Preliminary Step towards COVID-19 Inactivated Vaccine Development in Egypt.

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Archives of Clinical Trials

Original Article

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- Received Date: 13 Feb 2021
- Accepted Date: 23 Feb 2021
- Publication Date: 28 Feb 2021

Abstract

The current worldwide COVID-19 pandemic is causing severe human health problems, with high mortality rates and huge economic burdens requiring the urgent development of a safe and effective vaccine. Here, preclinical evaluation of an inactivated SARS-CoV-2 vaccine candidate (EgySerVac-20) is reported. Oropharyngeal swabs and nasopharyngeal aspirates obtained from Egyptian patients with laboratory-confirmed SARS-CoV-2 infection were isolated using Vero cells and were then genetically characterized. Vaccine inactivation was performed using diluted formaldehyde, followed by safety testing for the inactivated vaccine. To determine the high humoral immune responses against SARS-COV-2 infection, the safety and capacity of the vaccine prepared with alum adjuvant were tested. The immunogenicity and efficacy of the vaccine candidate was tested in vitro by a neutralization assay and in vivo using mouse models. Our results revealed a cytopathic effect which was observed 48 hours post infection and the viral particles were identified by rRT-PCR as SARS-CoV-2. Propagation of the isolated virus in ten serial passages on the Vero cells yielded a virus titer 7.5 log10 TCID50/ml. Complete inactivation of SARS-CoV-2 was observed at 37°C in 24 hours post treatment by diluted formaldehyde. Inactivated SARS-CoV-2 infected fluid safety was determined by absence of cytopathic effect by repeated passage in Vero cell line, indicating loss of virus infectivity. Virus inactivated by diluted formaldehyde showed no deaths or clinical symptoms in mice groups post intraperitoneal inoculation (0.5ml/mouse). EgySerVac-20 inactivated vaccine has safely induced high levels of neutralizing antibodies titers in mice, where 0.1 ml immunization dose showed protective efficacy against SARS-CoV-2 challenge in mice. This finding will support the future preclinical and clinical trials evaluation for our SARS-CoV-2 vaccine candidate in primates and human, respectively.

Introduction

The recently emerged severe acute respiratory syndrome coronavirus-2, commonly known as SARS-CoV-2, is an enveloped RNA virus with a single-strand positive- sense genome and is considered to be highly pathogenic, causing severe human illness with a reported high mortality rate [1]. COVID-19 pandemic has caused an enormous economic burden worldwide, more than 80 million cases with almost 2 million deaths have been reported globally (https://coronavirus.jhu.edu), which requires a collaborative effort to develop effective interventions to control its spread. Few treatment modalities such as Hydroxychloroquine and Remdesivir have been used to contain the adverse impacts of the viral attack, however, varied results have been detected and their effectiveness is still under investigation [2]. On the other hand, trials for effective SARS-CoV-2 vaccine have been reported so far to reduce disease severity and prevent respiratory infections [3]. More than 100 ongoing COVID-19 vaccines preclinical and clinical trials including live- attenuated virus, mRNA vaccines, viral vectors, inactivated virus were recently reported and documented in the clinicaltrials.gov database [3,4]. Inactivated vaccines have been reported to be safe and effective for the prevention of Influenza viruses and other infectious diseases [5,6]. The efficacy of two inactivated vaccines against SARS-CoV-2 developed by Sinovac Biotech and Sinopharm in China were

Citation: Saad MA, Saleh AA, Ryan I, et al. Preliminary Step towards COVID-19 Inactivated Vaccine Development in Egypt .Arch Clin Trials. 2021; 1(1):1-6.

assessed using mice, rats and other animal models, in which it is currently under phase 3 study [7,8].

