was performed on females and males per group after 42 days.*

2.6 Statistical analysis

Using the Graph Pad Prism 5.04 software, one-way ANOVA, and multiple Tukey comparison tests Post-hoc analysis. Data were expressed as the mean ± SE, compared to the command, for all analyses a p-value less than 0.05 was considered as a significant difference. The asterisks replicate significant differences with* for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

3 . Results

3.1 Confirmation of the purified inactivated virus

The vaccine strain virus on the fifth passage was filtered and further purified by dilution. The primary, master, and working seed lot of the strain were well-known and characterized according to the national guidelines for vaccine production.

As shown in figure 1, TEM analysis displayed that the purified inactivated viral particles were intact, representing the well-defined spike on the virus membrane (Fig 1).
Real-time PCR and virus-neutralization assay were conducted to confirm virus inactivation in three consecutive passages by formaldehyde. No CPE was observed in three continuous passages on Vero cell culture (Fig 2).

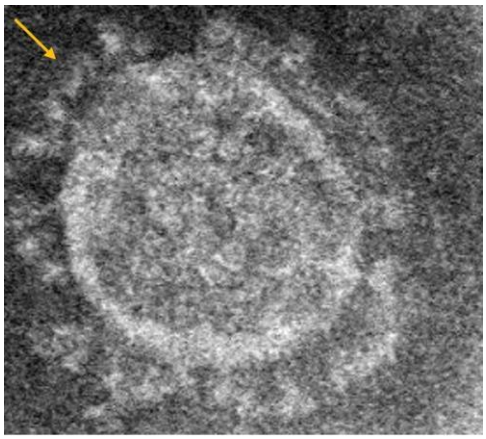
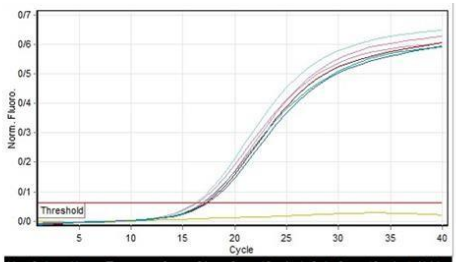


Fig 1: The structure of the isolated and cultured SARS-CoV-2 strain with spike proteins. The virus spike protein remained intact after inactivation and purification(yellow arrow).



No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
1		NTC	Unknown				
2		1day	Unknown	16/20			
3		2day	Unknown	17/06			
4		3day	Unknown	16/44			
19		4day	Unknown	17/22			
20		5day	Unknown	16/92			
21		6day	Unknown	17/45			

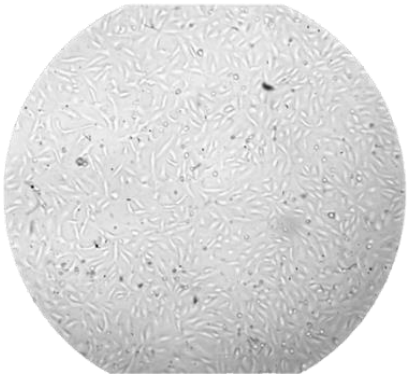


Fig 2: Inactivated virus in Vero cell culture and the absence of CPE after 5 days by microscopy evaluation.

Based on inactivation process, the third passage was performed for the inactivated virus and did not show any alive virus by Real-time PCR. As shown in figure 2, detailed studies of the virus genome from the first day to the five days of culture reveal the inactivated virus. There was no increase in viral genome titer and the amount of virus were completely constant in all days. In the samples examined by Real-time PCR technique, all samples had constant CT on different days and there was no increase in genome and virus replication.

3.2 Cellular and humoral immunological states in Mice

Important parameters that are determined in vaccine development as a criteria of immune response profile are IgG2a and IgG1 in mice. As shown in Fig 3, IgG1 and IgG2a antibodies were raised in all mice that received the formulated vaccine. There was a significant increase of IgG2a in groups that received 5 and 10 µg vaccines in comparison with group received PBS 2 weeks followed immunization ($P<0.01$). Two months followed vaccination, statistically significant increase in IgG2a level was observed in two doses vaccine in comparison to PBS group($P<0.001$).
Additionally, an enhancement in IgG1 antibodies level was observed in vaccine groups 2 weeks and 2 months followed post immunization in comparison to the BPS group which revealed the importance

of adjuvant in vaccine formulation (P<0.01 and P<0.001, respectively). The results of antibody levels in the experimental groups showed that injection of adjuvant-formulated vaccine significantly induced the level of specific antibodies on 2 weeks and 2 months after immunization in both selected doses (Fig 3).

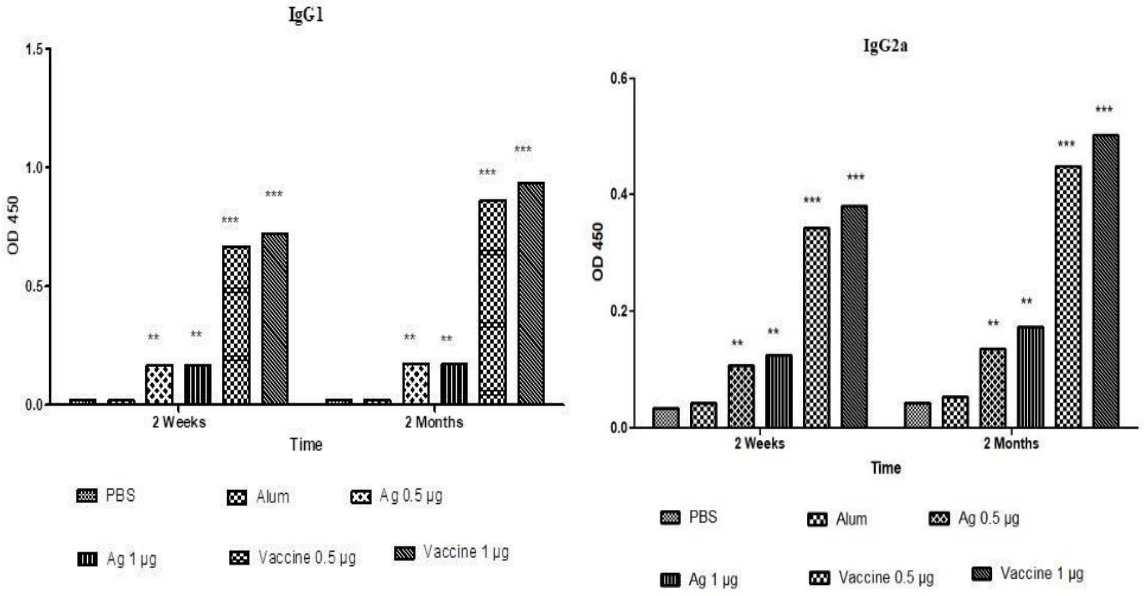


Fig 3: Benefits of the inactivated virus at different concentrations, adjuvant, and candid vaccine in terms of immunogenicity in mice. Immunizations were carried out two times during 14 days with two different Ag

alone or in combination with alum. The control groups received PBS. The blood sample was obtained at 2 weeks and 2 months' post-immunization and sera were diluted to Indirect-ELISA assay. The asterisks replicate significant differences with* for p<0.05, ** for p<0.01, and *** for p<0.001.

