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REVIEW ARTICLE

Comprehensive review of preclinical evaluation strategies for COVID-19 vaccine candidates: assessing immunogenicity, toxicology, and safety profiles

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ABSTRACT

Following the worldwide spread of Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), there is a vital requirement for safe and effective vaccines against Coronavirus disease 2019 (COVID-19). Therefore, several vaccine-candidate platforms have been designed, tested, and developed. Based on guidelines, preclinical studies are recommended to assess the safety and potency of COVID-19 vaccines in appropriate in vitro and in vivo settings. These studies provide essential information to describe the potential toxic properties of a vaccine and the formulation of vaccine agents during the preclinical trial phase. In toxicology studies, several factors must be considered, such as the appropriate animal species and strains, dosing timetable, mode of administration, time of sampling for biochemistry and antibody evaluation, and necropsy. Pharmacokinetic/ biodistribution studies are not usually required for infectious disease prophylaxis vaccines unless the vaccine contains a novel substance. Evaluating their biodistribution is crucial for newly developed vaccines, such as lipid nanoparticles –messenger RNA (LNP-mRNA), DNA, and Viral vectors in non-replicated (VVnr), or recombinant virus vaccines. The review highlights the importance of preclinical studies in assessing the safety and efficacy of vaccine candidates. This guidance is essential for researchers and manufacturers to design effective vaccines that can progress to clinical trials safely.

Keywords: Animals; COVID-19 vaccines; Immunogenicity; Toxicity tests

INTRODUCTION

A new strain of coronavirus, identified as severe acute respiratory syndrome coronavirus 2, was responsible for the Coronavirus disease 2019 (COVID-19) outbreak in China in December 2019. This virus has subsequently disseminated globally, resulting in a pandemic (1, 2). SARS-CoV-2 is an RNA-enveloped single-stranded virus whose entire genome encodes different amino acids and structural and non-structural proteins (NSPs). The open reading frames region encodes the nonstructural proteins, such as C30 endopeptidase, papain-like protease, and RNA replicase. In contrast, structural proteins are encoded by the surface or spike (S), envelope (E), membrane (M), and nucleocapsid proteins (N) genes (3).

The virus's spike (S) protein engages with the host cell by attaching to angiotensin-converting enzyme 2 (ACE2) receptors, resulting in a structural recon-

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figuration. Subsequently, the virus merges with the host cell membrane, facilitated by transmembrane serine protease 2 (TMPRSS2), thereby finalizing the process of infection (4). The development of a therapeutic vaccine targeting COVID-19, specifically formulated with proteins, has become essential in light of the pandemic (5).

Researchers are currently testing 183 vaccines on humans in clinical trials until March 30, 2023, with ten vaccines having been approved and licensed for general use by the World Health Organization (WHO). At least 199 preclinical vaccines are being tested on animals. The various platforms for COVID-19 vaccines include Protein subunits; Viral vectors in mignon-replicated (nr) and replicated; DNA / RNA, Inactivated viruses; virus-like particles, Live attenuated viruses; bacterial antigen-spore expression vectors, and Viral vectors in non-replicated (VVnr) + Antigen Presenting Cell (APC) (6).

The ongoing advances in biotechnology and vaccine immunology have led to the development of various innovative vaccines to prevent infectious diseases (7). On the other hand, improvements in the production process have resulted in innovative products and adjuvants. The novelty of products poses challenges for scientific and regulatory assessments of safety, potency, and quality (8, 9).

The diversity in product design, new methodologies, and knowledge expansion over time, mainly due to vaccine development in the COVID-19 pandemic, emphasize the importance of designing types and methods for preclinical evaluation. Preclinical assessment is critical in expanding vaccine candidates, and regulations regarding the preclinical evaluation of new platforms are sometimes inadequate. Established guidelines cover the general principles of preclinical evaluation of vaccines; however, supplemental documents are essential for newly developed vaccines to prevent undesirable side effects (7-9).