Successful experimental steps for isolating and characterizing SARS-CoV-2 from patients are crucial for vaccine development. To develop an inactivated SARS- CoV-2 vaccine candidate; isolation of the virus from Egyptian patients was performed, followed by propagation, genetic characterization and virus inactivation. An in vitro neutralization assay was performed in Vero cell line, and the efficacy and immunogenicity were tested in vivo in mouse models to evaluate the inactivated vaccine candidate for further preclinical and clinical trial approaches.

Materials and methods

Ethics Statement

Informed consent forms required for samples collection were approved by the ethics institutional review board (IRB) of the Egypt Centre for Research and regenerative medicine (Approval number 2/4-2020)

All experiments involving animals were conducted according to the ethical policies and procedures approved by the ethics committee of the Egypt Centre for Research and Regenerative medicine (Approval number CU II F 20 20)

Sample collection

Nasopharyngeal and oropharyngeal swabs were collected in 5 ml viral transport media from six COVID-19 patients with age < 45 years that were positively diagnosed using real time reverse transcriptase PCR (rRT-PCR). Swabs were transferred to Egypt Center for Research and Regenerative Medicine (ECRRM) and stored at 4oC for immediate virus isolation. All experiments with positive samples and infected cells were handled in ECRRM Biosafety Level 3 laboratory. All the recommendations, conditions and requirements of the WHO were maintained [9].

Virus isolation, Identification and Propagation

The epithelial cells of the African green monkey kidney (ATCC No. CCL-81) were used as our Vero cell line, for virus isolation. Theses cell lines were supplied by Veterinary Serum Vaccine Research Institute (VSVRI). The confluent monolayer of Vero cells was infected with 1 ml of suspected samples for about 45-60 minutes. DMEM, supplemented with 2% fetal bovine serum, was used as maintenance medium and the cells were incubated at 37oC. The cells were examined twice daily for cytopathic effects (CPE) formed by the inoculated virus. Three blind passages followed by seven successive serial passages were obtained [10]. Supernatants were collected for virus quantification by rRT-PCR. CPE, gene detection, and electron microscope methods were used to confirm virus replication.

Real Time RT-PCR detection

Total RNA was extracted using Viral RNA Extraction kit (Qiagen, CA) following manufacturer's instructions. NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), was used for checking the concentration and purity of the extracted RNA. One step rRT-PCR was performed using TaqPath™ COVID-19 CE-IVD RT-PCR Combo Kit (Thermofischer Scientific, USA) following manufacturer's instructions. The reaction was incubated in rRT-PCR ABI 7500 (Thermo Fisher Scientific, USA) at 50oC for 15 minutes for the reverse transcriptase step, 95oC for 10 minutes, followed by 45 cycles of 95oC for 15 seconds and 60oC for 30 seconds. Three primers/probes were used targeting ORF1ab, Nucleocapsid (N) and Spike (S) regions. Primers/probes specific for bacteriophage MS2 were used as a positive control. The cycle threshold value below 33 was considered to be positive. Results were considered valid when two of the three targeted genes and the MS2 showed positive readouts.

Whole Genome sequencing

Qubit RNA High Sensitivity Kit (Invitrogen, USA), was used to quantify the extracted RNAs from original (P1 stock) and tenth passage (P10 stock). Libraries were prepared using Ion AmpliSeq SARS-CoV-2 Kit (Thermo Scientific, USA) following the manufacturer's protocol. Ion-PI-Hi-Q Sequencing 200 Kit (ThermoScientific, USA) was used for the clonal amplification of the libraries. Purified libraries were qualified and quantified by Agilent Bioanalyzer and Qubit 4 Fluorometer (Thermo Scientific, USA). Sequencing of the libraries was performed on the Ionproton NGS platform (Thermo Scientific, USA). The Ion Torrent package (v.5.12) was employed for the assembly of virus sequences, and the genome mapping was performed using tmap program (v.512) against submitted SARS-CoV-2 whole genome sequences. Sequences are available at GenBank with accession number MT611450 (P1 stock) and MW010029 (P10 stock).

