Development, Analysis, and Preclinical Evaluation of Inactivated Vaccine Candidate for Prevention of COVID-19 Disease

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Abstract

A novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in the Chinese capital Wuhan in 2019. Since late 2019, SARS-CoV-2 has been responsible for a global pandemic that has resulted in 405,961,201 confirmed cases of infection and 5,789,567 deaths as of February 11, 2022, according to the World Health Organization (WHO).

Here we report the development of an inactivated SARS-CoV-2 vaccine candidate and show that its efficacy and safety in preclinical studies warrant further clinical evaluation. Efficiency and possible side effects of vaccine candidate on different doses were evaluated using histopathology, K18 hACE2 transgenic mice challenge, T-cell response, and serum neutralization test in Balb/c mice. All pilot production and propagation experiments of SARS-CoV-2 were performed in the Level 3 Negative Pressure Biosafety Facility (BSL3/ABSL-3) according to COVID-19 interim guidance of WHO.

All analytical studies have shown that the quality control parameters of the vaccine candidate meet the requirements for inactivated viral vaccines according to European Pharmacopeia. Vaccine candidate (KOCAK-19) has been authorized by the Turkish Medicines and Medical Devices Agency and has now entered phase 2 clinical development (NCT04838080).

Introduction

The new coronavirus SARS-CoV-2, the causative agent of 2019 coronavirus disease (COVID-19), belongs to the genus Beta coronaviruses. This genus also includes SARS-CoV and MERS-CoV (Qiang Gao, Linlin Bao, Haiyan Mao 2020). SARS-CoV-2 is closely related to severe acute respiratory syndrome coronavirus (SARS-CoV) and several bat coronaviruses. SARS-CoV-2 has a linear single-stranded positive-sense RNA genome of 30 kb in length encoding four structural proteins (spike (S), envelope (E), membrane (M), and nucleocapsid (N)), sixteen non-structural proteins, and several accessory proteins. Spike (S) is the binding domain and a major structural protein that elicits highly potent neutralizing antibodies (Nabs). SARS-CoV-2 binds to ACE2 receptors via the RBD (Receptor Binding Domain) of the spike protein to initiate membrane fusion and enter human cells. Some critical amino acid residues (AA) in the RBD were different between SARS-CoV-2 and SARS-CoV [1–4].

Neutralizing antibodies (Nabs) are key components of the protective immune response to viral infections, as they can bind to and block viral particles and help to serve APC (antigen-presenting cells) [5–7].

Recently, new SARS-CoV-2 variants have emerged and are circulating globally. Most of these variants acquired new mutations in their surface protein (Spike) [A]. Variants with several S mutations have been detected in Brazil, Denmark, South Africa, the UK, and the USA. Four SARS-CoV-2 variants of concern including B.1.1.7, B.1.351, B.1.617.2, and P.1. Variants B.1.1.7 and B.1.351 which were first reported in the UK and South Africa, respectively. B.1.351 variant includes a variety of spike protein mutations (K417N; E484K or N501Y) but has no major difference in transmission or severity. The Delta variant (B.1.617.2) has become the predominant variant in much of the world during the second half of 2021. As of December 1st, 2021, at least 27 countries in six continents have identified the Omicron variant, including the United States. Omicron is a new variant strain and has at least 50 mutations identified, with 32 of those mutations involving the spike protein when compared to the reference strain. Of these 50 mutations, 26 are unique, further building upon the 10 unique mutations seen in Delta and six from Beta. Very little is known about either infectivity or transmissibility at this time [8–9].

More than 300 vaccines are under development and 9 of them are approved for emergency use in various countries. The Food and Drug Administration (FDA) has approved the use of Pfizer/BioNTech, Moderna COVID-19, and Janssen Ad26.COV2. S (COVID-19) vaccines in a mass immunization program. Phase three clinical trials for Pfizer/BioNTech vaccines enrolled 43,661 participants, while Moderna vaccines involved 30,000 participants. The European Commission authorizes Novavax's vaccine. CoronaVac has been given conditional approval in China and for emergency use in Brazil, Chile, Turkey, Colombia, Indonesia, and Uruguay. BBIBP-CoV has already been licensed for use in China, UAE, and Bahrain and approved for emergency use in many other countries. Sinopharm's vaccine CNBG-WIIBP has been given the green light only for limited use in China and UAE. India also granted approval of Covaxin for emergency use only [8, 10, 11, 12].

Material Method

Development and preparation of KOCAK-19 vaccine candidate

A SARS-CoV-2 strain (Vial no.31242/12.05.2020) isolated from bronchoalveolar lavage specimens or throat swabs from a hospitalized patient in the recent COVID – 19 outbreaks by the Turkish Ministry of Health was used to develop a candidate vaccine. This strain was isolated from Vero cells, which are approved by WHO as a cell line to produce inactivated vaccines for human use. This primary seed was further amplified to create a master seed virus bank, a working virus bank, and a production virus bank in the Class III isolator cabinet in the BSL-3 facility.

Highly efficient propagation and high genetic stability are key characteristics for the development of an inactivated vaccine. It was found that the strain showed optimal replication and generated high virus yields on Green Monkey Kidney Cells (Vero CCL-81, ATCC) were cultured in Eagle's modified Essential medium (EMEM) (Sigma, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma, Germany) and L-glutamine. In the present study, the strain showed optimal replication and produced high virus yields. Here, we set out to determine the most

appropriate times post-infection to harvest. We determined that the titers depend on the cytopathic effect (CPE) caused by SARS-CoV-2 in Vero cells is most apparent at 48 h post-infection. The highest titer is at 62 h post-infection by crystal violet (0.1% w/v) (Sigma-Aldrich) staining (Fig. 1).

Initially, Vero cells CCL-81 were seeded in T-flasks (NEST, USA) at 2.105 cm^2 and incubated in a humidified incubator at 37°C with 5% CO_2 . Four days after seeding, confluent Vero cells (Fig. 2A) were trypsinized at a ratio of 0.25% trypsin-EDTA (Sigma, USA) and transferred to the 3.165 cm^2 BioFactory Chamber (NEST, USA). In addition, two T-175 flasks were infected with SARS-CoV-2 virus seed to produce a bioreactor inoculum. Infected Vero cells show typical CPE under the inverted light microscope at a magnification of 20x (Nikon Eclipse Ti2) (Fig. 2B). After the 4th day of incubation, the titer was determined by microtitration assay. Four days after the cells were seeded in the BioFactory chamber, they were passaged onto a microcarrier system (3 to 5 mg/L Cytodex-1, Cytiva, USA) in a 20-liter bioreactor (Fig. 2C). Vero cells were cultured in a 20-liter bioreactor at a temperature of $37 \pm 1^\circ\text{C}$. The pH of 7.2 was regulated by CO_2 and the addition of NaHCO_3 at 88 g/L, and dissolved oxygen was adjusted to 35–98% air saturation by continuous surface aeration. To produce the SARS-CoV-2 virus, the virus seeds were inoculated at a ratio of 0.05 MOI after washing the cells with DPBS (Sigma Aldrich, USA) solution, the pH was 94 maintained at 6.8–7.1, the pO_2 at 35–98% air saturation, and the temperature at 37°C . The rotation speed was 12–18 rpm and the angle was 6° – 7° depending on the different cell growing and viral incubation stages. Growth kinetic analysis of the 5th passage (P5) of the virus (Working Seed Lot) in Vero cells showed that the strain virus could replicate efficiently and reached a peak titer of 3.6×10^6 PFU after 56–72 hours at infection multiplicities (MOI) of 0.05 [6]. The virus mass was harvested at 62 h (Fig. 2D) after the inoculation and then clarified using $50 \mu\text{m}$ PES (Polyethersulphone) and $0.45 \mu\text{m}$ PES filter. Clarified harvest was inactivated with β -propiolactone (Sigma, USA) at a 1:1500 ratio at 2 – 6°C for 16–20 h, followed by benzonase-endonuclease (Merck, USA) treatment at room temperature, concentration by 100–300 kDa molecular weight ultrafiltration cassettes, and multimodal chromatographic (SEC and AEX) purification. During the inactivation process, the pH was maintained at 6.8–7.1. The final mass was prepared by adding 0.05% (v/v) Alhydrogel (Croda, Denmark) as adjuvant and dilution buffer containing Phosphate Buffer Saline (1x pH 7.2 PBS, Sigma Aldrich) with stirring at 20–30 rpm at 25°C for 2 hours [13].

