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Role of gamma irradiation and disaccharide trehalose to induce immune responses in Syrian hamster model against Iranian SARS-CoV-2 virus isolate

Farahnaz Motamedi-Sedeh[1]*, Akbar Khorasani[2], Mohsen Lotfi[3], Seyed Morteza Moosavi[1,4], Arash Arbabi[5], Seyedeh Maede Hosseini[5]

[1] Department of Veterinary and Animal Diseases, Nuclear Agriculture Research School, Nuclear Science and Technology Research Institute, Karaj, Iran; [2] Department of FMD Vaccine, Razi Vaccine and Serum Research, Education and Extension Organization (AREEO), Karaj, Iran; [3] Department of Quality Control, Razi Vaccine and Serum Research, Education and Extension Organization (AREEO), Karaj, Iran; [4] Iran Veterinary Organization, Mashhad, Iran; [5] MD Student, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.

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Abstract

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus is the causative agent of the emerging zoonotic respiratory disease. One of the most important prerequisites for combating emerging diseases is the development of vaccines within a short period of time. In this study, antigen-irradiated, inactivated SARS-CoV-2 viruses and the disaccharide trehalose were used to enhance immune responses in the Syrian hamster. The SARS-CoV-2 virus was isolated from tracheal swabs, confirmed by real-time polymerase chain reaction, and propagated on Vero cells. For inactivation, it was irradiated with 14.00 kGy gamma radiation. Evaluation of the antigenic properties of the spike protein subunit S1 showed that the antigens were intact after gamma irradiation. The gamma-irradiated and formalin-treated viruses were used to immunize hamsters in four vaccine formulations. Neutralizing antibodies increased significantly in all vaccinated groups three weeks after the second and third vaccinations. The concentration of secretory immunoglobulin A in the irradiated vaccine plus trehalose increased significantly in nasal lavage and nasopharyngeal-associated lymphoid tissue fluids three weeks after the second and third vaccinations. The lymphocyte proliferation test in the spleen showed a significant increase in all vaccinated hamsters, but the increase was greater in irradiated vaccine plus trehalose and irradiated vaccine plus alum. We can recommend the irradiated inactivated vaccine SARS-CoV-2 plus trehalose (intra-nasal) and another irradiated inactivated vaccine SARS-CoV-2 plus alum (subcutaneous) as safe vaccines against coronavirus disease of 2019 (COVID-19), which can stimulate mucosal, humeral, and cellular immunities. However, the protectivity of the vaccine against COVID-19 in vaccinated hamsters must be investigated in a challenge test to assess the potency and efficiency of vaccine.

Introduction

Coronaviruses are enveloped RNA viruses being widespread among humans, other mammals, and birds and cause acute and persistent infections. Viruses of the Coronaviridae family are large, enveloped virions with positive RNA genomes (27.00 - 32.00 Kb) and diameters of 100 - 120 nm. Severe acute respiratory syndrome (SARS) emerged as a new human disease worldwide in 2002. Another zoonotic coronavirus, Middle East Respiratory Syndrome (MERS), was transmitted by camels and caused the often fatal disease in humans in 2012.[1,2] Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of the new emerging respiratory zoonosis known as coronavirus disease of 2019 (COVID-19) from 2019. One of the most important prerequisite requirements for combating emerging and re-emerging zoonoses is the development of vaccines within a short period. The most recent example is the inactivated whole virus vaccine against SARS-CoV-2, which is still in use. The use of chemical substances, such as formalin and beta-propiolactone, is the common method for producing inactivated vaccines.[3]

However, the chemicals reduced immunogenicity due to the damage of antigenic epitopes. Although inactivation with formalin is well established, the process has serious drawbacks, *e.g.*, formalin is toxic and there are problems with residual traces in the final product. In addition,