Transmission electron microscopy

Infected Vero cells were scraped from the flask, pelleted and rinsed with 0.1M phosphate buffer saline (PBS) (Sigma Aldrich, Germany). First, the Vero cell monolayers were fixed in 2% formaldehyde in PBS with the collected cell supernatants for 1 hour. Second, an ultracentrifugation (25,000 rpm, 1 hour) was performed, then the samples were loaded on a carbon coating grid stained with 2% phosphotungestic acid for 30 seconds before being examined. Bar:100 nm.

Virus titration

The infectious dose ($TCID_{50}$) per ml was determined in Vero cell monolayers on 24 and 96-well plates. The CPE of the cells was tested on the serial dilutions of virus samples, incubated at 37oC for 4 days. $TCID_{50}$ assay was performed according to Ksiazek et al. [11]. The infectious titer was calculated using an in-house method adapted by Spearman and Kärber and expressed in $TCID_{50}$ units [12].

Virus inactivation and vaccine production

For vaccine preparation, the virus was propagated in Vero cells with a dilution of 1:100 (v:v) of the SARS-CoV-2 virus in serum-free medium. The cells were incubated at 370C for 72 hours. On day three post infection, supernatants with visible CPE were collected for cell harvesting. The infectious titer of the virus was determined using a 50% cell culture infectious dose as described above. At a lower centrifugation speed of 1000 rpm, cell harvest clarification and vaccine purification were done. SARS-CoV-2 was chemically inactivated with 37% diluted formaldehyde (Sigma-Aldrich, Germany) at 37 °C for 24 hours [13,14]. Inactivation of virus infectivity following treatment was confirmed by examining Vero cell infectivity [15]. The final formulations were prepared shortly before vaccination by addition of alum adjuvant (2% Alhydrogel; Accurate Chemical & Scientific Corp.) in a ratio of 1: 9 to

inactivated virus and named EgySerVac-20 vaccine.

Safety test

Safety assessment was performed according to Animal and Plant Health Inspection Service (USDA) and Institutional Animal Care and Use Committee (IACUC). A total of two groups (8 mice each) of 3 to 4-week-old Swiss albino mice were provided by VSVRI, one group was injected intraperitoneally with 0.5 ml of the inactivated vaccine that contain 5 mg of Al(OH)3 which represented about 25 - 8 human doses that recommended (0.2 - 0.8mg). The second group was injected with 0.5 ml phosphate buffered saline (PBS) as control group. Clinical or other adverse effects were observed [16].

Vaccine immunogenicity analysis

Three groups of 6-week-old/ Swiss albino mice (n=10) were immunized with the trail vaccine on day 0 at two doses. The first and second groups (Gr.1 & Gr.2) immunized with 0.1ml and 0.2ml, respectively via the intramuscular route, and re vaccinated with the same doses on day 7 [17]. The control group (Gr.3) was injected with physiological saline. Blood was obtained from the animals' tail veins, and an antibody neutralizing assay was performed to analyze the immunogenicity of the vaccine.

SARS-CoV-2 challenge test

Four weeks post initial dose, both vaccinated and control groups were anesthetized and challenged intranasally with 60 µl SARS-CoV-2 virus (106TCID50) according to animal care guidelines [17]. Seven mice from each group were anesthetized and sacrificed post challenge, four mice at 3 days and three mice at 7 days post virus challenge, the rest 3 mice were considered as positive control to any adverse or abnormal signs till end of study. The lungs were removed. RNA from the lung tissue was isolated using Qiagen's RNeasy min kit following the manufacturer's instruction. The extracted RNA was stored at -80°C for further rRT-PCR analysis as mentioned above (and virus titration in Vero cell culture as described by See et al. [18].

Collection of blood samples

Blood was collected from each mouse from the retro-orbital area at 0, 7 and 28 days post the first dose and 3, 7 days post challenge. Blood samples were allowed to clot for 30 minutes at room temperature then centrifuged to get rid of any cell debris, and the resulting sera were stored at -80 oC.