Since safety and efficiency are critical for vaccine development, all sterility, Mycoplasma sp., and endotoxin analyses were performed according to European Pharmacopeia 10 and acceptable levels were found. However, Vero Cell Banks and Virus Seed Lots were found to be free of mycoplasma by culture and NAT (Nucleic Acid amplification Technique) according to European Pharmacopeia 10. Mycoplasma NAT test is based on a nucleic acid amplification technique using a very sensitive touch-down PCR profile. The presence of mollicutes DNA in the test specimens is determined by an approx. 450 bp target sequence which is amplified and this amplicon is finally detected by polyacrylamide gel electrophoresis. No mycoplasma species' DNA was detected using specific primers. Bacterial endotoxin levels measured the gel-clot technique using amoebocyte lysate (LAL) from the horseshoe crab. Endotoxin is expressed in International Units (IU) of endotoxin. Our endotoxin levels always are detected below 12.5 IU/dose compared to standards in the final product.

In the present study, a purified inactivated SARS-CoV-2 virus vaccine candidate was prepared at a pilot scale to induce specific neutralizing antibodies in 6–8 weeks old Balb/c mice. Five immunization doses (1 μg , 4 μg , 6 μg , 10 μg per dose, and placebo) mixed with 0.05% (v/v) Alhydrogel adjuvant were administered to five groups of mice ($n = 10$). Partial and complete protection against challenge by observing an increase in serum neutralizing antibody levels pneumonia symptoms, and histopathological examination of the lungs. In transgenic mice, we observed protection against challenge and viral loads in the tissues [5, 6].

Virus titration by plaque assay and microtitration assay

SARS-CoV-2 viral titers were determined using a plaque assay. Serial 10-fold dilutions of virus-containing samples were inoculated into 12-well culture plates seeded with confluent Vero cells. 1 hour later, the inoculums were removed and then covered with 0.4% agarose MEM. After 3 days of culture in 5% CO_2 incubators at 37°C , the cells were fixed with 10% neutralized formalin for 1 hour at room temperature. After the fixation process, the agarose layer was removed with pipette tips and crystal violet solution (0.4%) was added to the wells. After the washing process, the wells were checked for plaque formation and the titer was calculated as Plaque Forming Unit (PFU/ml) [13].

Virus titers were also determined using microtitration assay and samples were serially diluted (from 1 log to 10 logs) in 96-well, clear bottom plates containing confluent monolayers of Vero cells. For each dilution, a total of five replicate wells were infected. The infected plates were incubated at 37°C and 5% CO_2 for 4 days, and then individual wells were visually inspected using an inverted microscope for the presence of virus-induced cytopathic effects (CPE) or crystal violet (0.1% w/v) (Sigma-Aldrich) staining macroscopic observation at each dilution as compared a negative, media-only control. The end-point titers (TCID_{50}) were calculated according to the Reed & Muench method based on four replicates for titration [14–15].

Validation of the inactivation

β -Propiolactone (BPL) modifies the structure of nucleic acids of the viruses after reaction with purine residues. BPL is widely used for inactivation viruses to produce inactivated vaccines [16]. SARS-CoV-2 with BPL at a ratio of 1:1500 for 16–20 h at 6°C followed by 2 h at 37°C was found effective inactivation of the SARS-CoV-2 which was validated in a sample from each batch of COVID-19 vaccine. 3 ml of inactivated SARS-CoV-2 was used to inoculate Vero cell monolayers in 25 cm^2 flasks, and negative control cells were prepared; the cells were then cultured at $36 \pm 1^\circ\text{C}$ for 4 days. Then, 5 ml supernatant of cells in the flask was inoculated onto two more Vero cell monolayers in 25 cm^2 flasks (5 ml each) and incubated at $36 \pm$

1°C for 4 days. This was the second passage. Then 5 ml supernatant of cells from the flask was inoculated onto more Vero monolayers in 75 cm² flasks and incubated for 4 days at 36 ± 1°C [13].

Transmission Electron Microscopy (TEM) sample preparation

Inactivated samples and infected Vero cells were mounted on pyroform-coated nickel grids, stained with 2% uranyl acetate, washed with sterile pure water, and visualized in a transmission electron microscope (Jeol, Tokyo, Japan) [8]. A specialized histologist and a virologist made the observations.

Nucleic acid extraction and first-strand cDNA synthesis

Total nucleic acids were extracted from 200 µL of clarified viral supernatants using the Mini Pathogen Nucleic Acid Kit (QIAGEN) according to the manufacturer's instructions and collected 50 µL elution volumes. The viral RNA was reverse transcribed using the Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Thermo Scientific, USA) using random hexamers according to the manufacturer's recommendations. The reaction mixtures were incubated for 60 min at 42°C, and the reaction was stopped by heating the mixture at 95°C for 5 min and chilling it on ice.

Sequencing

For whole-genome sequencing of SARS-CoV-2 samples, Quant-it RNA HS Assay kit (Invitrogen, USA) and Qubit fluorometer were used for measurement of isolated RNA samples. Library preparation was performed using the CleanPlex® SARS-CoV-2 Panel (Paragon Genomics) with isolated RNA samples following the kit's recommended instructions. Libraries were sequenced on the Illumina NextSeq (Illumina, USA) platform with a 2x loop kit with an average of 500,000 reads. The quality of raw data was examined with FastQC v.0.11.5 and low-quality bases and primers were trimmed using Trimmomatic v.0.32. Reads were aligned to the known SARS-CoV-2 genome (GenBank Access: MN908947.3) using the Burrows-Wheeler aligner v.0.7.1. Variants were detected using the Genome Analysis Toolkit-HaplotypeCaller (GATK) v.3.8.0 and analyzed on GenomeBrowse v2.1.2 (GoldenHelix). The genome sequences of the first viral sample obtained and the genome sequence of the last sample was aligned using BLAST+ [18].

Quantitative Real-Time PCR

rRT-RT assay rRT-PCR assay was performed using the One-Step RT -qPCR Master Mix, (Primer design, USA). The Primer design 2019-nCoV genesig® Advanced Kit is a Vitro diagnostic real-time reverse transcriptase PCR (RT-PCR) assay intended for the quantitative detection of nucleic acid from SARS-CoV-2. The assay utilizes real-time technology targeting the RNA-dependent RNA polymerase (RdRp). Each 20-µL reaction contained 10 µL 2X Master Mix (Genesig/primer design, USA), 2 µL 5 µmol/L probes, 20 µmol/L forward and reverse primer mix, 5 µL nuclease-free water, and 5 µL nucleic acid extract. We performed amplification in 96-well plates on a ROCHE Light Cycler 480 Real-Time PCR instrument (ROCHE). Real-time PCR conditions RT consisted of 10 min at 55°C for reverse transcription, 2 min at 95°C for Taq enzyme activation, and 40 cycles of 3 s at 95°C and 30 s at 55°C. Viral load (copy number/µl) is calculated based on the standard curve [19].