Preclinical studies encompass all features of product characterization, immunogenicity studies, safety, and toxicology testing in relevant animals before introducing to humans in clinical studies. Additionally, preclinical evaluation may be essential when product formulations and industrial methods are altered, product formulations and industrial processes are altered, or unexpected safety concerns have been reported from phase 1 and 2 clinical trials (7, 9). Potential safety issues for a vaccine candidate arise due to the toxicities of the product, impurities, and contaminants derived during the production process, or interactions of the vaccine antigen with the formulation agents (7). Furthermore, the vaccine-induced immune response can cause unwanted toxic side effects (10, 11). Preclinical studies help recognize potential vaccine immunogenicity and guide the selection of dose, dosing schedule, and administration method for clinical studies. Given the importance of preclinical studies in developing COVID-19 vaccines, a summary of the approved tests is provided. Food and Drug Administration (FDA) advises vaccine manufacturers to participate in communications with the national FDA to design the initial preclinical testing required for the COVID-19 vaccine candidate that can support the first in human clinical trials.

Literature search and selection of the articles. We carried out a narrative review of the literature on preclinical studies in COVID-19 vaccines. To be included, articles had to meet the following criteria: they had to be written in English, accessible in full-text format, wholly published, and directly relevant to our subject. Our team investigated PubMed and Scopus in December 2022 using the following keywords: (“Immunogenicity”) AND (“Toxicology”) AND (“Safety”) AND (“Vaccine”) AND (“COVID-19” OR “SARS Corona Virus” OR “SARS-CoV-2”) AND (“Preclinical” Or “Animal Studies “). We found 189 articles based on their titles, abstracts, and publication dates (we only included publications after 2000 because Severe acute respiratory syndrome (SARS) was detected in Southern China in November 2002. In addition, in December 2024, an extra search was performed using Google Scholar, PubMed, and Scopus to categorize recently published papers.

COVID-19 candides vaccines. This review presents a comprehensive examination of the historical development of vaccines targeting SARS-CoV-2 that have received approval from regulatory bodies. It delves into various aspects of preclinical tests of vaccine design, elucidating how these vaccines stimulate the immune system and confer immunity to the host. Historically, in August 2020, the Russian government declared the development of a vaccine, designated Sputnik V. Concurrently, the Chinese biotechnology firm Sinovac Biotech Ltd. initiated clinical trials following approval of preclinical tests for an inactivated virus vaccine, referred to as CoronaVac

during the period from April to July 2020. Around the same timeframe, clinical trials for two innovative vaccines (developed by biotechnology companies Moderna and Pfizer-BioNTech) commenced in the United States. In December 2020, the United States Food and Drug Administration granted emergency use authorization to two mRNA vaccines. Afterward, in February 2021, a viral vector vaccine produced by Johnson & Johnson also received emergency use authorization (12).

The analysis encompasses a range of platforms utilized in the expansion of current SARS-CoV-2 vaccines, including protein subunit vaccines (59 candidates; Nuvaxovid XBB.1.5 and VidPrevtyn Beta), DNA-based vaccines (17 candidates such as the nCoV vaccine by Zydus Cadila and INO-4800), RNA vaccines (43 candidate vaccines; like the Spikevax vaccine, BNT162b2 /Comirnaty, and CVnCoV), virus-like particle vaccines (7 candidates including CoVLP and RBD-HBsAg-VLPs-Covid), viral vector vaccines (16 candidates such as the Vaxzevria/AstraZeneca COVID-19 Vaccine, Convidecia, and Sputnik V), inactivated pathogen vaccines (22 vaccine candidates for instance CoronaVac and BBIBP-CorV), as well as nasal and attenuated virus formulations. Furthermore, the review addresses critical topics such as the assessment of vaccine efficacy, effectiveness against virus strains, conducted toxicological analyses, and their safety and immunogenicity findings. The conventional vaccine candidates identified by the World Health Organization encompass protein subunits, RNA-based vaccines, non-replicating viral vectors, inactivated viral agents, and DNA-based vaccines (6).