***Correspondence:**

Farahnaz Motamedi-Sedeh. PhD
Department of Veterinary and Animal Diseases, Nuclear Agriculture Research School, Nuclear Science and Technology Research Institute, Karaj, Iran
E-mail: fmotamedi @aeoi.org.ir

formalin leads to cross-linking and changes in the structural components and damages the antigenic structures, impairing and affecting the antigenicity of vaccines.[3] Other chemicals, such as beta-propiolactone, are also very dangerous. Therefore, an alternative to the chemical inactivation method must be found to produce an inactivated vaccine.[4] Recently, ionizing irradiation (gamma radiation by Cobalt -60, X-rays, and electron beams) has been considered as an alternative for vaccine development. Gamma rays are short electromagnetic rays having less effect on the antigenic properties of viral proteins compared to the chemical substances.[5] The method of inactivation by irradiation has some advantages over chemical inactivation for vaccine production.[6] The strength of gamma irradiation lies in the fact that viral replication can be eliminated while immunogenicity is maintained. As a result, the viral proteins are naturally presented to the immune system, facilitating the induction of both T-cell and humoral immunity.[7] The advantage of irradiated vaccines is that, because they are inactivated, they retain their immunogenicity even if they are not stored refrigerated, eliminating the need for a cold chain to maintain the vaccine's efficacy. The ability to store vaccines at room temperature or refrigerated (*versus* frozen storage) can significantly reduce the overall cost of vaccine transportation and distribution. The ability to distribute vaccines without a cold chain also improves access to vaccines in remote areas.[8] Another critical issue is the question of a cellular receptor. The SARS-CoV-2 could use the angiotensin-converting enzyme 2 (ACE2) receptor of humans, bats, or civets, but not that of mice.[9,10] Suitable small animal models are crucial for the study of viral pathogenesis, vaccines production, and anti-viral therapies development. Hamster models are very popular due to their affordability, availability, and simple genetic structure, and have been widely used to study the pathogenesis of the human coronavirus. Hamsters vaccinated against the SARS-CoV-2 virus were able to effectively induce memory B and T cells and were able to protect the animals from reinfection with the same strain.[11] Neutralizing antibodies (nAbs) against SARS-CoV-2 represent potential prophylactic and therapeutic options and could help vaccine development.[3,12] This study aimed to investigate the efficacy of gamma irradiation in inactivating emerging/re-emerging pathogens. In this study, we tested gamma irradiation and formalin-inactivated vaccine candidates for their immunogenicity and immune responses against the SARS-CoV-2 virus and demonstrated the efficacy of the vaccine in Syrian hamsters. Another goal of this research was to use the disaccharide trehalose as a protein protectant to enhance the immune response. Trehalose could protect protein molecules from the effects of cryo-damage and free radicals. Trehalose can stabilize proteins and prevent protein denaturation by keeping water molecules away from the surface of proteins when cells are in a dehydrated state. The dry state keeps the proteins in their folded state by replacing the water molecules and forming hydrogen bonds directly with the proteins and their structure. Also, trehalose acts as a natural stabilizer of life processes.[13]

Materials and Methods

Virus (isolation, real time-polymerase chain reaction, and multiplication on Vero cells). The isolated SARS-CoV-2 virus from tracheal swabs of an infected man (43 years old) in Karaj, Iran, in May 2020 on Vero cells was used for this study. The cycle threshold value of this sample in the real-time quantitative polymerase chain reaction (RT-QPCR) assay was 16. The tracheal swab samples were transferred to viral transport media, and transported under the cold chain to the cell culture laboratory and inoculated on Vero cells with Dulbecco's Modified Eagle Medium plus (Millipore Sigma, Darmstadt, Germany) 3.00% fetal calf serum (Millipore Sigma) and 1.00% penicillin-streptomycin solution (Millipore Sigma) including 10,000 units of penicillin and 10.00 mg mL^{-1} streptomycin at 37.00 ˚C and 5.00% CO_2. The cytopathic effect (CPE) was visible after 24 - 48 hr on Vero cells in the form of cell rounding and the development of syncytial cells. The virus samples were isolated and propagated in biosafety level III facilities at Razi Vaccine and Serum Research Institute, Karaj, Iran. The infected cells were harvested and confirmed using a RT-qPCR kit (AccuPower® SARS-CoV-2 RT-PCR Kit, Bioneer, Daejeon, South Korea). The infected cells were used to infect four interval passages on Vero cells. After the fourth passage of infected cells was harvested, it was used for RT-PCR assay with specific primers for the *spike* (S) gene. The PCR product of the S gene was sequenced and the nucleotide sequence was deposited in NCBI under the accession number of MW709393 as the SARS-CoV-2/human/IRN/ Alborz- IR /2020 surface glycoprotein (S) gene with a length of 3,822 bp.[12] The isolated SARS-CoV-2 virus was propagated on Vero cells and the infected cell suspension was centrifuged at 4.00 ˚C for 15 min at low speed to remove cell debris. The supernatant was collected as a virus broth and divided into three parts. The 20.00% disaccharide trehalose (1.00 M; Merck, Darmstadt, Germany) was added to the first part, while the second and third parts did not contain trehalose.