Histopathological analysis

Lung tissues from both mice groups (control and challenged) were fixed in 10% buffered formaldehyde and embedded in paraffin wax. Hematoxylin and eosin (H&E) staining was performed to observe the histopathological changes resulting from the isolated SARS-CoV-2 virus infection under the microscope [19].

Determination of neutralizing antibody titers

Heat-inactivation for serum samples was performed by incubation at 56°C for 30 minutes. Equal volumes of two-fold serial dilutions of serum samples and viral solution containing 100 $TCID_{50}$ of SARS-CoV-2 were mixed. The serum-virus mixture was incubated at 37°C for 1 hour in a humidified atmosphere with 5% CO_2. Post incubation, 100 µl from each dilution was added to duplicate wells on a plate with a semi-confluent monolayer of Vero cells. The plates were incubated for 5 days at 37°C in a CO incubator, before the cultures were inspected under a light microscope for the presence of CPE. The inverse of the last serum dilution that inhibited virus-induced CPE was employed to express the neutralizing antibody titers.

Results

Virus isolation and propagation

Vero cells were inoculated with COVID-19 samples collected from patients; the cells were then checked for CPE every day. At the first two passages, high CPE was observed in the infected cells as compared to the controls at 96 – 120 hours post inoculation. However, at the third passage, CPE developed in the Vero cell within 72 hours of inoculation. Firstly, it appears as rounded cell formation following 30 to 40 hours post infection and distinct CPE in cells monolayer infected by SARS-CoV-2 following 48 hours of infection, with plaque formation in cell monolayer. Nuclei aggregates from a few cells were seen in the monolayer (Figures 1 a & 1b). During the terminal stage of CPE, maximum degeneration of cells for the whole monolayer was observed with large gaps throughout the monolayer with areas of dead cells that fused to form plaques. Plaques were formed during 60 to 72 hours following infection. Marked detachment of cells from surface monolayer and cellular degeneration were recorded 72 - 96 hours post inoculation (Figure 1c). Control non infected monolayers did not show any changes throughout the observation (Figure 1d). For virus propagation, ten consecutive passages were given and virus replication was confirmed using rRT- PCR with a Ct value of range 28-30, Virion particles belonging to the Coronaviridae Family were observed by electron microscopy. Oval, spiky particles with a 100 nm diameter were viewed by the electron microscopy using negative staining technique (Figure 2a).

Determination of Virus Titer

Titration of the virus isolate revealed gradual increase in the virus titer through the successive passages (Table 1). The virus titer was 5 log^{10} $TCID_{50}$ at the 4th passage and reached 6.5 log^{10} $TCID_{50}$ by the 8th passage. At the final passage, the virus titer reached 7.5 log^{10} $TCID_{50}$.

Whole Genome sequencing

Results of the WGS of the virus showed more than 99.5% nucleotide similarity to USA/VA-CDC-6377/2020 strain (MT325612.1) and USA/FL-BPHL-0259/2020

strain (MT704077.1) and 100 % nucleotide similarity with the other SARS-Cov-2 strains submitted at GenBank database. For genetic stability evaluation, P1 stock and P10 were sequenced and the results showed that their sequence were 99.98% identity with no amino acid modification.

SARS-COV- 2 inactivation and mouse safety test

The P9 stock in Vero cells was used for virus inactivation process. Formaldehyde inactivation was confirmed by two passages into Vero cells. No CPE was observed in the inactivated virus infected cell monolayers after two passages. Quantitative RT-PCR performed at different time points confirmed the absence of amplification of virus genomes. The titer of the infected fluid was 107.5 $TCID_{50}$ / ml before the inactivation process and the total protein content of the inactivated virus as determined by the Lowry method was 341µg/ml.