In a comparison between the formulated vaccine with aluminum hydroxide and the antigen formulation without the aluminum hydroxide adjuvant (Alum), it was found that the adjuvant-formulated vaccine showed a suitable antibody titer after two months, and the vaccine has proper stability to stimulate humoral immunity and, therefore, cellular immunity.

To further discover antigen effects on T-cell cytokines, splenocytes were obtained 21 days' post-immunization. In evaluations of related cytokines, a significant difference in IL-6 levels was observed between the vaccine with 0.5 µgr Ag (p<0.001), aluminum hydroxide (p<0.01) and the PBS group, and there was statistically significant difference in the two antigen concentrations (p<0.01) (Fig 4). Furthermore, IL-4 increased in vaccine groups compared to the other groups, although these enhancements were not statistically significant (p>0.05).

Based on the presented results, no significant difference was observed between the two vaccine concentrations in TNF-alpha level in immunized mice, but there was a statistically significant difference between the mice receiving the formulated vaccine and PBS ($p < 0.05$). There was also a statistically significant difference in IL-10 level between the vaccinated mice with 1 µg Ag and PBS groups ($p < 0.05$).

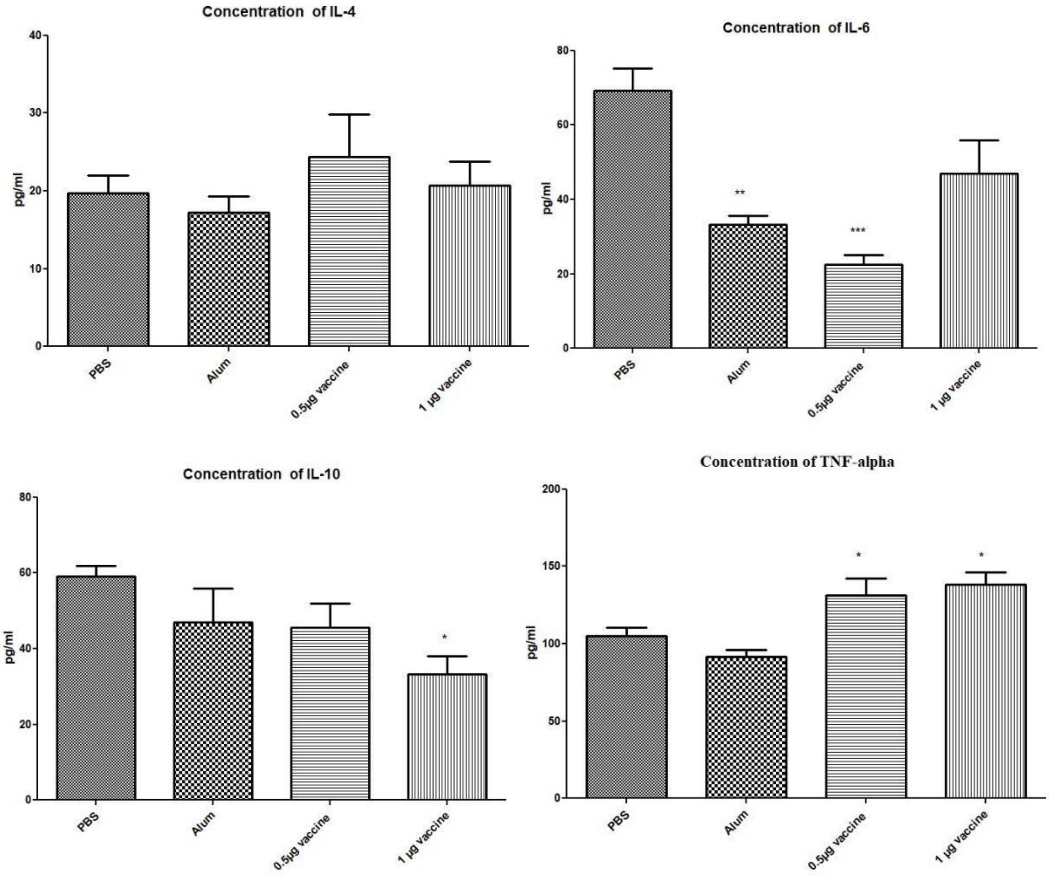


Fig 4: Bar graph showing a change in the proportion of IL4, IL10, IL6, and TNF-α of isolated spleens in examined mice in four examined groups which include PBS, Adjuvant received group(Alum), Vaccine with low dose (0.5 µgr) and Vaccine with high dose (1 µgr) . The asterisks replicate significant differences with* for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

No significant difference was observed in the number of T cells in the vaccine, antigen, and control groups two weeks after immunization. Although two months after immunization, there was a relative difference in the vaccinated group with the control group, mainly observed in both CD4+ ($p< 0.05$) and CD8+ ($p<0.05$) cells, presented in figure 5.