SARS virus inactivation using gamma irradiation and formalin. The SARS-CoV-2 virus stocks were irradiated with a Cobalt-60 Irradiator, Gamma Cell 220 (MDS Nordion, Ottawa, Canada), at a dose rate of 0.93 Gy per sec and an activity of 3,985 Curie to inactivate viral infectivity and SARS-CoV-2 virus genomic RNA at the Nuclear Science and Technology Research Institute, Tehran, Iran. Gamma-ray doses of 2.00, 4.00, 5.00, 8.00, 10.00, and 15.00 kGy were administered to infected

samples frozen on dry ice. Each dose was repeated in three rounds.[5,13,14] Virus titration of all irradiated samples was performed using the 50.00% tissue culture infectious dose ($TCID_{50}$) method. The third virus stock was inactivated with 0.04% formalin (v/v) at room temperature for 30 hr (final concentration: 4.00 : 10,000). The inactivated virus stocks (irradiated and treated with formalin) were concentrated by ultrafiltration and 8.00% polyethylene glycol 6,000. The concentrated and inactivated viruses were dialyzed against phosphate-buffered saline (PBS) and the protein concentration was quantified with Nano-Drop (ND-1,000 UV/Vis, Thermo Fisher Scientific Inc., Toronto, Canada).

Safety test and antigenicity evaluation by enzyme-linked immunosorbent assay (ELISA). The infectivity of the irradiated, inactivated SARS-CoV-2 virus was determined after inoculation of Vero cell monolayers at 37.00 ˚C for 48 hr and then, it was sub-cultured in four blind cultures on fresh Vero cells and the viral titration was determined by the $TCID_{50}$ method.[5,13] In addition, the antigenic properties of the irradiated and native virus samples were tested with the ELISA for the S protein. The SARS-CoV-2 S protein is one of the most important surface glycoproteins. It consists of S1 and S2 domains and binds to host cell receptors (ACE2). This protein plays an important role in viral infection and is involved in the fusion of viral envelope with cell membrane. The ELISA method was used to check the antigenic properties of the irradiated virus samples in comparison with the control virus. The SARS-CoV-2 anti-spike protein S1 monoclonal antibody (Invitrogen, Waltham, USA) and goat anti-mouse immunoglobulin (Ig) G (H+L; horseradish peroxidase [HRP] conjugate; Invitrogen) reconstituted in 1.00 mL PBS (pH: 7.20), to obtain 1.00 mg mL^{-1} stock solution, were used. The receptor-binding domain (RBD) of S1 can bind specifically to ACE2, the receptor on the target cells. The antigenicity of SARS-CoV-2 viruses was assessed by ELISA, whereby serial dilutions (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, and 1/512) of irradiated and non-irradiated viral antigens were first prepared in bicarbonate buffer (pH: 9.60) as a coating buffer. A volume of 100 µL of each antigen dilution was added to each well of the 96-well micro-plate in duplicate and incubated overnight at 4.00 ˚C. The suspension of Vero cells was used in duplicate as a negative control. Subsequently, all wells were washed five times with wash buffer including PBS + 0.05% Tween 20 (Merck). Two-hundred µL of blocking buffer (PBS + 5.00% skim milk) was added to each well and stored at 37.00 ˚C for 2 hr to block the wells, after which they were washed three times. The GT263 (1.00 mg mL^{-1}; Invitrogen) was diluted 1.00 : 1,000, and 100 µL of the dilution was added to each well and stored at 37.00 ˚C for 1 hr, after which the wells were washed three times. The H+L (HRP conjugate; Invitrogen), reconstituted in 1.00 mL PBS (pH: 7.20), to obtain 1.00 mg mL^{-1} stock solution, was stored at – 20.00 ˚C. The working solution (1.00 : 10,000) was prepared and 100µL of the working solution was added to each well for 1 hr at 37.00 ˚C. The 3, 3', 5, 5'-Tetramethylbenzidine (100µL) was added to each well in the dark at room temperature for 30 min. Finally, a stop solution (1.25 mM sulfuric acid; Merck) was added and the optical density (OD) was read at 450 nm using an ELISA reader (MR4 Plus; Hyperion, Miami, USA).