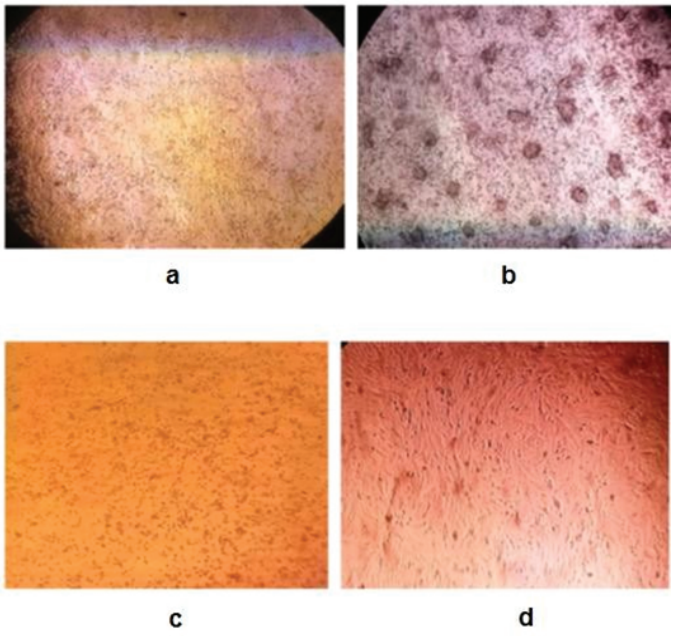

Figure 1. **Virus isolation on Vero cell.** (a) Vero cells monolayer infected by SARS-CoV-2 following 48 hours of infection, with rounded cell formation following 30 to 40 hours post infection. (b) Aggregated nuclei of few Vero cells. (c) Plaque formation in cell monolayer with marked detachment of cells following 60 to 72 hours post infection. (d) Control non infected Vero monolayer X100.

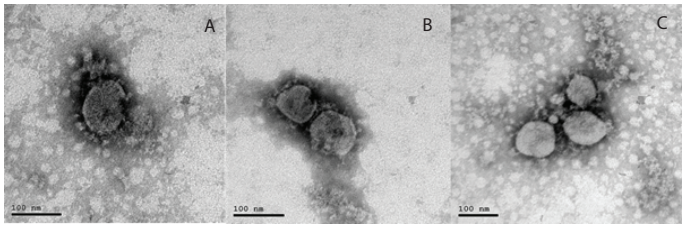

Figure 2. Electron microscope analysis. (a) Electron micrograph of Virion Particle belonging to the Coronaviridae Family. (b&c) Electron micrograph (187,000-fold magnification) of purified inactivated SARS-CoV-2 candidate vaccine after staining with uranyl acetate. Spike protein S appears on viral surface.

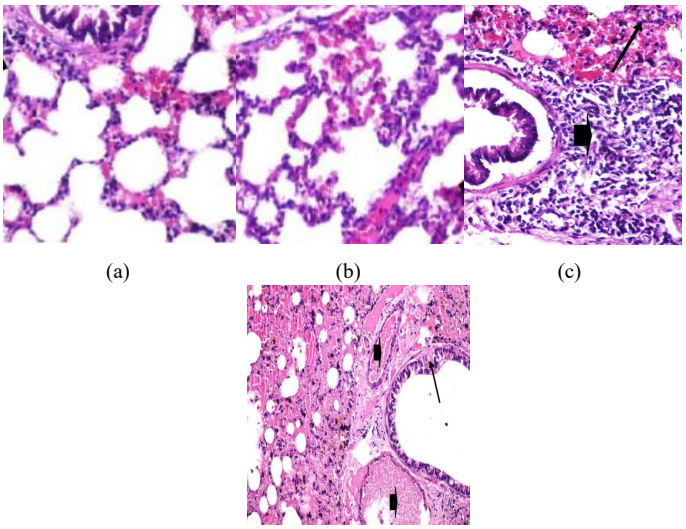

Figure 3. Histopathological evidence of protective efficacy post challenge. (a & b) vaccinated mice 3 &7 days post SARS-COV-2 challenge showing normal lung tissues with scattered inflammatory cells. (c& d) Unvaccinated mice lung at 3&7 days post challenge test showing diffuse thickening in the interstitial tissue, congestion peri-alveolar blood capillaries and lymphocytic cells infiltrations. (H&EX200)

An electron micrograph of the purified inactivated virus confirmed that virus particles demonstrated well-defined surface spikes (Figures 2b & 2c). No mortality or morbidity was observed in mice inoculated intraperitoneally with high doses of the vaccine throughout the experiment.