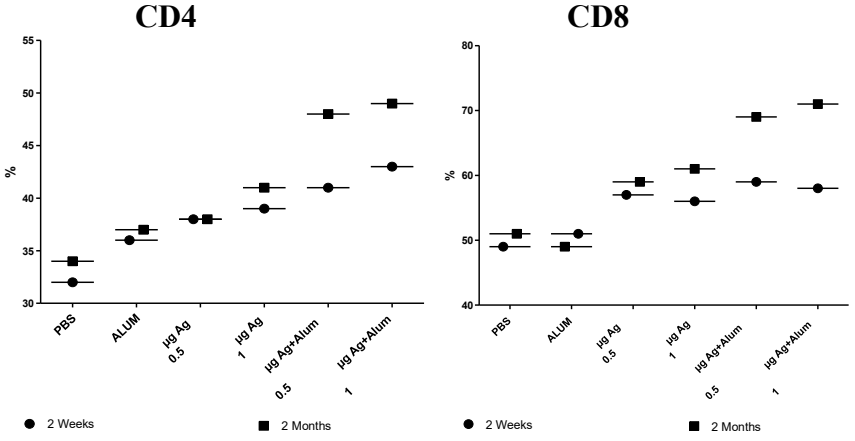


Fig 5: The immune lymphocyte T cells subsets (CD4+, CD8+) in the peripheral blood of Mice. Flow

Cytometric evaluation of CD4+, CD8+ immune cell subsets were assessed 2 weeks and 2 months after the immunization.

Based on the presented results, no significant difference was observed between the two vaccine concentrations in immunized mice in GB concentration, but there was a statistically significant difference between the mice receiving the formulated vaccine and those of receiving antigen ($p < 0.01$). As shown in figure 6, there was also a statistically significant difference between GB level in Ag received mice and PBS groups ($p < 0.01$). There was also a statistically significant difference between vaccinated mice and PBS groups ($p < 0.001$) (Fig 6).

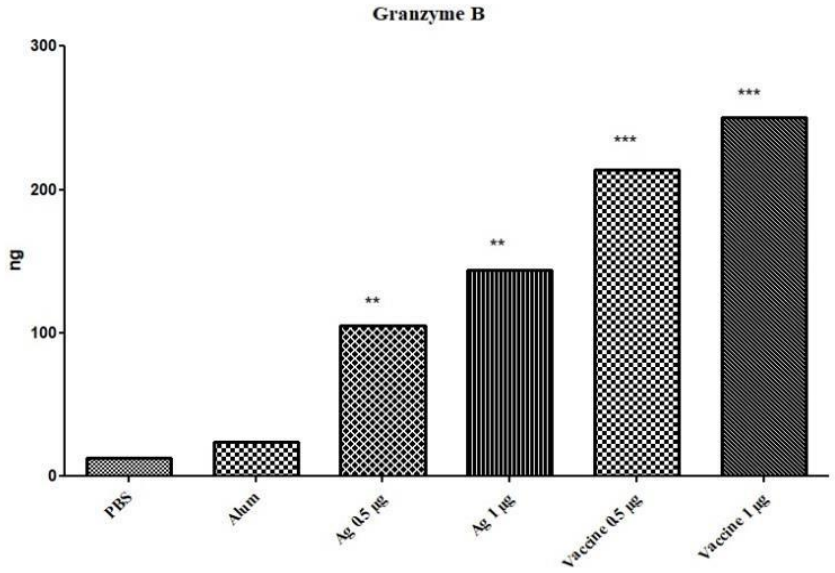


Fig 6: Cellular immunity induction was evaluated by Granzyme B activity in different vaccine preparations. Spleens were isolated from the immunized mice 21 days' post-immunization. The asterisks replicate significant differences with* for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

Based on cVNT results, the inactivated vaccine in both doses was able to induce a neutralizing effect against SARS-CoV-2 up to a dilution of 1:512 and 1:1000. Nevertheless, CPE in cell culture was observed in adjuvant recipient different serum dilutions with Virus 100 TCID50.

3.3 Immunological and Protective States in Rhesus monkeys

Before immunization, ten samples were taken from the end of the nose of the monkeys using sterile swabs for nasal sampling. The samples were tested for RdRP and E genes of SARS-CoV-2 and the CFR610 gene as the housekeeping gene by RT-PCR. All examined samples were negative for Covid-19 genes. Additionally, serum levels of PTH (268.8 ± 2.87 pg/ml) and vitamin D (28.2±0.21ng/ml) were checked for further investigations, which were in the normal range.

The serum levels of SARS-CoV-2 anti-spike IgG antibody titers in vaccinated Rhesus monkeys were monitored every week post-immunization. The results showed that antiSARS-CoV-2 spike IgG quickly significantly increased in the sera of vaccinated animals and vaccinated animals followed challenge in comparison to the PBS and adjuvant ($p < 0.001$) (Fig 7). Additionally, immunogenicity study results demonstrated an excellent humoral response with stable high titers of antibodies induced against SARS-CoV-2 spike protein in the groups vaccinated with two 5-µg doses of the antigen formulated with the adjuvant during the five months following the second dose.