Vaccine formulation. The first vaccine formulation was irradiated SARS-CoV-2 viruses and the second vaccine formulation was irradiated SARS-CoV-2 viruses plus 20.00% disaccharide trehalose (1.00 M). Trehalose can stabilize proteins as a cryoprotectant and free radical scavenger and acts as a natural stabilizer of life processes.[15-18] The third and fourth vaccine formulations were formalin-inactivated viruses and irradiated viruses mixed with alum, respectively. Alum compounds, such as aluminum hydroxide (Merck) or alhydrogel (Merck), increase the uptake and presentation of the antigen by antigen-presenting cells and enhance monocyte recruitment at the site of inoculation.

Syrian hamster immunization by four inactivated SARS-CoV-2 vaccines. Four regimens of inactivated SARS-CoV-2 vaccines were formulated and used to immunize male Syrian hamsters weighing 100 ± 10.00 g. Forty-two Syrian hamsters were purchased from the Razi Vaccine and Serum Research Institute, Karaj, Iran, and divided into seven groups of six animals each (Table 1). The first group was used as pre-immune animals and sampling was done before immunization. The four other groups of animals were immunized with irradiated inactivated SARS-CoV-2 antigen (intra-nasal), irradiated inactivated SARS-CoV-2 antigen + 20.00% trehalose (intra-nasal), formalin-inactivated SARS-CoV-2 antigen + alum (subcutaneous), and irradiated inactivated SARS-CoV-2 antigen + alum (subcutaneous). The negative control group vaccinated with 100µL sterile PBS (intra-nasal) and negative control group injected with 100µL sterile PBS (subcutaneous) were also included. The vaccination was administered *via* two routes (intra-nasal and subcutaneous injection into the neck) as one prime and two booster doses three weeks apart. The blood samples were taken from the orbit of anesthetized animals three weeks after each vaccination. Each hamster was anesthetized and then, blood was collected from the inner corner of the eye using a sterile Pasteur pipette. The blood tubes were stored overnight at 4.00 ˚C and then, the sera were separated by centrifugation at 600 g for 10 min at 4.00 ˚C. The complement compounds in the isolated sera were inactivated at 56.00 ˚C for 30 min. The sera were tested for the presence of antibodies against the SARS-CoV-2 virus using the SARS-CoV-2 nAb Detection Kit (Abeomics Inc., San Diego, USA). The spleen lymphocytes of the hamster groups were cultured and stimulated by homologous inactivated antigens to perform the spleen lymphocyte proliferation

assay as a cellular immunity 3 weeks after the second and third vaccinations (three animals in each group were used for each collection day). The schedule for vaccination of the animals is shown in Table 1. The animal study was reviewed and approved by Tehran University of Medical Sciences, Tehran, Iran. For this research, all institutional and national guidelines adopted by the horizontal legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU, as amended by Regulation 2019/1010) were approved for implementation.

Neutralizing antibody detection. The SARS-CoV-2 nAb Detection Kit (Abeomics Inc.) contains the key reagents required for the functional detection of nAbs to SARS-CoV-2 in sera. It is a colorimetric kit for measuring the neutralizing activity of antibodies in sera after binding of the SARS-CoV-2 S protein (RBD) to the ACE2 receptor. The recombinant SARS-CoV-2 S protein (RBD) was pre-coated onto the 96-well micro-titer plate. The procedure was performed according to the manufacturer's instructions. The presence of nAbs in the serum samples was detected by a decrease in OD, indicating inhibition of binding between spike (RBD) and ACE2. Percent inhibition was calculated for each sample using the following formula:

Inhibition (%) = 1 – OD of *sample*/*OD* of *negative control*) × *100*

Mucosal immunity assay. The hamster IgA ELISA kit (MyBioSource, San Diego, USA) as a quantitative sandwich ELISA kit was used to determine the level of IgA in original serum, tissue samples, or other fluids. The fluids of nasal cavity and nasopharyngeal-associated lymphoid tissue (NALT) of the vaccinated hamsters were collected three weeks after the second and third vaccinations to determine the IgA content using the ELISA method. Three hamsters *per* group were randomly scarified. The nasal cavity was then washed and the alveoli were rinsed with 1.00 mL of sterile PBS plus complete Roche protease inhibitor. Fluids were then collected from the nasal cavity and bronchoalveolar lavage was used to detect mucosal antibodies (IgA) with the hamster IgA ELISA kit.