Preclinical pharmacology studies: primary pharmacodynamics study. A vaccine's primary pharmacodynamics study assesses its immunogenicity and effectiveness. Such studies are conducted to regulate the suitable dose for initiating preclinical and clinical studies, typically during the research phase of pharmaceutical development (13).

In addition, the pharmacodynamics study might consist of the basic pharmacology of any used adjuvant. Appropriate animal model immunization studies are essential to provide sufficient proof of concept information supporting subsequent clinical phases. Furthermore, immunogenicity information helps verify the product's immunological aspects, select the immunogenic doses without toxic effects, determine timetables, and choose methods of administration for human use (14).

Preclinical immunogenicity studies should evaluate the appropriate immune response. This encompasses humeral evaluation, including seroconversion rates, average antibody titers, and cell-mediated induced immune responses in the immunized animals. Depending on the specific vaccine and its components, different approaches might be required for the immunogenicity testing of vaccines and adjuvants (15).

The animal species assortment for the study design should be based on the preferred clinical immune response and anatomical and physiological relevance to humans. Appropriate rodents include *Mus musculus* (most commonly Balb/C and C57BL/6 mice), Golden hamster or Syrian hamster (*Mesocricetus auratus*), Guinea pig (*Cavia porcellus*), Rattus norvegicus (*Sprague dawley* and Wistar rats). Non-rodents such as Rabbits (*Oryctolagus cuniculus*) and non-human primates, for instance, Rhesus macaque (*Macaca mulatta*) and Cynomolgus macaques (*Macaca fascicularis*), have been recommended for primary pharmacodynamics studies (9, 16, 17).

In developing a COVID-19 vaccine candidate, evaluating the humoral immunity involved measuring the specific Ab production response to SARS-CoV-2 Spike, receptor-binding domain (RBD), and N Ag following vaccination. This was achieved using particular antibody kits for S, RBD, and N during the study period, in addition to assays for receptor-binding domain-specific serum antibodies and Angiotensin-converting enzyme 2 (ACE 2) binding inhibition (18, 19). The plaque reduction neutralization test (PRNT) is considered the “gold standard test” to assess the virus-neutralizing potency of serum or antibody samples. However, various SARS CoV-2 neutralization assays, such as conventional virus neutralization test (cVNT), surrogate virus neutralization test (sVNT), ELISA-based test, pseudovirus-based neutralization titers (pVNT) that measure inhibition of binding between RBD / Spike and ACE2 in immunized animal serum, are also regarded as essential tests (10, 11, 15, 20).

Evaluating specific Immunoglobulin A (IgA) responses might be more suitable for developing mucosal vaccines. T-cellular-mediated responses may also play a central character in the immunogenicity assessment of certain vaccine types. Therefore, measuring IgG1 and IgG2a levels is recommended

to evaluate COVID-19 vaccines in rodents. Several murine studies proved that B cells, regulated by Interleukin-4 (IL-4), secret the IgG1 at the same time, while interferon-γ drives the expression of IgG2a, indicating the response of helper cells (Th2 or Th1) (21, 22).

SARS-CoV-2 specific T-cell immunity can also play a significant role in pathogenesis or defense against infectious viruses and might affect the humoral immune reaction. Investigating the particular proliferation of isolated lymphocytes in the presence of viral antigen in immunized animals by interferon-gamma enzyme-linked immunospot (IFN-γ ELISPOT) or flow cytometry, as well as evaluating CD8 (cytotoxic lymphocyte cells) in terms of phenotype, specific function, and cytokines profiles including tumor necrosis factor α (TNF-alpha), interferon-γ (IFN- γ) and IL- 2, 4, 5, 6, 12, 13, 17 and 21 in various models such as inbred mice, monkeys, and the hamsters in both sexes can assess the cellular immunity (18-20). Activated CD4 T cells are essential for stimulating B-cells, which subsequently leads to the production of antibodies and cytokines. Vaccine-associated enhanced respiratory disease (VAERD) has been observed in some patients and is often due to the activation of CD4 type 2 helper T-cells (interleukin-4, -5, or -13) following respiratory virus infections. However, VAERD is typically not observed in the presence of a CD4 Th1 response (characterized by IFN-γ, IL-2, and TNF- α) without a concurrent Th2 response (20, 23, 24).