SDS-PAGE and WESTERN BLOTTING

β-Propiolactone inactivated SARS-CoV-2 virus samples were separated using the 10% polyacrylamide gels described by Laemmli and visualized using the Commosie Blue staining kit (BIO-RAD). Another SDS -PAGE (Bio-Rad TGX Mini, Bio-Rad, Hercules, CA, USA) gel was transferred to a 0.2 µM PVDF membrane according to the manufacturer's protocols (Bio-Rad Transblot Turbo; Bio-Rad, Hercules, CA, USA). After blocking with 5% non-fat dry milk in TBST (10 mM Tris, 150 mM NaCl, 0.5% Tween-20, pH8) for one hour, the membranes were incubated overnight at 4°C with antibodies against SARS-CoV N (Sino Biological, Wayne, PA, USA; 40588-T62) and SARS-CoV S (Sino Biological, Wayne, PA, USA; 40589-T62). Membranes were washed in TBST and incubated with an HRP-conjugated rabbit secondary antibody (Cell Signaling, Danvers, MA, USA; 7074) for 1 hour at room temperature. Membranes were then washed, developed with ECL, and imaged on a Bio-Rad Chemidoc imaging system. The molecular weights of the full-length S and N proteins are approximately 190 and 48, respectively.

SARS-CoV-2 Spike Protein quantity detection by Sandwich-ELISA method

SARS-CoV-2 Spike Protein was quantified by sandwich ELISA using the SARS-CoV-2 (2019-nCoV) Spike Protein ELISA kit (Creative Biolabs, Life Technology, NY, USA) according to the manufacturer's instructions. The SARS-CoV-2 (2019-nCoV) Spike Protein ELISA kit is based on a solid-phase sandwich enzyme immunoassay. The wells of the plate strips are coated with a monoclonal antibody specific for SARS-CoV-2 spike protein and nucleoprotein. Since the 10000 pg/ml standard serves as a high standard, we diluted the samples hundreds to thousands of times. We determined the optical density of each well at 450 nm. The results were analyzed using EPOCH Gen 5 software. We calculate the spike protein levels in a dose with a standard curve.

Humoral immunogenicity analysis and T-cell response analyses of the vaccine candidate by neutralization assay and ELISPOT

An ideal serological assay should measure the binding of neutralizing antibodies to the SARS-CoV-2 spike protein. For this purpose, Balb/c mice were randomly divided into five groups (10 animals in each group) and injected with the experimental vaccine at five different doses (0 µg, 1 µg, 4 µg, 6 µg,

and 10 µg per dose, mixed with alum adjuvant) on the day 0th and 21st [7], blood was collected from the mice at 0th, 7th, 14th, 21st and 35th days after the first immunization. Each animal was injected with 0.2 ml of the test sample.

Serum from each group of mice to be tested was diluted 1:2 in advance and inactivated in a 56°C water bath for 30 minutes. The serum was diluted 1:2 by a 2-fold dilution series to the required concentration, and an equal volume of a challenge virus solution containing 100 TCID₅₀/50µl viruses was added. After neutralization in a 37°C incubator for 1 hour, a 1 x 10⁶/ml cell suspension was added to the wells (0.1 ml/well) and cultured in a CO₂ incubator at 37°C for 4 days. The CPE observation was used to calculate the neutralization endpoint (conversion of serum dilution to logarithm) according to the Karber method [14], i.e., the highest serum dilution that can protect 50% of the cells from infection by 100 TCID₅₀ viruses is the antibody potency of the serum. Each of the samples was performed in the well-plate at four repeats. A neutralization antibody potency of 1:8 is negative, while one of > 1:8 is positive [15, 17].

Three animals for 4 and 6 µg group were euthanized on day 35th and splenocytes were collected to evaluate T-cell response by IFN-γ ELISPOT assay.

IFN-γ ELISPOT Assay

The ELISPOT assay constitutes an ideal tool in the investigation of Th1 / Th2 responses. ELISPOT assay was performed with Murine IFNγ ELISPOT Kit (Abcam, USA) according to the manufacturer's instructions. A total of 2.5 x 10⁵ splenocytes per 100 µl were stimulated at 37°C for 48 h with MOI 0.05 of the SARS-CoV-2 and controls (splenocytes from PBS-inoculated Balb/c mice); culture media alone (background); concalvine A (conA) 0.5 µg/mL (cell viability). IFN-γ-secreting cells were revealed by adding streptavidin-alkaline phosphatase and ready to use BCIP/NBT substrate. Incubate the plate for 5–15 minutes monitoring spot formation visually throughout the incubation period to assess sufficient color development. Spots were counted under a stereomicroscope. Spot-forming units (SFUs) per million cells were represented after background subtraction from unstimulated cells.

Repeated dose toxicity studies in ferrets

Some studies have demonstrated that ferrets are a suitable mammalian model for SARS CoV-2, which efficiently replicates in its upper respiratory tracts. To examine whether the KOCAC-19 can cause toxicity or local reactions in the ferrets. Ferrets (n = 6) received 8 µg of KOCAC-19 th on the 0th, 8th, and 15th days via the intramuscular route (IM). Three ferrets received physiologic saline solution (0.9% NaCl) on 0th, 8th, and 15th days via the same route (IM). Clinical evaluations included local reactions of injection locations, temperature, weight loss, fever, dyspnoea, lethargy, anorexia, and grooming. Monitoring of ferrets was performed twice a day for two months. Also, biochemical and hematological examinations were carried out.

Challenge studies in transgenic K18 hACE-2 mice

K18 hACE-2 Transgenic male mice (Jackson Lab., USA) were randomly divided into three groups (13 animals in each group) and intraperitoneally (IP) injected with the experimental vaccine at three different doses (0 µg, 4 µg, and 6 µg per dose, mixed with alum adjuvant) on the 0th and 21st [7]. All groups of mice were challenged by intranasally 10⁴ TCID₅₀/50µl live viruses SARS- CoV-2, which was administered into both nasal passages using a micropipette in an isolator (220 Pa negative pressure) on day 35th. Symptoms (weight loss, fever, dyspnoea, lethargy, anorexia, and grooming) and death were monitored to day 45 after the first immunization.

Three animals for each group were euthanized three days following the challenge. Lungs, nasal turbinate, trachea, and brains were collected for virus isolation and virus load detection by real-time RT-PCR kit (Primer design, USA).

Experimental Method in Balb/c mice

To evaluate the efficacy and safety of the prepared vaccine, 50–60 days old, 20 female Balb/c mice were randomly divided into 6 groups. Animals were kept in IVC cages (22°C, 55–65% relative humidity), all groups were separated, they were fed with ad libitum and animals had access to food and water whenever they wanted. Group N1 animals received virus only (n = 2), group N2 received no application (n = 2), group G1 received virus and 1 µg vaccine (n = 4), group G2 received virus and 6 µg vaccine (n = 4), group G3 received virus and 10 µl vaccine (n = 5), group G4 received 4 µl vaccine only (n = 2). The vaccine was administered on days 0 and 21. On day 35, N1, G1, G2, and G3 received intranasally 10⁴ TCID₅₀ live viruses SARS-CoV-2, which was administered into both nasal passages using a micropipette in an isolator cabinet (220 Pa negative pressures). All animals were sacrificed on day 45 for histopathological examination.

Animals were subjected to routine necropsy, organ tissue samples were collected in 10% formalin solution and subjected to routine tissue examination and embedded in paraffin blocks. Sections 3–4 µm thick were collected from each block using a rotary microtome (Leica RM2255, Germany), stained with hematoxylin & eosin (H&E), and analyzed under a light microscope (Olympus BX50, Japan). All histopathological examinations were double-blinded and scored by each pathologist and their mathematical mean was accepted as the true value.