Spleen lymphocyte proliferation response. The spleen lymphocytes of the vaccinated hamsters were collected aseptically 3 weeks after two booster vaccinations. The single spleen lymphocyte suspensions were prepared and incubated in 96-well plates at 5.00 × 10^4 cells per well in RPMI 1640 plus (Millipore Sigma) 10.00% fetal calf serum at 37.00 ˚C and 5.00% CO_2. Cells were stimulated with 50.00 µL phytohemagglutinin (50.00 µg mL^{-1}; positive control), 2.50 µg mL^{-1} inactivated SARS-CoV-2 antigen (specific antigen stimulation), and no antigen (negative control) in triplicate. After 48 hr, the cell proliferation ELISA, the BrdU assay (colorimetric kit; Roche, Mannheim, Germany), was performed according to the manufacturer's instructions. The BrdU kit allows accurate assessment of cell proliferation by measuring BrdU incorporation into newly synthesized cellular DNA. Absorbance was measured at 450 nm and the stimulation index was calculated as the mean OD value of three wells corresponding to cells stimulated with an antigen divided by the mean OD value of the triple-negative control wells.[17,19]

Statistical analysis. Comparisons of mean values for nAb titration, lymphocyte proliferation, and mucosal immunity were performed with SPSS Software (version 16.0; SPSS Inc., Chicago, USA), using one-way test (ANOVA) and Duncan's multiple range tests in hamster experiment with a sample size of forty-two Syrian hamsters. All values were expressed as mean ± standard deviation, and a significant *p* value was considered ≤ 0.05 statistically.[17]

Results

Inactivated SARS-CoV-2 virus. To produce inactivated SARS-CoV-2 viruses, two frozen concentrated virus stocks, one with trehalose and the other without trehalose, were irradiated with increasing doses of gamma radiation (2.00 - 15.00 kGy) from a Cobalt-60 Gamma Irradiator, and virus infectivity was assessed using a $TCID_{50}$ method. Using the 50.00% endpoint titers of the irradiated and unirradiated samples (Table 2), a dose-response curve (Fig. 1) was generated using OriginPro Software (version 6.1; Taksan, Tehran, Iran) and the D_{10} value, *i.e.*, the gamma radiation dose required to reduce viral infectivity by one log_{10} of viral virus infectivity, was calculated.[20,21] Based on the dose-response curve and the log-linear regression equation (*Y= 5.826 - 0.591X*), the D_{10} value was set to

Table 1. The samples collection and vaccination schedule against severe acute respiratory syndrome coronavirus 2 virus (n = 6 in each group and vaccine dose = 100 µL).

Groups	Routes	Vaccination days	Sampling days for nAb titration	Sampling days for SLP assay	Sampling days for IgA titration
PI	_	_	1	1	1
C	IN	1, 21, 42	21, 42, 63, 84	21, 42, 63, 84	21, 42, 63, 84
I.V.T	IN	1, 21, 42	21, 42, 63, 84	21, 42, 63, 84	21, 42, 63, 84
F.V.A	SC	1, 21, 42	21, 42, 63, 84	21, 42, 63, 84	21, 42, 63, 84
I.V.A	SC	1, 21, 42	21, 42, 63, 84	21, 42, 63, 84	21, 42, 63, 84
NC (PBS)	SC	1, 21, 42	21, 42, 63, 84	21, 42, 63, 84	21, 42, 63, 84
NC (PBS)	IN	1, 21, 42	21, 42, 63, 84	21, 42, 63, 84	21, 42, 63, 84

PI: Pre-immune; I.V: Irradiated vaccine; I.V.T: Irradiated vaccine plus trehalose; F.V.A: Formalin inactivated vaccine plus alum; I.V.A: Irradiated vaccine plus alum; IN: Intra-nasal, SC: Subcutaneous; NC: Negative control; PBS: Phosphate-buffered saline; SLP: Spleen lymphocyte proliferation; nAb: Neutralizing antibody.

Table 2. Mean ± SD of virus titration in irradiated and un-irradiated virus samples.