The protective efficacy of vaccines is evaluated in non-human primates or hamsters following immunization. A challenge will be conducted via intratracheal–intranasal or endobronchial routes with an appropriate dose of virus, administered 2-6 weeks after the completion of vaccination (Fig. 1). The main goal is to deliver pathogens to the airways to measure virus levels in nasal and pharyngeal secretions and to evaluate humeral/cellular responses that mimic those seen after natural SARS-CoV-2 infection (20, 24). The potential immune responses for protection at the time of the challenge and later, can be assessed by viral PCR and subgenomic RNA in Bronchoalveolar lavage (BAL) fluids, as well as nasal, anal, and pharyngeal secretions, or from prepared swab samples (20, 24, 25). Afterward, vital and target organs such as the liver, heart, spleen, kidneys, brain, injection site, eyes, lymphoid nodes, and lungs should be collected for further histopathological and immunohistochemical (IHC) assessments (7, 26) (Tables 1 and 2).

Secondary pharmacodynamics and safety pharmacology. According to the International Council for Harmonisation (ICH) S7A and S7B guidelines, the primary objective of safety pharmacology research is to evaluate the effect of pharmaceutical products on the cardiovascular, central nervous, and respiratory systems. This evaluation is generally required before the products are administered to humans. When necessary, supplementary safety pharmacology evaluations may also be performed during later clinical development. Preclinical safety evaluations are vital prior to the commencement of human clinical trials for a new vaccine candidate, particularly when the candidate incorporates a novel product type that lacks existing preclinical and clinical data. These preclinical safety pharmacology assessments are designed to evaluate the potential adverse effects of the vaccine candidate in relation to its intended application in human populations (9, 47).

However, for some products, conducting preclinical safety studies before human clinical trials may not be necessary if there is sufficient evidence to characterize the product's safety from alternative sources. For example, suppose a COVID-19 vaccine is developed using a licensed vaccine platform technology, and the vaccine's contents are well-characterized. Data from repeat dose toxicity and biodistribution studies may be utilized. Vaccine manufacturers are required to compile these findings and offer a credible justification for their reliance on this information in lieu of performing preclinical safety studies (8, 10, 11).

Pharmacokinetics / bio-distribution studies: absorption, distribution, metabolism, and excretion. Pharmacokinetic studies are generally not a requirement for infectious prophylaxis vaccines. However, if the vaccine formulation contains a novel substance, such as an innovative adjuvant, pharmacokinetic / biodistribution studies may become necessary. This is particularly true for newly developed vaccines incorporating lipid nanoparticle-containing messenger RNA (LNP-mRNA), DNA, or recombinant virus vaccines; their biodistribution must be thoroughly assessed (25).

This assessment must be completed before the beginning of the first clinical trial or before a large-scale clinical trial commences. Biodistribution should be examined using sensitive detection methods, and the