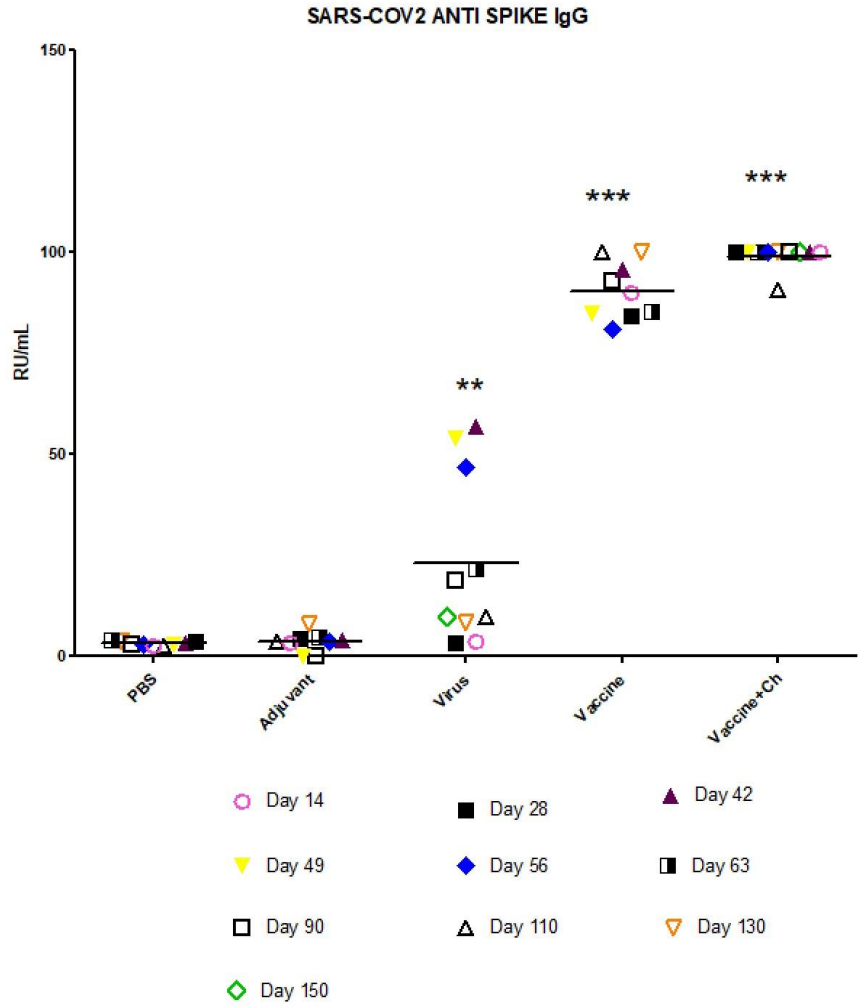


Fig 7: Time course of spike antigen-specific IgG response to COVID-19 Osvid-19 vaccine in rhesus monkeys immunized. ELISAs were performed on serum from subjects, at different time points (X-axis, days). Serum IgG levels are proportional to ELISA optical density values (Y-axis). PBS received group as control, Adjuvant received group, Virus received group (Challenge without vaccination), Vaccine group (two dose vaccination without challenge), and Vaccine+ Ch (two doses vaccination and challenge with virus). Each symbol represents average ELISA data for a single subject at a single time point. The asterisks replicate significant differences with* for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

After the inactivated SARS-CoV-2 vaccine administration, sVNT detected different levels in the immunized groups 14 to 150 days' post-immunization (Fig 8). According to manufacturer instructions values >2.5µg/mL were considered as positive, which in vaccinated groups following two immunization doses indicated positive results and remained at a high level until about five months later. As presented on the graph of the vaccinated groups, neutralizing antibody levels significantly increase after the second dose and challenge ($p<0.001$).

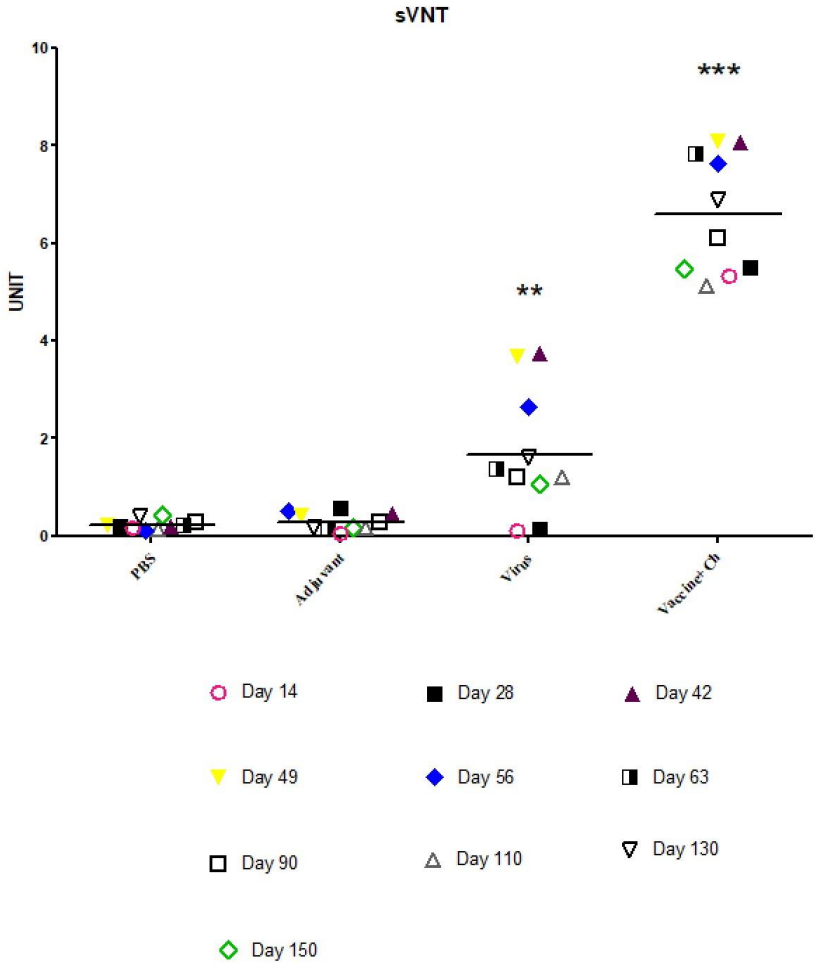


Fig 8: Short- and long-term effects on virus-neutralizing antibody in rhesus monkeys in different groups

(negative: ≤ 0.8, positive: ≥ 2.5, borderline: 0.8-2.5). PBS received group as control, Adjuvant received group, Virus received group (Challenge without vaccination), and Vaccine+ Ch (two doses vaccination and challenge with virus). The asterisks replicate significant differences with* for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

The total T-cell population of monkeys two months after immunization showed that the percentage of total T-cells in the vaccine group (64.2 ±1.0 %) was higher than the control group (36.7 ± 1.9 %), which stimulated cellular immunity and the production of cytotoxic CD8+ lymphocytes. So, no significant alteration was observed in CD4+ and CD8+ cells and total T-cells in weeks after immunity in different groups.

The production of cytokines IL-1β and IL-6, IFN-γ, and TNF-α in the vaccinated group showed a significant increase compared to the control groups, which indicates the ability of the formulated vaccine to induce a wide range of Th1 and Th2 cytokines. Increased

IFN-γ secretion after immunization in both vaccine and adjuvant groups compared with the control group indicates the importance of adjuvant in stimulating cellular immunity. However, two months after immunization, this increase did not show any statistical difference in the adjuvant group compared to the beginning of the study. Contrarily, in the vaccine group showed a high level of IFN-γ until the end of the study; this suggests that the combination of antigen and adjuvant promotes long-term immunity by increasing IFN-γ–secreting T cells, consistent with cellular immunity outcomes. This pattern is also consistent with IL-1β and IL-6 levels. The stimulated production of TNF-α days after immunization with the antigen and adjuvant combined, lasting at least five months later, indicates the superior immunogenicity of the antigen and adjuvant combination compared to the

antigen or adjuvant alone (Fig 9). Additionally, increased IFN-γ secretion after immunization in both the vaccine and adjuvant groups compared to the PBS group indicates the importance of adjuvant in stimulating cellular immunity.