Gamma irradiation (kGy)	Log of virus (without trehalose)	Log of virus (plus trehalose)
0	5.93 ± 0.21	5.94 ± 0.24
2	4.56 ± 0.06	4.59 ± 0.15
4	3.31 ± 0.16	3.47 ± 0.15
5	2.75 ± 0.11	2.75 ± 0.07
6	1.93 ± 0.08	1.87 ± 0.06
8	> 1.50	0.81 ± 0.03
10	> 1.50	> 1.50
15	> 1.50	> 1.50

1.70 kGy. Based on the D_{10} value, the initial titer ($10^{5.93}$ per mL), and the 5.93 $\log_{10}$ reductions in titer to achieve complete loss of viral infectivity, the minimum dose required for complete inactivation (the optimal inactivation dose) was calculated using the formula of *D_{10} value × 5.93* and set at approximately 11.00 kGy. Despite the calculated inactivation dose (11.00 kGy), three virus stocks were irradiated with three gamma radiation doses to confirm virus inactivation, including 11.00, 12.00, and 14.00 kGy. Subsequently, all three irradiated virus strains were used to evaluate virus inactivation by four blind cultures on Vero cells as a safety test. The complete inactivation of the virus was confirmed by the absence of virus replication and absence of CPE after four serial passages in Vero cells (safety test). Since no virus infectivity could be detected in the monolayers infected with 14.00 kGy, the irradiation dose of 14.00 kGy could be considered sufficient for complete virus inactivation.

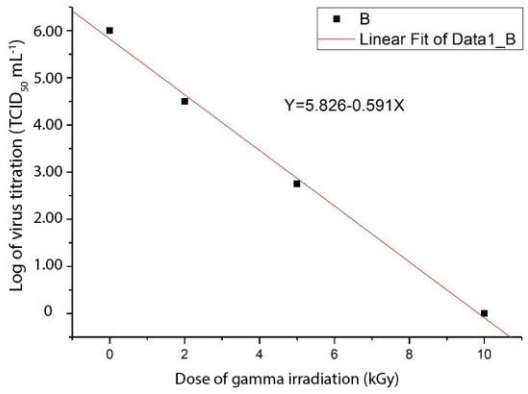

Fig. 1. Dose-response curve of gamma irradiated severe acute respiratory syndrome coronavirus 2 virus samples. $TCID_{50}$: 50.00% tissue culture infectious dose.

Evaluation of irradiated SARS-CoV-2 virus antigen by ELISA. The SARS-CoV-2 virus samples irradiated with 12.00 and 14.00 kGy gamma rays were used to evaluate the antigenicity of the S protein (S1 subunit). Based on the ODs of all samples at 450 nm, the results of the indirect ELISA to evaluate the antigenic properties of the S protein subunit S1 of the SARS-CoV-2 virus showed that the virus samples irradiated with gamma rays (12.00 and 14.00 kGy) have no significant difference in antigenic properties compared to the non-irradiated virus samples ($p > 0.05$). However, the OD of the irradiated and non-irradiated virus samples plus 20.00% trehalose was higher than that of the samples without trehalose. It is possible that the disaccharide plays an important role in maintaining the properties of proteins, which of course should be further investigated when evaluating the inactivated virus antigen in animal models.

Neutralizing antibody response. After infection with SARS-CoV-2, patients develop specific IgG and IgM antibodies. A subset of these antibodies against spike (RBD) can block viral infection and entry into cell through a process called neutralization. A strong correlation has been observed between the number of RBD-binding antibodies and amount of SARS-CoV-2 blocked by nAbs in patients.[22] The percentage inhibition of spike (RBD)-ACE2 binding indicates the titration of nAbs, as shown in Figure 2A. Percentage inhibition of spike (RBD)-ACE2 binding showed a significant increase in nAb titers three weeks after the third vaccination ($p < 0.05$). All vaccination groups showed a significant increase in nAbs three weeks after the second and third vaccinations (groups 6 and 7), and 6 weeks after third vaccination compared to the PBS groups ($p < 0.05$).

Concentration of IgA. Nasal lavage and NALT fluid from vaccinated hamsters were used to assess mucosal immunity with the quantitative sandwich ELISA kit to determine IgA concentration. The IgA concentration is shown in Figure 2B. While circulating IgA is mostly monomeric and consists predominantly of the IgA1 subclass, secretory IgA (sIgA) is dimeric and consists of IgA1 and IgA2 subclasses. The SARS-CoV-2 mainly infects the upper respiratory tract (URT). Mucosal immune responses are thought to be triggered in the NALT (*via* nasal epithelium and tonsils), serving as induction sites for the mucosal immune system.[23,24] The important effector molecules at the mucosal sites are antibodies, having two main sources, including translocation of circulating IgG into the mucosa and local production of IgA.[25] In this study, sIgA in nasal washings and NALT fluids was investigated. The results showed that the concentration of sIgA in irradiated vaccine plus trehalose increased significantly three weeks after the second and third vaccinations ($p < 0.05$).