Results

Vaccine preparation

Microcarrier-based production in disposable wave bioreactors (SARTORIUS Biostat RM20, Germany) was very efficient for vaccine production. Disposable bags are easily maintained, and aseptic connections of bags are very useful for dangerous pathogens. Cells are growing very fast and healthy in appropriate conditions (rocking, pH, angle, gases control). All manipulations are carried out aseptically and safely using with bio-welder and bio-sealer (SARTORIUS, Germany). However, 0.01–0.05 MOI was found optimal multiply of infection ratio to infect Vero CCL-81 cells. Biowelder and biosealer (SARTORIUS, Germany) were used to connect pipes and transferring of inoculums, collect samples, and harvest

SARS-CoV-2 Spike ELISA

We found the SARS-COV-2 Spike protein quantity is equal to 7.53 ng/ml in 1:75 dilution of the main bulk of the vaccine candidate. Each dose of the vaccine candidate contains about 562 ng viral spike protein in 6 µg vaccine (Table 1). Also, we determine the Alum-antigen binding capacity before and after the adjuvant binding process by quantitative SARS-COV-2 Spike protein ELISA and found about 95–99% in different trials. Alum-antigen binding is important for immunogenicity and long-time stability of viral proteins in + 2 to + 8°C.

Table 1
SARS-COV-2 Spike protein quantities

And standards	Absorbance	Concentration pg/ml
STD 1 10000 pg/ml	2,896	10840,095
STD 2 5000 pg/ml	1,645	5486,138
STD 3 2500 pg/ml	0,853	2486,339
STD 4 1250 pg/ml	0,406	1014,814
STD 5 625 pg/ml	0,246	554,92
STD 6 312,5 pg/ml	0,145	293,464
STD 7 156,25 pg/ml	0,103	195,136
Negative	0,078	139,697
Main bulk 1/75 dilution	2,14	7529,486

Virus titration by Plaque assay and microtitration

Virus titration of 3rd passage of the virus (master seed lot) was reaching a peak titer over 3.4×10^6 PFU/ml or 9.9×10^6 TCID₅₀/ml by 56–72 h post-infection (hpi) at multiplicities of infection (MOI) of 0.01. Growth kinetic analysis of the 5th passage of virus stock (working seed lot) in Vero cells showed that the stock virus could replicate efficiently and reached a peak titer 3.6×10^6 PFU/ml or 1×10^7 TCID₅₀/ml by 56 – 72 h post-infection (hpi) at multiplicities of infection (MOI) 0.05 (Fig. 3). Viral kinetics of the KOCaK-19 strain at different multiplicities of infection (MOI) was given in Fig. 4A and 4B.

Quantitative RT-PCR Genesig/Primerdesign qRT-PCR (RdRp gene) kit results are correlated with viral titer results at 56–72 h. qRT-PCR is a fast way to the determination of harvesting time (Fig. 5). However, the preparation of viral stocks' viral titers should be determined before and after the storage in liquid nitrogen and inoculation.

Inactivation validation

No CPE was observed for three sub passages of inactivated virus bulk. Inactivation was validated by passaging the treated samples 3 generations (n = 3) without the appearance of CPE. β-propiolacton (BPL) inactivation provides a rapid and safe method for the inactivation of viral particles. If there are any live residual viruses and infective RNA in the bulk, Vero cells must be infected and cytopathic effect (CPE) must be observed under the inverted light microscope.

Sequencing

Sequence distance matrix of first, third, and, ninth passages of SARS-COV-2 in Vero cell culture. All these passages correlate to SARS-COV-2 NC 045512 isolate at a ratio of 97.891 to 99.117% in the NCBI database. It was determined that the 1st passage, 4th passage, and 9th passage of SARS-CoV-2 genome sequences matched 100%, and all the mutations in the first sample were also observed completely in the other samples. Some negligible deletions were observed in the sequences, not in the affected structure of the virus. The variations were found to correspond to the genomic positions 313, 5554, 8782, and 17259 (ORF1ab gene), 21784 and 22468 (S gene), 28878 (N gene), 28144 (ORF 8 gene), 26873 (M gene), and 29742 (3' UTR).

SDS-PAGE and WESTERN BLOTTING

It is observed that Spike, Nucleoprotein, and Membrane proteins are approximately 190, 48, 26 kDa, respectively after the purification step by SDS page (Fig. 6).

SARS-CoV-2 N and SARS-CoV-2 S proteins are determined by incubation with anti-SARS-COV-2 N, S antibodies and, HRP conjugated rabbit antibodies in Western Blot. It is observed that Spike and Nucleoprotein bands are approximately 190 and 48 kDa in Western Blot (Fig. 7A and 7B).

Transmission electron microscopy (TEM)

TEM analysis verified the viral replication in Vero cells and showed intact, oval-shaped particles with diameters of 90 to 110 nm of the virus after the final purification step (Fig. 8).

Vaccine candidate immunogenicity analysis and microneutralization assay (MNT)

The preliminary results in mice showed that the Nab titers reached the peak post-priming and boosting in 21 days. The geometric mean titers were respectively < 1/4, 1/39, 1/1/ 595, 1 /791, and 1/1048 in placebo, 1 µg, 4 µg, 6 µg, and 10 µg groups (Fig. 9). Statistical significance was tested with the ANOVA corrected for multiple comparisons in vaccinated and placebo groups and then P values are shown for comparisons that resulted in statistical significance ($P < 0.05$). No statistical significance was observed in 4 µg, 6 µg, and 10 µg vaccinated groups.

SARS-CoV-2-specific cellular responses were assessed in 4 and 6 µg vaccinated animals by IFN- γ ELISPOT. T-cell immune responses in 4 µg ($n = 3$) and 6 µg dose group ($n = 3$) and control group ($n = 3$) were analyzed at day 35 following first immunization by an IFN- γ -based ELISPOT assay (Fig. 11). The statistical significance was assessed using an ANOVA test p values less than 0.01 were considered to be statistically significant. Error bars represent mean $\pm$ standard deviation.

K18 hACE-2 Transgenic Mice Challenge, findings of viral loads of tissues

6–8 weeks old, non-vaccinated K18 hACE-2 transgenic mice exhibited a significant decrease in body weight at 3–5 dpi compared with vaccinated K18 hACE-2 transgenic mice. More than 50% of non-vaccinated K18 hACE-2 transgenic mice died at 5–7 dpi. All vaccinated mice survived during the study (Fig. 12A, 12B, and 12C).

More viral RNA copy number was detected in all unvaccinated transgenic mice than vaccinated groups' tissues. Especially lung and trachea have more viral RNA load than the brain and nasal turbinate in unvaccinated controls (Fig. 13A, 13B, 13C, and 13D). Viral loads are significantly less in all vaccinated animals than control animals at 3 days post-infection. Error bars represent mean $\pm$ standard deviation.

Repeated dose toxicity studies in ferrets

The most common adverse reaction was injection site swelling, which was mild and limited. Fever, inappetence, and weight loss were not observed in Balb/c mice and transgenic ferrets. When the inactivated vaccine candidate ($n = 6$) and placebo ($n = 3$) was applied in 8 µg doses three times, It was well tolerated in all dose groups with no vaccine-related serious adverse effects. Two months after the first application, biochemical and hematological parameters were found in the normal range compared to the placebo group (Fig. 14A, 14B).

Macroscopic Findings:

The weight gain percentage of Balb/c mice inoculated with the lowest and highest doses of the vaccine candidate were significantly more than control groups after the challenge (Fig. 15). Although there were no significant differences between the groups in the macroscopic examination, depending on the euthanasia method applied (decapitation), bleeding areas were observed in the head, neck, and back regions, and in some animals, blood accumulation/collections in the lungs due to blood aspiration were observed.