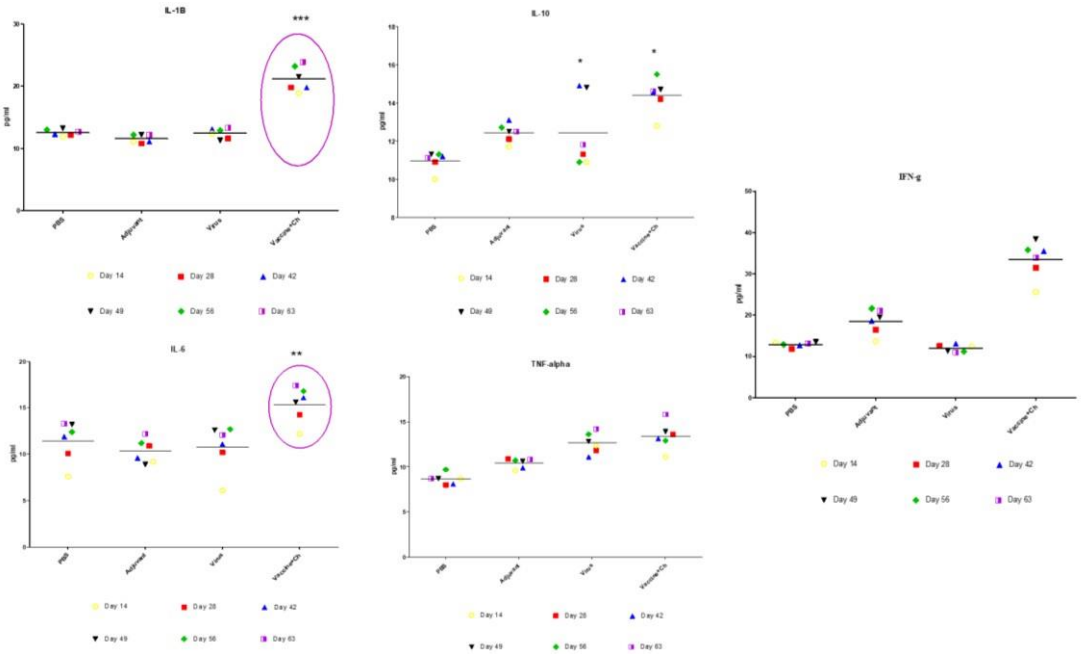


Fig 9: Evaluation of cytokines in different groups in Rhesus during time points of study in different groups which received two doses PBS (Control), two doses Adjuvant, Challenged with virus as natural infectious(Virus), and two doses Vaccine with challenge(Vaccine+Ch). The asterisks replicate significant differences with* for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

Three days after the virus challenge, only one of the three animals in the vaccine groups had detectable subgenomic RNA in the BAL fluid compared to the control group. By day
3, none of the vaccinated animals in the 5 µg dose group had identifiable subgenomic RNA in nasal swab samples. According to the statistical analysis, the peak of subgenomic RNA levels during days 2 to 7 in both vaccinated groups compared to the control group was significantly lower in both BAL fluid samples and evaluated swabs. After seven days, no viral RNA was detected in any of the swab samples prepared from the anus, nose, and trachea, while in the control group, the samples were positive for up to 14 days in tracheal, nasal samples and BAL fluids.

Table 2: The subgenomic RNA detection in nasal(N) , pharynx (P) , and anal(A) swab samples isolated in different days.

Sample No	Three days after challenge			Seven days after challenge			Fourteen days after challenge			Twenty one days after challenge		
	N	P	A	N	P	A	N	P	A	N	P	A
Vaccine + challenge	27	35	35	30	31	-	29	29	-	-	-	-
Vaccine + challenge	27	32	35	32	31	-	-	-	-	-	-	-
Vaccine + challenge	29	29	34	34	-	-	-	28	-	-	-	-
Virus	29	31	33	31	34	37	30	30	35	29	30	-
Virus	29	30	32	30	33	38	30	30	34	29	30	-
Virus	29	31	32	31	34	37	30	30	35	29	29	-

The intramuscular injection of the vaccine was clinically well tolerated in macaques with no unscheduled deaths; no local or systemic adverse clinical signs and no effect on body weight or food

intake were reported in the vaccine group. The body temperature peaked at 38.9-40.5°C between 12 to 48 hours following the start of immunization. Additionally, fever at slightly milder temperatures was observed in Rhesus monkeys following viral challenge, peaking at 40.6°C.
Histopathologic evaluation and the lung CT scan of the immunized monkeys (two FHD with a 21-day interval) seven days following the viral challenge failed to detect any abnormality in the lung parenchyma or thoracic structures. Additionally, no radiographic evidence of VAERD disease was observed (Fig 10).

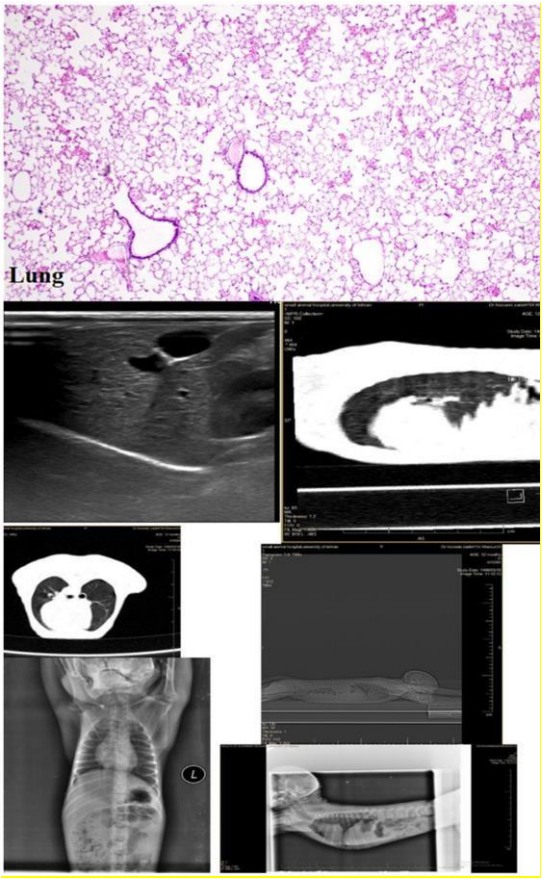


Fig 10: CT-Scan, Chest X-Ray, and histopathology of isolated organs to confirm the virus effect on lung tissue in two doses vaccine received groups 7 days followed challenge. As shown in figures, the alveoli's of isolated lungs followed challenge were intact without vaccine associated enhancement respiratory disease symptom in CT-Scan and histopathological evaluations.