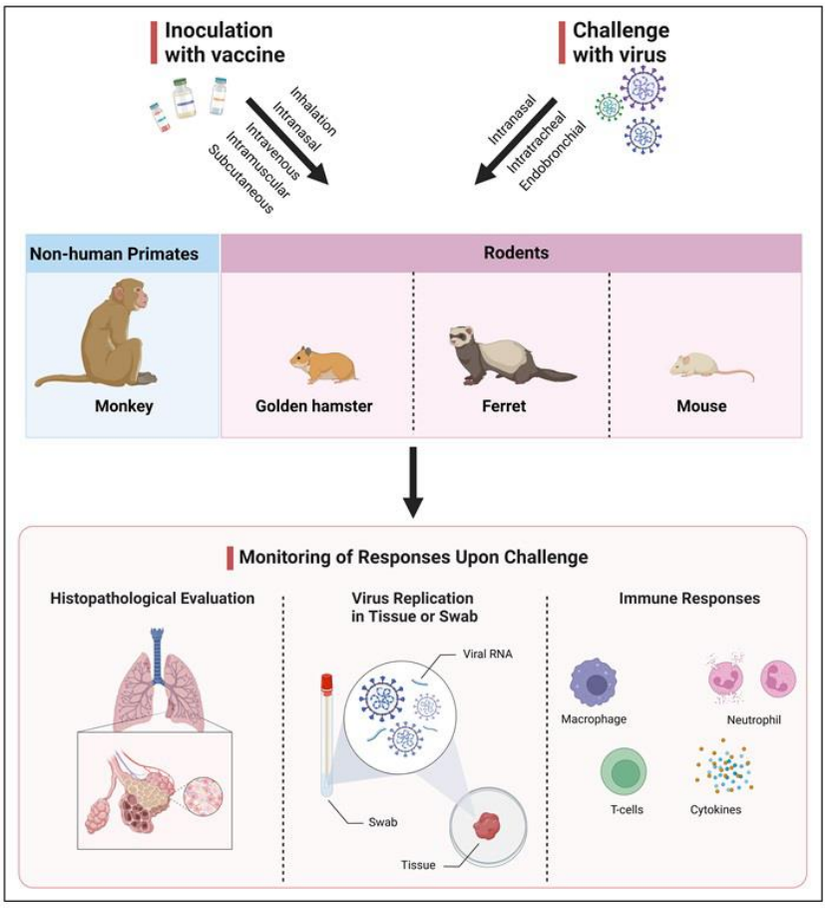

Fig. 1. The protective efficacy of vaccines is assessed in non-human primates, hamsters, mice, and ferrets following their immunization with the vaccine. Animals are given varying doses of the vaccine formulation to evaluate the vaccine's ability to protect against viral infection and disease. This could be a single intramuscular or intranasal immunization or a series of two or three intramuscular administrations according to different schedules. Following immunization, the animals are exposed to SARS-CoV-2 via intranasal, intratracheal, or endobronchial inoculation and monitored for clinical signs of the disease. The primary objective is to introduce the pathogen into the airways for measurement. Vital organs, especially the lungs, are collected for histopathological examination and to quantify viral replication. In addition, viral subgenomic RNA (sgRNA) levels are measured in pharyngeal, anal, and nasal swabs using RT-qPCR, alongside evaluations of humoral and cellular immunity.

rationale for choosing these particular assays must be definitive. Researchers should evaluate plasma and tissue pharmacokinetics and tissue distribution following IM, SC, IV, or ID injections with the total human dose in appropriate animal models, typically including rats and New Zealand white rabbits. These assessments will be conducted for both single- and multiple-dose, focusing on blood and a predetermined array of organs/tissues. A qualified branched DNA (bDNA) multiplex method or Quantitative Whole-Body Autoradiography (QWBA) can be used for these studies. Quantitative polymerase chain reaction (PCR) based analyses have been the most frequently used nucleic acid recognition techniques and are considered the most reliable methods for measuring plasmid or nucleic acid levels in biodistribution and integration studies (20, 25, 48).

Toxicology studies. Preclinical toxicity studies should provide adequate information to recognize and characterize a vaccine's potential toxic properties. In designing animal toxicology studies, deliberation must be given to selecting proper animal species and strains, dosing timetable, administration routes, sampling timing for biochemistry and antibody evaluation, and necropsy. The toxicity valuation of the vaccine formulation agents can be evaluated through dedicated toxicity studies or as part of

Table 1. Comparative overview of some COVID-19 vaccines assessed for immunogenicity effect in different animal models.