Histopathologic Findings:

Histopathologic changes observed in animals are given in Table 2. In the histopathologic evaluation, the main differences between groups were observed especially in the lung, heart, abdominal lipid tissue, and spleen.

Table 2. Histopathologic changes observed in animals

Tissue	Histopathology	N1 (CoV)		N2		G1 (CoV+1µl vaccine)					G2 (CoV+6µl vaccine)				G3 (CoV+10µl vaccine)					G4 (4µl vaccine)	
		1	2	1	2	1	2	3	4	5	1	2	3	4	1	2	3	4	5	1	2
Lung	Perivascular lymphoid infiltration	+	++	-	-	++	++	++	-	++	-	-	++	++	+		+	++	++	-	-
	Peribronchial lymphoid infiltration	-	++	-	-	++	++	++	-	++	-	-	+++	++	++	++	+	++	+	+	-
	Bronchial epithelial hyperplasia/hypertrophia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-
	Hemorrhage	+	-	-	-	++	-	-	-	++	-	-	++	-	+	-	-	-	++	-	++
	Emphysema	+	+	-	-	+	-	-	-	-	-	-	-	++	+	+	+	+	-	+	+
	Alveolar macrophage infiltrations	-	+	-	-	-	+	-	-	+	-	-	++	-	-	-	-	+	-	+	++
	Intraalveolar mononuclear cells infiltrations	-	+	-	-	-	-	-	-	+	-	-	+++	-	-	-	-	+	-	+	+
	Intraalveolar polymorph cell infiltration	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Interstitial pneumonia	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Fibrinoid necrosis in small vessels	-	++	-	-	+	+	-	++	-	-	-	++	-	-	-	-	-	-	-	-
	Per diapedesis hemorrhage	-	+	-	-	-	-	-	++	-	+	-	-	-	-	-	-	-	-	+	+
	Fibrin inside the alveoli	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	++
	Thrombosis	-	-	-	-	-	-	-	-	++	-	-	-	-	-	+	-	-	-	+	+
	Tumor	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Hyperemia	-	-	-	-	-	++	-	++	-	++	-	++	++	+	-	-	+	-	-	-
	Bronchiectasis	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-
	Hyaline accumulation in the bronchus	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	++
CNS	Meningeal lymphoid infiltration	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	+	+	-
	Micro thrombosis	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
	Hemorrhage	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
	Perivascular lymphoid infiltrations	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-
Heart	Zenker degeneration/necrosis	+	++	-	-	-	+	++	-	+	++	+++	+++	-	++	+	+	++	++	++	-
	Mononuclear cell infiltration	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Hemorrhage	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Abdominal lipid tissue	Lymphoid infiltration	-	-	-	-	+++	++	-	-	+	+++	-	-	+++	+	++	++	-	-	++	++
	Abdominal fat necrosis	-	-	-	-	-	-	-	-	-	-	-	-	+++	-	-	+	-	+	-	-
	Granuloma	-	-	-	-	-	-	-	-	-	-	-	-	-	+	++	+++	++	-	-	-
Liver	Parenchymatous degeneration	+++	+++	++	++	+++	++	+	+++	+++	+	++	+++	++	++	+++	+++	+++	++	+++	-
	Fatty degeneration	-	-	+	+	+	+	-	-	+	-	+	+	+	+	++	+	+	-	+	-
	Hepatitis	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Hemorrhage	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spleen	Lymphoid hyperplasia	+	++	-	-	+	++	+	++	++	++	+++	-	++	++	++	+++	++	++	++	++
	Increased megakaryocytes	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+	++	+++	+	-	-
	Congestion	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+++	+	++	-	-
Kidney	Interstitial nephritis	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	-
	Hyperemia	-	-	-	-	-	-	-	-	-	-	-	-	-	+	++	+	++	+	-	-

In the lungs, the most significant findings were the perivascular and peribronchial mononuclear cell infiltrates, especially in animals that received vaccination and they were more prominent in the 6 and 10 µL vaccine groups (Fig. 16A, 16B). And only in G3 bronchial epithelial hyperplasia/hypertropia is observed. Hemorrhages were observed in all groups in which we assumed blood aspiration due to the decapitation process. Interstitial pneumonia is only observed in one animal (N2) which only received a virus. Thrombosis in small vessels and inside alveoli and hyaline accumulation inside alveoli were observed especially in G4 in animals (Fig. 16C, 16D) only received 4 µL vaccination.

In the central nervous system, some meningeal lymphoid infiltrations and per diapedesis bleeding were observed in 5 animals; N1-1, G2-1, G2-4, G3-5, and G4-1 (Fig. 16E), and hemorrhage, micro thrombosis, and perivascular lymphoid infiltrations are observed only in G2-3 (Fig. 16F).

Varying degrees of hemorrhages, myofiber degenerative changes (Zenker degenerations), and necrosis, mononuclear cell infiltrations were detected in the heart (Fig. 16G). Only the animals in the group that did not receive any treatment or virus application (N2) heart tissues were healthy. The

degenerative changes were distinct, especially in G2 (Fig. 16H) and they are generally located on the left side of the heart.

Widespread parenchymatous degeneration and varying degrees of fatty degeneration were detected in the livers of all animal groups. Only hepatitis and hemorrhages were observed in one animal (N1).

In all groups which received intraperitoneal vaccination varying degrees of changes were observed in the abdominal lipid tissue. G3 animals which received 10 μ L vaccination demonstrated the severest cases. Lymphocytic infiltrations, macrophages, necrosis, granulomatous changes, and granulomas are observed (Fig. 16I). In one animal (G2-4) where these changes were close to the pancreatic tissue, degenerative changes and lymphocytic infiltrations were observed in the pancreatic tissue.

In the splenic tissue of animals, lymphocytic hyperplasia was significant in all groups with vaccinations (Fig. 16J). Increased megakaryocytes in the spleen are observed in all animals.

In kidneys, hyperemia and interstitial nephritis were only observed in groups G3 and G4, and small degrees of lymphocytic infiltration especially in the renal pelvis were determined.

Discussion

In the development of the vaccine candidate, the candidate strain generated the highest virus yields in Vero cells CCL-81 and had no amino acid variations within 9 passages, suggesting its good genetic stability. We observed only some deletions in the full genome sequence but not affected the amino acid structure of the basic structure of the virus. Pavel et. al. [19] found the variations that genomic positions 1397 and 11083 (ORF1ab gene), 23876 (S gene), 26688 (N gene), 29563 (ORF 10 gene), and 29742 (3' UTR). Also, Gao et al. [6] reported that some genetic alterations between in the passaging levels of virus, but not affecting to immunity. In this study, we found the variations that genomic positions 313, 5554, 8782 and 17259 (ORF1ab gene), 21784 and 22468 (S gene), 28878 (N gene), 28144 (ORF 8 gene), 26873 (M gene), and 29742 (3' UTR). These differences may be affected immunity to wild-type virus strains slightly, but it will become clear after the cohort studies are carried out on large scale.

Due to high viral yield, microcarrier size and concentration, Vero cell seeding quantity, multiply of infection ratio, time and duration of viral infection, maintenance of pO₂, pH, bioreactor angle and shaking, and time of harvesting are critical factors in the upstream process. The pO₂ saturation must not be decreased below 35% and pH should be maintained around 7 to avoid the aggregation and lyses of viral proteins and support cell growth and viral production. 56–72 h was found optimal harvesting time in these production conditions. Our data agrees with a study of Manenti et al. [15] in which SARS-CoV-2 replicated rapidly in Vero CCL-81 cells after an initial eclipse phase and increased gradually, peaking at 56 h post-infection.