In the control group, however, histopathologic evaluation seven days following the virus challenge detected a well-demarcated 9*4*6 mm air-filled cyst-like structure peripherally situated at the dorsal aspect of the right cranial lung lobe. The legion was consistent with pulmonary bleb formation, and further histopathological evaluation revealing inflammatory cell infiltrates in inter alveolar septum, the hemorrhage, the mucus secretion, and the thickening of the alveolar wall were observed in unvaccinated group followed challenging that confirmed interstitial pneumonia (Fig 11). The preliminary results of immunizing the Rhesus macaques as an appropriate animal model using two doses of 5 µg formulated purified antigen with a 21-day interval showed increased levels of neutralizing antibodies that remained at a high level for at least 150 days' postimmunization. Following active immunization with the vaccine, the Rhesus monkeys were protected from the SARS-CoV-2 challenge, and no adverse reactions were observed in the lung CT scan and histopathological evaluation of the lungs seven days following the challenge.

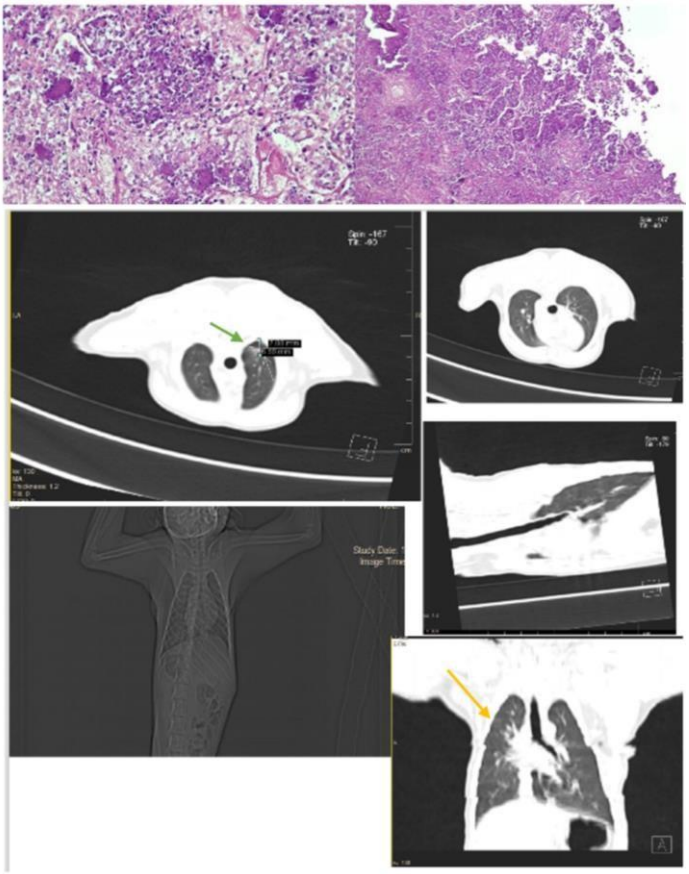


Fig 11: CT-Scan, Chest X-Ray and histopathology of isolated organs to confirm the virus effect on lung tissue in control groups 7 days followed challenge. In the control group, following the virus challenge detected a well-demarcated 9*4*6 mm air-filled cyst-like structure peripherally situated at the dorsal aspect of the right cranial lung lobe (green arrow). The inflammatory cell infiltration in inter alveolar septum, the hemorrhage, the mucus secretion, and the thickening of the alveolar wall was observed in unvaccinated group followed challenging in lung CT-Scan (yellow arrow) which confirmed by histopathological evaluations.

3.4 Single-dose and repeated dose toxicity study in Rats and Monkeys

The intramuscular injection of vaccine with maximum tolerable selected dose was clinically well tolerated in animals with no unscheduled deaths; also no irreversible effects on body weight or water and food intake were reported in the four stages studied, including before immunization, 7 and 14 days after the first injection, and at the end of the study. Treatment with formulated vaccine had a transient effect on temperature in the examined animals following administration.

Reversed decreased the weight gaining in the one case of male and female rats were recorded seven days following the first dose of vaccine inoculation and minimal erythema observation in injection sites due to 48 h followed immunization were consistent with an inflammatory response to the adjuvant and/or vaccine administration. Based on histopathological evaluation of vital organs in rats with 10FHD, no specific changes were observed in liver, kidney, heart, and lung.

The bronchioles were a normal appearance, and the hemorrhage, inflammatory cell infiltrations, and the alveoli's wall thickness in the evaluated lungs were not observed. The kidney histopathological examination showed a normal appearance in the glomerulus epithelial cells. After vaccine injection, the infiltration rate of lymphocytes was in a normal range, also tissue density and hyperemia were not observed. The adjacent cortex epithelial cells had not any significant differences in all the proximal and distal tubules.

Based on figure 12 left, hepatic parenchyma with hepatocytes and sinusoids ('lobular' region), which were interspersed with central venules and portal tracts were normal. The hepatic cell patterns and infiltration were in normal condition (Fig 12 left).
No abnormal finding was observed in the Rhesus monkeys injected with a vaccine concentration three times the FHD in the single-dose toxicity experiment. Compared with the control group, no significant abnormalities in body weight, clinical symptoms, and necropsy and histopathological analysis were found in the vaccine group. These results suggested that the maximum tolerable dose of vaccine was greater than 5 μg when the vaccine was injected intramuscularly. Histopathological findings demonstrated minimal infiltration of inflammatory cells in monkeys in the high-dose group in the injection site. However, these changes were also significantly improved at the end of the recovery period (Fig 12 right).