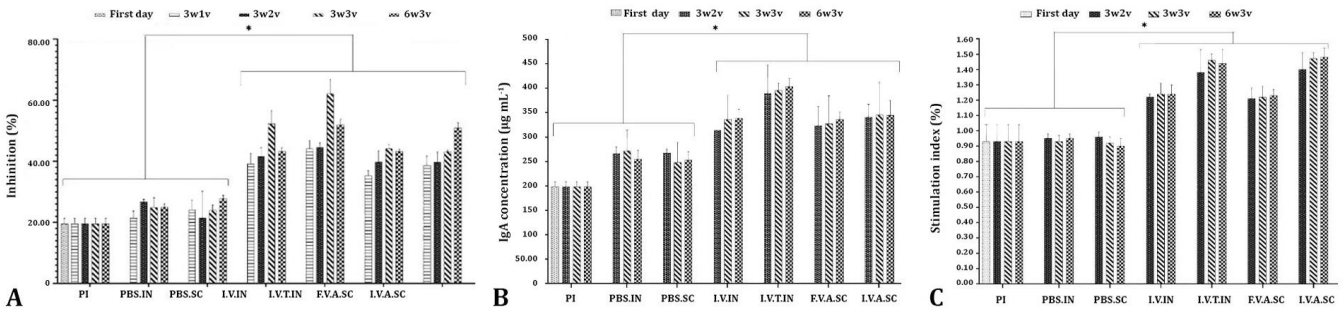

Fig. 2. A) The inhibition percent of the spike-angiotensin-converting enzyme 2 (ACE2) binding, indicating the neutralizing antibody titration; **B)** IgA concentration (µg mL^{-1}); and **C)** The spleen lymphocyte proliferation results being showed as stimulation index. PI: Pre-immune; I.V: Irradiated vaccine; I.V.T: Irradiated vaccine plus trehalose; F.V.A: Formalin inactivated vaccine plus alum; I.V.A: Irradiated vaccine plus alum; IN: Intra-nasal, SC: Subcutaneous; NC: Negative control; PBS: Phosphate-buffered saline; wa1v: Weeks after first vaccination; wa2v: Weeks after second vaccination; and wa3v: Weeks after third vaccination. *: $p < 0.05$.

Cellular immunity. The T cells against coronaviruses persist in human and can be detected for months and/or years after infection. Memory T cells are directed against conserved regions of coronaviruses and are therefore largely cross-reactive. Therefore, T cell-based vaccination approaches against COVID-19 are of realistic utility.[26] In this study, the spleen lymphocyte proliferation assay was performed using a colorimetric BrdU kit, and the stimulation index of spleen cells is shown in Figure 2C. A significant increase in lymphocyte proliferation in the spleen was observed in all vaccinated hamsters ($p < 0.05$), but the increase was greater with two irradiated vaccines plus trehalose and alum.

Discussion

The SARS-CoV-2 virus has caused the most severe pandemic in the world. The development of a safe and effective vaccine is critical to manage the disease. We have optimized an inactivated virus vaccine using the gamma irradiation method as an alternative to classical chemical inactivation methods. Previous studies have shown that gamma irradiation can induce immunogenicity more effectively than conventional inactivation methods.[9,27-29] Gamma irradiation preserves antigen composition, can reduce free radical damage due to water radiolysis, and can be used in a frozen state.[20,30] Gamma irradiation is a cost-effective and faster method of virus inactivation used for the production of many inactivated virus vaccines, such as human and avian influenza vaccines. The current study reports the efficacy of a viral vaccine produced with gamma irradiation instead of a virus inactivated with formalin.