However, the latest report shows in the preparation of vaccines, BPL inactivation provides a rapid and safe method for the inactivation of viral particles [20]. Also, intact viral particles are apparent by transmissible electron microscopy (TEM). In TEM analysis showed intact, oval-shaped particles with diameters of 90 to 110 nm of the virus after the final purification step. Our data agrees with that of Jureka et. al. [13], in which TEM analysis showed 90–110 nm virus in purified bulk.

Quantitative SARS-CoV-2 Spike antigen ELISA test verified SDS gel antigen quantities with working BSA standards in SDS gel electrophoresis. Also, qRT-PCR results from the Genesig Advanced COVID-19 RT-PCR kit gave information about the viral titers and viral protein quantity with invert microscopy examination to decide harvesting time of viral bulk.

We demonstrated that both nucleoprotein and spike (full-length and cleaved) of SARS-CoV-2 in all downstream process steps are detectable by western blot in these samples.

The two-dose immunizations with median doses (4 μ g and 6 μ g) at days 0/21, were found efficient. SARS CoV-2 vaccine candidate induces high levels of Nab titers, T-cell response and efficiently protects them in Balb/c mice. Also, these two-dose immunizations with 4 μ g and 6 μ g doses at days 0/21, were found efficient in K18 hACE-2 transgenic mice challenge studies compared to the placebo group. SARS-CoV-2 vaccine candidate efficiently protects them in suitable animal models against challenge. Viral loads in vaccinated animals' tissues (Lung, trachea, nasal turbinate, and brain) are much less than in the unvaccinated group.

We used the ANOVA method to compare the log-transformed antibody titer depending on the normality test results that showed normal distribution. When the comparison among all five groups showed a significant difference between vaccinated and non-vaccinated (Placebo) groups, we then did pair-wise comparisons. Median doses (4 and 6 μ g) showed a good immune response and not any gross and pathological findings. SARS CoV-2 vaccine candidate is efficiently produced, genetically stable, and safe in Balb/c mice, K18 hACE-2 transgenic mice, and ferrets. Clinical evaluations included temperature, weight loss, fever, dyspnea, lethargy, anorexia, and grooming. The most common adverse reaction was injection site swelling, which was mild and limited. Fever, inappetence, and weight loss were not observed in Balb/c mice and transgenic ferrets [1, 6].

During the study, no differences in body weight, body temperature, or clinical pathology were observed or detected in Balb/c and K18 hACE-2 transgenic mice inoculated with the lowest and highest doses of the vaccine candidate, compared with control groups [1, 6].

No obvious systemic toxicity was observed in any animal species during the dosing period and at the end of the 2-week recovery period. Local skin irritation reactions related to alum adjuvants were noted on injection sites, but they disappeared within a week [6].

The results showed that the vaccine candidate stimulated immoral immune responses and presented no toxicity in animals tested. In conclusion, a study on the safety and immunogenicity of the inactivated, whole virus vaccine showed the vaccine stimulated a strong humoral immune response. In this study, the preliminary results in mice showed that the Nab titers reached the peak post-priming and boosting in 21 days. Titers in 1, 4, 6, and 10 µg groups found about 1/39, 1/ 595, 1/791, and 1/1048, respectively.

Even the placebo and vaccination of Balb/c mice were challenged with 10^5 TCID₅₀ /50 µL doses of SARS-CoV-2 by the intranasal route, lethality and clinical pneumonia were not reported. SARS-CoV-2 showed good binding for human ACE2 but limited binding to mice ACE2. The SARS-CoV-2 virus showed slight pneumonia without apparent clinical signs and replication was remained in limit in other epithelial tissues [6].

Based on the results presented here, a Phase I clinical trial of candidate COVID-19 vaccine is currently in six months evaluation progress (NCT04838080), and a Phase II of the clinical trial has recently been initiated. These clinical trials have been designed using the same aluminum adjuvant formulation described here, with two different groups of 4, and 6 µg dose groups to evaluate the appropriate dose for further clinical application.

About the histopathologic examination of the organs on Balb/c mice, there were not any reports we found that performs systemic examinations. The published report generally investigates changes in lung tissue and olfactoric bulbs in Balb/c mice [1, 22].

In all groups that received intraperitoneal vaccination, varying degrees of changes were observed in the abdominal lipid tissue. These findings were similar to the findings of Portuondo et. al. [23] where they observed palpable nodules at the injection site in all animals injected with aluminum hydroxide adjuvants. Meningeal lymphoid infiltrations observed in animals were similar in cases in humans with focal leptomeningeal inflammation described by Lee et. al. [24]. However, we could not detect any reports regarding the changes observed in Balb/c mice. The most significant finding was varying degrees of hemorrhages and Zenker degenerations in the heart of animals that receive treatment and/or virus application. These changes were similar to the findings reported in Syrian hamsters [21, 25] and humans [26]. The findings of mild focal myocardial degeneration in Syrian hamsters after virus application were similar to our findings and observed more intensely in non-vaccinated animals than vaccinated animals. Widespread parenchymatous degeneration and varying degrees of fatty degeneration were detected in the livers of all animal groups. Detecting these changes in all animals gives rise to the thought of changes due to ad libitum feeding, type of feed, or inactivity inside the cages. The changes observed in the kidneys were limited to 2 animals from different groups, and due to small populations, we thought they could be negligible and no literature data in Balb/c to compare was determined.

In the literature about Balb/c mice generally, lung tissue changes were observed in aged mice [1, 22, 27]. When the necropsies were performed our mouse population was aged between 90–100 days. According to Hongjing et. al. [1], modified virus applications similar to our modification can cause some changes in the young Balb/c mouse at early ages, and this finding was like our findings in the lung tissues. The increased lymphocytic infiltration especially in the vaccinated group was thought to be due to the immune response gained by the vaccine. Small doses of vaccination were observed to cause vascular changes but to report that much larger populations, where statistical analyzes can be performed should be used. Like in the lung tissue increased lymphocytic hyperplasia in the splenic tissue of vaccinated animals was thought to be due to the immune response gained by the vaccine. Increased megakaryocytes in the spleen are observed in all animals which we think is a result of repetitive blood collections for titrations.

Conclusion

In the presented study the preclinical development of an inactivated COVID-19 vaccine candidate is described. Initial results describing the immunogenicity of the COVID-19 vaccine candidate, are promising, and it is particularly encouraging to measure neutralization antibodies, T-cell response, and weight losses in Balb/c mice. Also, protection against challenge was demonstrated in transgenic mice compared to the control group. P values are shown for comparisons that resulted in statistical significance ($P < 0.05$) between vaccinated groups and placebo. Median doses (4 and 6 µg) showed a good humoral and T-cell immune response and not any gross and pathological findings. We provide evidence for the safety of inactivated COVID-19 vaccine candidates in Balb/c mice and ferrets did not observe infection enhancement and immunopathological changes in two doses vaccinated Balb/c mice and tree doses vaccinated ferrets. SARS CoV-2 vaccine candidate is efficiently produced, genetically stable, and safe in Balb/c mice, K18 hACE-2 transgenic mice, and ferrets. Vaccine candidate (KOCaK-19) has been authorized by the Turkish Medicines and Medical Devices Agency and has now entered phase 2 clinical development (NCT04838080).

Abbreviations

SARS-CoV-2

Severe Acute Respiratory Syndrome Coronavirus 2

MERS-CoV

Middle East Respiratory Syndrome Coronavirus

qRT-PCR

Quantitative Real-Time Polymerase Chain Reaction

TEM

Transmissible Electron Microscopy

SEC

Size Exclusion

AEX

Anion exchange

BPL

β-Propiolactone

CPE

Cytopathic Effect

TCID₅₀

50% Tissue Culture Infectious Dose

hACE-2

Human Angiotensin-Converting Enzyme 2

mRNA

Messenger RNA

RBD

Receptor binding domain

Nab

Neutralizing antibody

MOI

Multiply of Infection

Declarations

Ethics declarations

The Animal Local Ethics Committee of Kocak Pharmaceuticals approved all animal experiments in this study (No. 2020-5).