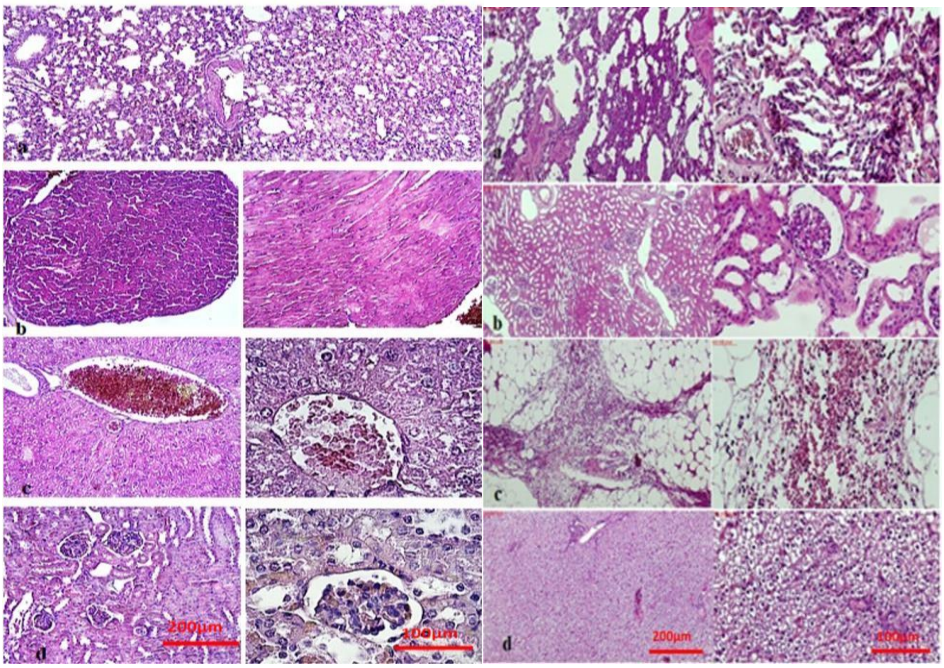


Fig 12: Left No specific changes were observed in different Rats groups; lung(a), heart(b), liver (c)and kidney (d) showed no signs of toxicity in the group receiving 10-fold dose of vaccine. Scale bars represent 200 and 100 μm.

Right The histopathological evaluations in the vital organs (lung (a), kidney (b), injection site(c) and liver (d)) isolated from Monkeys after the vaccine administration. Hematoxylin and eosin staining was performed for slides containing tissues isolated from the organ. Scale bars represent 200 and 100 μm.

4 DISCUSSION

An efficacious vaccine with a low or medium dose is could be considered to be an important economic strategy to overcome viral pandemics, especially when there are no approved drugs to combat emerging viruses such as SARS-CoV-2. In addition to potency and efficacy, the safety of a vaccine is a critical factor for public acceptance (25)(26).
Several approaches based on conventional vaccine technologies (inactivated, live attenuated, subunit) as well as next generation vaccine platforms (mRNA/DNA, viral vectored) have been investigated and approved to develop SARS-CoV-2 vaccine candidates (27)(28).
Here, we report the two dose vaccination regimen of inactivated SARS-CoV-2 vaccine candidates induce a strong immune response and protection of monkeys from the infection of SARS-CoV-2. A beneficial feature of inactivated vaccines compared to the other vaccine platforms beside low cost price and side effects is that vaccine-induced immunogenicity occurs against whole viral proteins

including spike, envelop, nucleoprotein, and other proteins. Several Vero cell-based vaccines have been certified and utilized to produce licensed vaccines against different viral diseases including poliovirus, rotavirus, and influenza virus(29) . It is well known that Vero-E6 cells produce a high viral titer of SARS-CoV-2 compared to other cell lines, and this characteristic is attractive for mass production(30). Subsequently, in the current study, inactivation of the propagated virus was performed using formaldehyde because it has been approved as an inactivating agent and widely used for the inactivation of several types of viruses (31).

Based on previous studies by Kalnin et al. (32) and Gao et al. (33), neutralizing antibodies (NAbs) and specific IgG responses are observed from the second week post immunization in vaccinated groups. Similar to the previous preclinical studies of inactivated vaccine candidates for SARS-CoV-2 have shown specific NAbs in animal models vaccinated with two-dose immunizations(34).

Consistent with Prompetchara report(35), in the present research, SARS-CoV-2 inactivated vaccine candidate stimulated a mixed response of IgG2a and IgG1. This implies that this SARS-CoV-2 vaccine candidate could induce humoral immune responses (Th1/Th2) and therefore could reduce concerns about vaccine-associated enhanced respiratory diseases (VAERD) induced by Th2-bias vaccine modality(36).

In this study, it was found that vaccine candidate could produce respectable cell immune response by assessment the *Granzyme B*, this finding is consistent with the results of other studies by Kar T et al(37) and Zhang BZ et al.(38). In this study, the cytokine profile analysis results showed that TNF-α, IL-6, IL-10, IL-4, and *Granzyme B* were significantly higher in the formulated vaccine groups 21 days followed postimmunization than in the other groups. Yang et al. have recently reported that *Granzyme B* as a key cytotoxic effector molecule is released by effector $CD8^+$ T cells(39) . The effectiveness of the inactivated vaccine in the production of virus-neutralizing antibodies in serum samples and activation of *Granzyme B* showed that the candidate vaccine could induce high humoral and cellular immunity in candidate of vaccine.

One of main concerns about the SARS-CoV-2 candidate vaccine is whether it can cause antibody dependent enhancement and might progress severe pathological changes in the lung when vaccine received individuals after being exposed to wild-type virus. For the evaluation of protective effect of vaccine, challenge in monkeys carried out by proved wild type virus which two dose vaccine with 5 μg inactivated and purified antigen revealed the high safety and protective profile. No mortality was reported in the experimental animals throughout the experiment. A slight increase in body temperature was observed, fever at slightly milder temperatures was also observed in rhesus monkeys following viral challenge(39).

Based on the results, the production of cytokines *IL-1β*, *IL-6*, *IL-10, IFN-γ*, and *TNF-α* in the vaccinated rhesus group showed a significant increase compared to the control groups, which indicates the ability of the formulated vaccine to induce a wide range of cytokines Th1 and Th2. Increased IFN-γ secretion after immunization in both vaccine and adjuvant groups compared with the PBS group indicates the importance of adjuvant in stimulating cellular immunity in the face of antigen and adjuvant. However, 5 months after immunization, this increase does not show a statistically different difference in the adjuvant group compared to the beginning of the study, but in the vaccine group, it shows a high level until the end of the study. This suggested that the combination of antigen with adjuvant promotes longer-term immunity by increasing *IFN* gamma-secreting T cells, which is consistent with cellular immunity outcomes(40).This pattern was consistent with *IL-1* beta and *IL-6* levels. An elevated level of *IL-6* is extremely consistent in COVID-19 disease which followed challenge with SARS-Cov2 virus, an elevation in IL-*6* in vaccinated groups was observed.