Vaccine Candidate	Vaccine Platform	Animal Model	Antigen & Dosage	Evaluated Factors	Reference
BBIBP-CorV	Inactivated SARS-CoV-2 Vaccine candidate	Balb/C mice; Guinea pigs; Wistar rats; Chinchilla rabbits; Cynomolgus macaques *(Macaca fascicularis)*	Balb/C mice: 2, 4, 5 µg/ inoculation; Guinea pigs: 2, 4, 5 µg/ inoculation; Wistar rats: 2, 4, 5 µg/ inoculation; Chinchilla rabbits: 2, 4, 5 µg/ inoculation; Cynomolgus macaques: 2, 4, 5 µg/ inoculation	VNT	(10)
NRC-Vacc	Inactivated SARS-CoV-2 Vaccine candidate	Balb/C mice; Albino Wistar rats; Syrian hamsters; Guinea pigs	Balb/C mice, Syrian hamsters, Albino Wistar rats: 3, 6, 15, and 30 µg; Guinea pigs: 10, 20, 50, and 100 µg/dose	Body weight IgG TNF-α, CRP, IL-1, IL-6, and IL-10 CBC, Plasma D-dimer, SGOT, SGPT, Albumin, Urea, Creatinine, Calcium, Ferritin	(15)
BNT162b2	mRNA vaccine	Balb/C mice; Rhesus macaques *(Macaca mulatta)*	Balb/C mice: 0.2, 1, 5 µg; Rhesus macaques: 5, 17 µg /Kg	IgG1, IgG2 IFN-γ, TNF-α, IL-4, IL-5, IL-13, GM-CSF, IL-6, IL-18, IL-1β, MIP-1β, MCP-1 CD4, CD8	(20)
BIV1-Cov Iran	Inactivated SARS-CoV-2 Vaccine candidate	Balb/C mice; NZW rabbit; Rhesus macaques *(Macaca mulatta)*	Balb/C mice: 3, 5 µg; NZW rabbit: 5 µg; Rhesus macaques: 3, 5 µg	IgG1, IgG2a IFN-γ, TNF-α, IL-4, IL-17 Gzm B cVNT	(27)
BBV154	Replication-defective chimpanzee adenovirus (ChAd)-vectored vaccine	Balb/C mice; Swiss albino mice; Wistar rat; NZW rabbit	$5 \times 10^{9-11}$ VP	SARS-CoV-2 spike specific IgM, IgG, IgA, IgG1 and IgG2/IgG3 IFN-γ or TNF-α IL-10, IL-4 $TCID_{50}$ sVNT	(28)
PTX-COVID19-B	mRNA vaccine	Balb/C mice; C57BL/6 mice	Balb/C mice: 1 and 10 µg C57BL/6 mice: 1 and 10 µg	IFN-γ, TNF-α, IL-2, IL-4, IL-5 CD3, CD4, CD8, CD44, CD62L mVNT Serum neutralization using pseudovirus ELISPOT	(29)
AZD1222 or ChAdOx1 nCoV-19	Adenovirus-based vaccine	CD-1 mice	1×10^{9} viral particles (30 µL)	Body weights Mortality/moribundity Serum S1 Sequential Sandwich Injection site, spleen and bone marrow (sternum and femora-tibial joint) histopathology, Platletes, WBC and Neutrophiles	(30)
Swt-2P and SOmicron-6P	Omicron-specific mRNA vaccine	Balb/C mice; Rhesus macaques *(Macaca mulatta)*	Balb/C Mice: 1, 5, 10 µg of Swt-2P and SOmicron-6P mRNA; Rhesus macaques: 20, 100 µg of SOmicron-6P	S-specific IgG neutralization assay using VSV-based Omicron pseudovirus VNT	(30)

Table 1. Continuing...