The monoclonal anti-S1 spike protein antibody was used by ELISA method to compare the antigenic properties of the irradiated and non-irradiated virus samples. The S protein of virus, containing the major neutralizing epitopes in the RBD and the N-terminal domain, has been shown to be the most promising immunogen. Thus, most recently approved vaccines use the full-length S virus or the whole virus (inactivated) as a target antigen.[31] In this study, assessment of the antigenic properties of S protein using ELISA showed no significant differences between irradiated and non-irradiated SARS-CoV-2 virus samples with and without trehalose. However, the OD of the irradiated and non-irradiated virus samples with trehalose was higher than that of the samples without trehalose. This could be related to the fact that the disaccharide plays an important role in maintaining the properties of proteins, which of course should be further investigated when evaluating the inactivated virus antigen in animal models.

Offersgaard *et al.* reported that increasing the dose of inactivated SARS-CoV-2 improves the induction of nAbs in tests on mice. Although a second immunization in both mice and hamsters resulted in a large increase in nAbs titers compared to the single immunization.[32,33] However, higher levels of nAbs are associated with a lower risk of symptomatic infection, and immune protection is dependent on nAbs levels.[10,34] In this study, a gamma-irradiated vaccine against SARS-CoV-2 was used as an inactivated vaccine, and the nAb response showed a significant increase in four vaccinated hamster groups as early as three weeks after the first vaccination. However, the highest peak antibody titer was not reached until three weeks after the third vaccination. Therefore, we can suggest that the gamma-irradiated inactivated SARS-COV-2 vaccine can be used in three doses, one primary and two booster doses three weeks apart.

The IgA can be enriched up to three-fold in URT secretions compared to the IgG, whereas IgG is the most common isotype in the blood and lower respiratory tract. The higher concentration of sIgA compared to the IgG has been shown to result in greater avidity and neutralizing capacity.[35-38] The IgA can be expressed on mucosal surfaces in both monomeric and dimeric forms as sIgA and occurs in two isotypes in humans, with IgA1 being present in both systemic and mucosal secretions and IgA2 being predominantly present in the mucosa.[38] Although, both clinical and animal studies for influenza and SARS-CoV-2 have reported that sIgA is induced at oral and nasal mucosal surfaces following intra-muscular vaccination.[38]

One of the most informative methods to evaluate effective immune responses against SARS-CoV-2, whether by natural infection or intra-nasal immunization, is the determination of mucosal sIgA in nasal secretions or saliva, being synthesized by IgA-secreting plasma cells. In this study, the concentration of sIgA in nasal washings and NALT was investigated. It increased significantly when the irradiated vaccine plus trehalose was administered *via* intra-nasal route three weeks after the second and third vaccinations. Previous experience with SARS-CoV-1 and MERS suggests that T cells may be the most important immune response for disease control.[39] In contrast to antibodies, cytotoxic T cells persist against coronaviruses and can be detected several months after infection. Therefore, an ideal SARS-CoV-2 vaccine should stimulate both B- and T-cell immunities to provide optimal protection against severe COVID-19. Gamma irradiation as a superior inactivation method can preserve T cell immunogenicity compared to the other inactivation methods. [40] Mullbacher *et al.*, previously shown that alphaviruses and bunyaviruses cannot be rendered infectious by gamma irradiation and yet are capable of eliciting cytotoxic T cell responses.[8,41,42] Gamma irradiation can be used to produce an experimental influenza vaccine and gamma-irradiated influenza virus preparations have been reported to promote T-cell immunity.[41] The irradiated viral proteins are naturally presented to the immune system, facilitating the induction of both T-cell and humoral immunities. In the current study, splenic lymphocyte proliferation was increased in all vaccinated hamsters and it was more pronounced in the irradiated vaccine groups. However, after performing a challenge test against the SARS-CoV-2 live virus in vaccinated hamster with the irradiated inactivated vaccine SARS-CoV-2 plus disaccharide trehalose and another irradiated inactivated vaccine SARS-CoV-2 plus alum, we can discuss the potency and efficiency of both vaccines against COVID-19. In addition, a cytokine assay should be carried out in the future study to determine the exact immune cells.

The irradiated inactivated SARS-CoV-2 vaccine plus disaccharide trehalose can be administered intra-nasally. Another irradiated inactivated vaccine SARS-CoV-2 plus alum can be administered subcutaneously and is a safe vaccine against COVID-19 that can stimulate mucosal, humeral, and cellular immunities. However, performing the challenge test against COVID-19 in vaccinated hamsters requires a discussion regarding the potency and efficiency of the vaccine.

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Conflict of interest

The authors declare that they have no conflict of interest. The funders had no role in the collection, analyses, or interpretation of data, as well as in the writing of the manuscript, or decision to publish the results.

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