Antigen with adjuvant by stimulating the production of *TNF-α* in the days after immunization until about two months later indicates the appropriate effects of combining antigen with adjuvant compared to empty adjuvant or immunogenicity following exposure to antigen. The *IFN-γ* is known to boost and stimulate cytokines in the immune system which several innate immune cells, such as macrophages, and acquired immunity, such as T lymphocytes increase this cytokine activates. The source of this *IFN-γ* could be macrophages, CD8 + and Th1, and even type 2 lung pneumocytes. The

main function of *IFN-γ* is the complete activation of cellular and innate immunity. Macrophage cells and other immune cells activated by *IFN-γ* perform their functions well, which includes increased phagocytosis and increased lethality of CD8 lymphocytes(40)(34). Increased *IFN-γ* secretion after immunization in both vaccine and adjuvant groups compared with the control group indicates the importance of adjuvant in stimulating cellular immunity in the face of antigen and adjuvant.

The results of serum cytokines production in the immunized monkeys propose that the vaccination preferentially induces a Th1responses. The results also indicate that the inactivated vaccine candidate could increase the cellular immune response. So that, the production of INF-γ can increases the activity of natural killer (NK) cells and inhibits replication of the virus.

Antigen with adjuvant by stimulating the production of *TNF-α* in the days after immunization until about two months later indicates the appropriate effects of combining antigen with adjuvant compared to empty adjuvant or immunogenicity following exposure to antigen. The production of *TNF-α* appears to be effective in predicting the body's initial response to vaccine and may be a predictor of a humoral response to vaccination(41)(42).

Based on the literature, it has been shown that *TNF-α* has regulatory role to increase APC function, dendritic cell maturation and IL-12 production. In addition, *in vivo* and *in vitro* studies have shown that TNF-α, with the help of IL-12, is synergistic in the production of IFN-γ and the conversion of the Th response to Th1(40)(3).

Prescribed dose and immune schedule study are very important in related animal models for initial clinical trials. The number of doses administered to the test animals based on guidelines should be equal to the number of doses planned to be administered in humans. So based on similar platforms, in animal study two usual mentioned doses with enough efficacy and without toxicology effects were chosen for the nonclinical studies. In this study, the preliminary results in Rhesus as an appropriate animal model showed that the NtAb titers reached the peak post-priming and boosting in an interval of 21 days for at least 150 days using 5 µg formulated purified Ag. Following active immunization of the vaccine, the rhesus monkeys were protected from the challenging SARS-CoV-2, and no adverse reactions were observed in lung CT-Scan and histopathological evaluation of lungs seven days followed challenge.

Toxicity study was also performed in SD rats and Rhesus monkeys and the results would support immunization of adults at a high dose and boosting two or three times if wanted.

The single dose vaccine with maximum tolerable dose injection in Rats and Rhesus's showed that vaccine was tolerable in animal. Local reactions with enhancement in body temperature in some immunized monkeys and rats were observed in used high dose which can be concluded that the vaccine is tolerated by experimental animals and can be injected intramuscularly without any considerable adverse effects with acceptable humoral immunity manifestations (40).

5 CONCLUSSION

This study demonstrates that a two-dose vaccination regimen using 5-µg dose of the vaccine candidate with aluminum hydroxide as adjuvant induces a significant immune response and provides effective protection in rhesus monkeys challenged with SARSCoV-2 due to at least five months. Hence, we were able to develop a whole virus inactivated SARS-CoV-2 vaccine that based on obtained results in preclinical studies is tolerable, safe, and immunogenic in preclinical trials which can entrance in human clinical trial to supporting the booster doses. However, it will be essential to test produced vaccine candidate for their immunogenicity, safety and efficacy in human.

Abbreviations

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; cVNT, conventional virus neutralization test; COVID-19, coronavirus disease-2019; WHO, world health organization; ACE2, angiotensin-converting enzyme 2 ; RNA, ribonucleic acid ; BPL, βpropiolactone; RT-PCR, real-time polymerase chain reaction ; CPE, cytopathic effects ; SEM , scanning electron microscope ; NGS, next-generation sequencing ; PVDF, polyvinylidene fluoride ; TCID50, median tissue culture

infectious dose ; DMEM , dulbecco's modified eagle medium ; MOI, multiplicity of infection ; TFF, tangential flow ultrafiltration; PBS, phosphate-buffered saline ; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis ; ABSL3, animal biosafety level 3 ; SD, Sprague dawley ; Ig, immunoglobulin ;sVNT , surrogate virus neutralizing test; CBC , complete blood count; BALF , bronchoalveolar lavage fluid; CT , computed tomography; TMB, tetramethylbenzidine ; TNF-α, tumor necrosis factor alpha; IFN, Interferon ; RPMI 1640, Roswell Park Memorial Institute ; FBS , Fetal bovine serum ; CRP, C-reactive protein ; ; PTH, parathyroid hormone ; PFU, plaque-forming unit; PE , phycoerythrin ; HRP, horseradish peroxidase; SD, standard deviation ; NTAb, neutralizing antibodies test ; IL, Interleukin; FHD , full human dose; VAERD, vaccine-associated enhanced respiratory disease; TEM, transmission electron microscope; CFSE, carboxyfluorescein succinimidyl ester.

Declarations Ethics approval and consent to participate

Animals were treated according to national and international ethical guidelines. The study was approved by the Ethics Committee of NRC (Ethics code No: IR.BMSU.REC.1399.373), and all procedures and experiments were performed according to the approved protocols.

Consent for publication

Not applicable.

Availability of data and materials

The data used to support the findings of this study are available either online or from the corresponding author upon request

Competing interests

The authors declare that they have no competing interests.

Authors contribution

Conceptualization: ZE, SDMN, RD, AAP, Methodology: ZE, SDMN, RD, AAP, MJM, SMH, MG, HAH, AR, Formal analysis: ZE, MS, MGH,MS, HK, Investigation: ZE, SDMN, RD, AAP, HK, Writing – original draft: ZE, SDMN, Writing – review and editing : All authors, Visualization: AR, MS , ZE, Funding acquisition: HK and RD. All authors read and approved the final manuscript.

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Conflict of interest

None

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