Immunogenicity in mice

A single dose (Gr.1) of the vaccine was immunogenic as a detectable titer of serum neutralizing antibody response was observed in mice that received one dose (0.1ml) containing 106 TCID50 and equivalent of 34 µg of total protein. A second dose of vaccine induced a boost in neutralizing antibody titers and the antibody response in mice (Gr.2) that received double dose (0.2ml) was greater than that seen in mice that received one dose at 7- and 14-days post vaccination (Table 2). All of the vaccinated animals had higher serum neutralizing antibody titers than non-vaccinated mice in group 3 that were challenged with 106 TCID50 of SARS- CoV-2.

Table 1. Titer of isolated SARS-COV2 in Vero cell culture

Passage No.	1	2	3	4	5	6	7	8	9	10
Virus titer ($Log^{10}TCID_{50}$)	-	-	-	5	5	5.5	6	6.5	7.5	7.5

Table2. Mean neutralizing-antibody responses to the EgySerVac-20 inactivated vaccine

	Neutralization titer					
Groups	0 d	7d*	14d	28d**	3d /c	7d/c
1	<1:10	1:60*	1:213	1:640**	1:960	1:1280
2	<1:10	1:80*	1:320	1:640**	1:1280	1:1280
3	<1:10	<1:10	<1:10	<1:10**	1:10	1:20

Gr.1: vaccinated group with 0.1 ml of inactivated vaccine; Gr.2: vaccinated group with 0.2 ml of inactivated vaccine; Gr.3: unvaccinated challenged group * Mice were reimmunized with booster dose; **Mice were challenged (c) with 106 50% tissue culture infectious doses ($TCID_{50}$) SARS- CoV2 intranasally.

Table 3. Virus replication upon challenge

		Lungs		Vero cell cultures	
Mice groups	Days post challenge	No. infected/ No. tested	Viral RNA	No. infected / No. tested	Mean virus titer*
unvaccinated	3	4/4	+VE	4/4	2.5
	7	3/3	+VE	3/3	5.4
Vaccinated (0.1ml/dose)	3 7	0/4 0/3	-VE -VE	0/4 0/3	<1**
Vaccinated (0.2ml/dose)	3 7	0/4 0/3	-VE -VE	0/4 0/3	<1**

*Virus titers are expressed as log10 TCID50/ ml of homogenized lung tissue
** Virus not detected by absence of CPE in infected Vero cell monolayer cultures

Efficacy in mice

No clinical signs were observed in vaccinated groups compared to the unchallenged control group. The unvaccinated group showed rough hair with arched back 3 days post challenge. EgySerVac-20 inactivated vaccine was highly efficacious in mice at the two dosage levels. The challenge virus replicated to titers

$10^{2.5}$ $TCID_{50}$/ml and $10^{5.4}$ TCID50in Vero cell culture infected with homogenized

lung tissues from mice at 3- and 7-days post infection, respectively. Replication of the challenge virus was not detected by rRT-PCR in the lung tissues of the vaccinated mice and no CPE was observed in the cell culture monolayer (Table 3).