Availability of data and material

The key information and data generated and/or analyzed during this study were included in this article.

Human tissue, human participants, and human data

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

References

1. Gu H, Chen Q, Yang G, He L, Fan H, Deng YQ, Wang Y, Teng Y, Zhao Z, Cui Y, Li Y, Li XF, Li J, Zhang NN, Yang X, Chen S, Guo Y, Zhao G, Wang X, Luo DY, Wang H, Yang X, Li Y, Han G, He Y, Zhou X, Geng S, Sheng X, Jiang S, Sun S, Qin CF, Zhou Y. Adaptation of SARS-CoV-2 in BALB/c mice for testing vaccine efficacy. *Science*. 2020 Sep 25;369(6511):1603–1607. DOI: 10.1126/science.abc4730. Epub 2020 Jul 30. PMID: 32732280; PMCID: PMC7574913.
2. Tang X, Wu C, Li X, Song Y, Yao X, Wu X, et al. On the origin and continuing evolution of SARS-CoV-2. *Natl Sci Rev* 2020;7. <https://doi.org/10.1093/nsr/nwaa036>.
3. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterization and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 2020;395. [https://doi.org/10.1016/S0140-6736\(20\)30251-8](https://doi.org/10.1016/S0140-6736(20)30251-8).
4. Andersen KG, Rambaut A, Lipkin WI, Holmes EC, Garry RF. The proximal origin of SARS-CoV-2. *Nat Med* 2020;26. <https://doi.org/10.1038/s41591-020-0820-9>.

5. Spruth M, Kistner O, Savidis-Dacho H, Hitter E, Crowe B, Gerencer M, et al. A double-inactivated whole virus candidate SARS coronavirus vaccine stimulates neutralizing and protective antibody responses. *Vaccine* 2006;24. <https://doi.org/10.1016/j.vaccine.2005.08.055>.
6. Gao Q, Bao L, Mao H, Wang L, Xu K, Yang M, Li Y, Zhu L, Wang N, Lv Z, Gao H, Ge X, Kan B, Hu Y, Liu J, Cai F, Jiang D, Yin Y, Qin C, Li J, Gong X, Lou X, Shi W, Wu D, Zhang H, Zhu L, Deng W, Li Y, Lu J, Li C, Wang X, Yin W, Zhang Y, Qin C. Development of an inactivated vaccine candidate for SARS-CoV-2. *Science*. 2020 Jul 3;369(6499):77–81. DOI: 10.1126/science.abc1932. Epub 2020 May 6. PMID: 32376603; PMCID: PMC7202686.
7. Wang H, Zhang Y, Huang B, Deng W, Quan Y, Wang W, et al. Development of an Inactivated Vaccine Candidate, BBIBP-CorV, with Potent Protection against SARS-CoV-2. *Cell* 2020;182. <https://doi.org/10.1016/j.cell.2020.06.008>.
8. Kumar, A., Dowling, W.E., Román, R.G. et al. Status Report on COVID-19 Vaccines Development. *Curr Infect Dis Rep* 23, 9 (2021). <https://doi.org/10.1007/s11908-021-00752-3>
9. Lou F, Li M, Pang Z, Jiang L, Guan L, Tian L, Hu J, Fan J and Fan H (2021) Understanding the Secret of SARS-CoV-2 Variants of Concern/Interest and Immune Escape. *Front. Immunol.* 12:744242. <https://doi.org/10.3389/fimmu.2021.744242>.
10. Mohamed NA, Solehan HM, Mohd Rani MD, Ithnin M, Che Isahak CI (2021) Knowledge, acceptance and perception on COVID-19 vaccine among Malaysians: A web-based survey. *PLoS ONE* 16(8): e0256110. <https://doi.org/10.1371/journal.pone.0256110>.
11. COVID-19 Vaccines Authorized for Emergency Use, FDA 21.01.22 <https://www.fda.gov/emergency-preparedness-and-response/coronavirus-disease-2019-covid-19/covid-19-vaccines#authorized-vaccines>
12. Amanat F, Strohmeier S, Meade P, Dambrauskas N, Muñhlemann B, Smith DJ, et al. (2021) Vaccination with SARS-CoV-2 variants of concern protects mice from challenge with wild-type virus. *PLoS Biol* 19(12): e3001384. <https://doi.org/10.1371/journal.pbio.3001384>.
13. Jureka A, Silvas J, Basler C. Propagation, Inactivation, and Safety Testing of SARS-CoV-2. *Viruses* 2020;12. <https://doi.org/10.3390/v12060622>.
14. Kärber G. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Naunyn Schmiedebergs Arch Exp Pathol Pharmacol* 1931;162. <https://doi.org/10.1007/BF01863914>.
15. Manenti A, Maggetti M, Casa E, Martinuzzi D, Torelli A, Trombetta CM, et al. Evaluation of SARS-CoV-2 neutralizing antibodies using a CPE-based colorimetric live virus micro-neutralization assay in human serum samples. *J Med Virol* 2020;92. <https://doi.org/10.1002/jmv.25986>.
16. P, Morgeaux S. Inactivation of DNA by beta-propiolactone. *Biologicals*. 1995 Sep;23(3):207 – 11. doi: 10.1006/biol.1995.0034. PMID: 8527119.
17. Muruato AE, Fontes-Garfias CR, Ren P, Garcia-Blanco MA, Menachery VD, Xie X, et al. A high-throughput neutralizing antibody assay for COVID-19 diagnosis and vaccine evaluation. *Nat Commun* 2020;11. <https://doi.org/10.1038/s41467-020-17892-0>.
18. Pavel STI, Yetiskin H, Aydin G, Holyavkin C, Uygut MA, Dursun ZB, et al. Isolation and characterization of severe acute respiratory syndrome coronavirus 2 in Turkey. *PLoS One* 2020;15. <https://doi.org/10.1371/journal.pone.0238614>.
19. Dhama K, Khan S, Tiwari R, Sircar S, Bhat S, Malik YS, Singh KP, Chaicumpa W, Bonilla-Aldana DK, Rodriguez-Morales AJ. 2020. Coronavirus disease 2019–COVID-19. *Clin Microbiol Rev* 33:e00028-20. <https://doi.org/10.1128/CMR.00028-20>.
20. Xia S, Duan K, Zhang Y, Zhao D, Zhang H, Xie Z, et al. Effect of an Inactivated Vaccine Against SARS-CoV-2 on Safety and Immunogenicity Outcomes. *JAMA* 2020;324. <https://doi.org/10.1001/jama.2020.15543>.
21. Zeiss CJ, Compton S, Veenhuis RT. Animal Models of COVID-19. I. Comparative Virology and Disease Pathogenesis. *ILAR J* 2021. <https://doi.org/10.1093/ilar/ilab007>.
22. Dinno KH, Leist SR, Schäfer A, Edwards CE, Martinez DR, Montgomery SA, et al. A mouse-adapted model of SARS-CoV-2 to test COVID-19 countermeasures. *Nature* 2020;586:560–6. <https://doi.org/10.1038/s41586-020-2708-8>.
23. Portuondo DL, Batista-Duharte A, Ferreira LS, de Andrade CR, Quinello C, Téllez-Martínez D, et al. Comparative efficacy and toxicity of two vaccine candidates against *Sporothrix schenckii* using either Montanide™ Pet Gel A or aluminum hydroxide 1/adjuvants in mice. *Vaccine* 2017;35. <https://doi.org/10.1016/j.vaccine.2017.05.046>.
24. Lee MH, Perl DP, Nair G, Li W, Maric D, Murray H, Dodd SJ, Koretsky AP, Watts JA, Cheung V, Masliah E, Horkayne-Szakaly I, Jones R, Stram MN, Moncur J, Hefti M, Folkert RD, Nath A. Microvascular Injury in the Brains of Patients with Covid-19. *N Engl J Med*. 2021 Feb 4;384(5):481–483. DOI: 10.1056/NEJMc2033369. Epub 2020 Dec 30. PMID: 33378608; PMCID: PMC7787217.
25. Chan JF-W, Zhang AJ, Yuan S, Poon VK-M, Chan CC-S, Lee AC-Y, et al. Simulation of the Clinical and Pathological Manifestations of Coronavirus Disease 2019 (COVID-19) in a Golden Syrian Hamster Model: Implications for Disease Pathogenesis and Transmissibility. *Clin Infect Dis* 2020. <https://doi.org/10.1093/cid/ciaa325>.
26. Diaz GA, Parsons GT, Gering SK, Meier AR, Hutchinson I V., Robicsek A. Myocarditis and Pericarditis After Vaccination for COVID-19. *JAMA* 2021. <https://doi.org/10.1001/jama.2021.13443>.
27. Zhang Y, Huang K, Wang T, Deng F, Gong W, Hui X, et al. SARS-CoV-2 Rapidly Adapts in Aged BALB/c Mice and Induces Typical Pneumonia. *J Virol* 2021;95. <https://doi.org/10.1128/jvi.02477-20>.