Histopathological evidence of protective efficacy post-challenge

Histopathological findings did not vary within either one dose (0.1ml) or double dose (0.2ml) vaccinated groups among tissues collected post challenge (Figure 3). In addition to the reduction in viral titers detected in the lungs, histopathological findings in the lungs of immunized mice indicate that EgySerVac-20 vaccine produced protection from SARS-CoV-2 after 3- and 7-days post challenge. The unvaccinated control animals that lacked detectable levels of SARS-CoV-2 neutralizing antibodies, had severe lung lesions, mice lung at 3 & 7 days post challenge test showing diffuse thickening in the interstitial tissue, congestion peri- alveolar blood capillaries and lymphocytic cells infiltrations (Figure s3c & d). Vaccinated groups that had detectable levels of serum neutralizing antibodies to SARS-CoV-2 at the time of challenge were protected from severe lung lesions (Figures 3a & 3b) with only mild scattered inflammatory cells.

Discussion

Novel COVID-19 has shown a rapid spread since December 2019 causing a huge outbreak in China [20]. In order to develop diagnostic tools, therapeutic approaches, and an effective vaccine, the virus should be isolated and profoundly studied. The development of vaccines with high immunogenicity and safety is necessary to control the pandemic COVID-19 and prevent further infection spread. Different approaches have been previously reported for the development of human SARS vaccine candidates, including inactivated whole virus vaccine [21]. The whole-genome sequence of isolated SARS-CoV-2 strain was closely related to most available sequences retrieved from the GenBank database, representing to some extent circulating SARS-CoV-2 populations. Moreover, the preliminary steps in the inactivated vaccine production, were based on how our isolated virus adapted to the optimal growth conditions, inactivated and purified.

SARS-CoV-2 had been reported to grow effectively on Vero cells [22, 23], this provided an optimal cell model for the rapid vaccine development. Propagation of isolated virus on Vero cell monolayers yield a titer of 7.5 log10 after ten passages with distinct CPE within 72 hours post infection, that finding, in which virus was efficiently replicated in Vero cell cultures reaching a peak titer of 6 to 7 log10 TCID50by 3 or 4 days. SARS-CoV CPE with monolayers of Vero cells were recorded previously three days after the first blind passage [10]. Zhu et al. [20] finding showed no specific CPE observed on the Vero E6 cells until 6th day after inoculation.

Inactivated whole virus vaccine has been reported as the most efficient method to induce neutralizing antibodies, against SARS-CoV-2 infection [7]. In this approach, formaldehyde is used as an inactivating agent [17]. When electron microscopy was performed post inactivation, it showed intact, oval-shaped particles (90 to 150 nm in diameter), having crown-like spikes; this represents a viral pre-fusion state confirming the effectiveness of diluted formaldehyde in inactivating the virus. These results come in line with the previously reported well defined spikes on the virus structure observed upon using transmission electron microscopy. These spikes showed no change in structure resulting from the inactivation procedures [7].

Our results revealed an increase in antigen neutralizing antibody responses in the vaccinated mice with EgySerVac-20 using two different doses, protecting them against SARS-CoV-2 infection, and that came in line with previous inactivated vaccines development studies (BBIBP-CorV, PiCoVacc, and BBV152) [7,8,24]. A robust neutralizing antibody titer was detected in the pre-clinical animal models used for BBIBP-CorV and PiCoVacc inactivated vaccine assessment [7,8]. Prior studies showed that low levels of neutralizing antibodies are enough to stop viral replication in mice following their exposure [25,26]. The alum adjuvant used with EgySerVac-20 is the most frequently used vaccine adjuvant and has an extensive safety record with primarily Th2-biased humoral responses via neutralizing antibodies [27].

Our data also demonstrate complete protection against SARS-CoV-2 challenge by inhibition virus replication in lung tissue post challenge test. The absence of histopathological findings in the lungs of the vaccinated mice groups confirmed the aforementioned results. The same finding was recorded in mice experimentally infected with SARS-COV and SARS-COV-2 virus as virus challenge was successfully established in animal models [28]. Thus, EgySerVac-20 inactivated SARS-CoV-2 vaccine described here provided potential solution to fight against COVID-19 pandemic and has desirable properties which supports further studies for preclinical and clinical trials.

Declaration of competing interest

The authors have declared no conflict of interest

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