Figures

Blue staining indicates cells are alive thus no CPE, Clear indicates viral CPE.

Figure 1

Viral CPE (Vero cells seeded at 5×10^4 cells/well with SARS-CoV-2 5th passage log₁₀ dilutions). Each of the samples were performed in the well-plate at four repeat. After 4 days in culture 96 well plates were fixed and stained with formaldehyde and crystal violet, respectively. Plates were washed in water, dried and scanned. The end-point titres (TCID₅₀) were calculated according to the Reed & Muench method based on four replicates for titration

Figure 2

Propagation of SARS-CoV-2 in VERO CCL-81 cell culture. **A.** Healthy and confluent VERO cells in T-175 Flasks. **B.** 62.h after the inoculation of SARS-CoV-2 in T-175 Flasks. **C.** Healthy and confluent VERO cells on Cytodex-1 microcarriers in Biostat RM 20L Bioreactor. **D.** 62.h after the inoculation of SARS-CoV-2 on Cytodex-1 microcarriers in Biostat RM 20L Bioreactor.

Figure 3

Quantification of SARS-CoV-2 in CCL-81 cell culture. Plaque assays were performed on harvested virus at 62 h post-infection, fixed, and stained with crystal violet to visualize. A, B, C, D, E wells are ten-fold dilutions of virus and F well is the negative control.

Figure 4

Viral kinetics of the KOCAC-19 strain at different MOIs. **A.** Growth kinetics titers as a PFU/ml **B.** Growth kinetics titers as a TCID₅₀/ml

Abs Quant/2nd Derivative Max

Settings

Channel	465-510	
Color Compensation	Off	
Program	cycling	Units
Mode	High Confidence	
Subset Name	viral titre	

Results

Inc	Pos	Name	Type	CP	Concentration	Standard	Status
☒	B7	copy 10^5	Standard	18.31	2.58E5	2.00E5	
☒	B8	copy 10^3	Standard	25.05	1.20E3	2.00E3	
☒	B9	copy 10^1	Standard	29.88	2.58E1	2.00E1	
☒	B11	negative	Negative Control				
☒	D9	SARS-COV-2 smp	Unknown	12.83	2.04E7		E
☒	D10	SARS-COV-2 smp	Unknown	12.64	2.37E7		E
☒	D11	SARS-COV-2 smp	Unknown	12.08	3.69E7		E

E - Extrapolated concentration in standard curve

Figure 5
qRT-PCR results for SARS-COV-2 harvest samples by cultivated in Vero CCL-81 cell line.

Figure 6

SARS-COV-2 SDS gel electrophoresis against known BSA (100 ng and 250 ng/25 μ l) standards

Figure 7

A. SARS-COV-2 Spike bands, aprox.190 kDa B. Nucleoprotein bands, approx. 48 kDa in Western Blotting

Figure 8

A. Negative stain transmission electron microscope (TEM) images of SARS-CoV-2 virus particles in a Vero cell vesicle at different magnifications. B. TEM images of oval-shaped particles with diameters of 90 to 110 nm of the virus after the final purification step at different magnifications.

Figure 9
Mouse neutralization antibody (NAb) levels with different doses by two-dose immunization. Mice were injected intraperitoneally (IP) route by using two-time immunization (D0/D21), and the NAb levels at 35 days after the first immunization were tested by the microtitration method (n = 10).

Figure 10
Microneutralization analysis of Balb/c mice Nab(s) was carried out in 96 well plate coated by Vero CCL-81 cells. The assay were performed on 0,7,21, and 35 days after the first immunization, neutralized by 100 TCID₅₀ SARS-CoV-2 virus, incubated 4 days, fixed and stained with crystal violet to visualize. First 10 well columns are belongs to two-fold dilutions of Nab, 11th well columns are the negative control and 12th well columns are positive virus controls. Each of the samples were performed in the well-plate at four repeat

Figure 11

The ELISPOT assay were performed on 35th days after the first immunization in three Balb/c mice (each of 3 animals).

Figure 12

K-18 hACE 2 transgenic mice placebo, 4µg and 6 µg groups (each of 10 animals) mean weight changing graphs. Five animals in placebo group died at 5 – 7 dpi. No any symptoms in both of the vaccinated groups

Figure 13

Viral loads were performed using SARS-CoV-2 qRT- PCR 3th days following the challenge in three K-18 hACE transgenic mice (each of 3 animals).

Figure 14

A. Hematological parameters in control and 8 μg doses vaccinated ferrets **B.** Biochemical parameters in control and 8 μg doses vaccinated ferrets after end of the 2 months of first injection

Figure 15

Mean weight gain percentage of Balb/c mice vaccinated with different doses of vaccine candidate during to two months. Error bars represent $\pm$ standard deviation.

Figure 16

A) Changes in the lungs. Hematoxylin-eosin, G1-1, Perivascular and peribronchial lymphocytic infiltrations (arrow). **B)** Changes in the lungs. Hematoxylin-eosin, G1-4, Fibrinoid necrosis in the small vessels (arrow). **C)** Changes in the lungs. Hematoxylin-eosin, G2-3, Hyperemia, and perivascular lymphocytic infiltrations (arrow). **D)** Changes in the lungs. Hematoxylin-eosin, G4-2, Thrombosis in small vessels (arrow). **E)** Changes in the central nervous system. Hematoxylin-eosin, G2-4, Per diapedesis bleeding in the meninges (arrowhead). **F)** Changes in the central nervous system. Hematoxylin-eosin, G2-3, Perivascular lymphoid infiltrations, and micro thrombosis (arrow). **G)** Changes in the heart. Hematoxylin-eosin, G2-2, Widespread Zenker degenerations (arrow). **H)** Changes in the heart. Hematoxylin-eosin, G2-1, Widespread Zenker degenerations (arrow). **I)** Changes in the abdominal fat tissue. Hematoxylin-eosin, G4-4 Granulomatous changes; lymphocytic infiltrations, macrophages, and small necrosis (arrow). Wide calcified necrosis in a granuloma (star). **J)** Changes in the spleen. Hematoxylin-eosin, G2-2, Lymphocytic hyperplasia throughout spleen (yellow arrow) and increased megakaryocytes (black arrow).