PastoCoAd	Recombinant adenovirus type 5 (rAd5) containing the full-length spike protein (rAd5-S)	Balb/C mice; NZW rabbit	Balb/C mice: first-dose rAd5-S (low; 5×10^7 VPs), the second-dose rAd5 RBD-N (high; 10^8 VPs); NZW rabbit: first-dose rAd5-S (low; 5×10^8 VPs), the second-dose rAd5 RBD-N (high; 10^9 VPs)	IgG1, IgG2a IFN-γ, TNF-α, IL-2, IL-5, IL-6 Cytotoxic T cell assay, VNT	(31)
RAZI-COV PARS	Recombinant Spike protein	Balb/C mice; Syrian hamsters; Pirbright Guinea pig; NZW rabbit	Balb/C mice: 0.5 µg (low), 1 µg (middle), 2 µg (high); Syrian hamsters: 1 µg (low), 2 µg (middle), and 3 µg (high); Pirbright Guinea pig: 2 µg (low), 4 µg (middle), 5 µg (high); NZW rabbit: 4 µg (low), 6 µg (middle), 8 µg (high)	IgG1, IgG2 IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-17 Human ACE2 Protein (hACE-2) Binding Assay VNT	(32)
Osvid-19	Inactivated SARS-CoV-2 Vaccine candidate	Balb/C mice; Rhesus macaques *(Macaca mulatta)*	Balb/C mice: 5, 10 µg / 100µL; Rhesus macaques: 5µg/ 500 µL	IgG2a, IgG1, IgG2a/IgG1, TNF-α, IL-10, IL-6, IL-4, CD4, CD8 anti-Spike antibody GB cVNT, sVNT IgG2a,	(33)
Spikevax Or mRNA-1273	mRNA vaccine	Syrian hamsters; Rhesus macaques *(Macaca mulatta)*	30 µg, 100 µg mRNA-1273	IgG1, IgG2a/IgG1 IFN-γ, IL-2, TNF-a; IL-4, IL-5, IL-10, IL-13 CD4, CD8	(34)
Nanocovax	Protein Subunit Vaccine	Balb/C mice; Syrian hamsters; Northern pig-tailed macaques *(Macaca leonina)*	25, 50, 75, 100 µg	PRNT sVNT	(35)
MRT5500	mRNA vaccine	Balb/C mice; Cynomolgus macaques *(Macaca fascicularis)*	Balb/C mice: 10 and 50 µL mRNA/LNP formulation (0.4 µg); Cynomolgus macaques: 500 µL mRNA/LNP formulation (5 µg)	IFN-γ, IL-13 PRNT PsVNa	(36)
AZD2816 vaccine	ChAdOx1 vectored vaccine	Balb/C Mice	10^8 infectious units of ChAdOx1 vector	IgG ELISpot and ICS staining, IFN-γ, TNF-α, IL-4, IL-10, CD3, CD4, CD8 mVNT	(37)
SpikoGen	Advax-CpG55.2-adjuvanted SARS-CoV-2 spike protein vaccine	Balb/C mice	1µg rSP	IgG1, IgG2a, IgG2b and IgG3, IFN-γ, IL-4, IL-17 CD4, CD8	(38)
ZyCoV-D	DNA plasmid-based COVID-19 vaccine	Balb/C mice; Guinea pig; NZW rabbit	Balb/C mouse, Guinea pig: 25 and 100 µg of DNA vaccine; NZW rabbit: 500 µg of DNA vaccine	IgG IFN-γ ELISPOT assay Micro-neutralization test (MNT)	(39)

Table 1. Continuing...

MVA-SARS-2-S	COVID-19 candidate vector vaccine	Balb/C mice	10^7, 10^8 PFU recombinant MVA-SARS-2-S	RBD-Specific IgG, IgG IFN-γ, TNF-α PRNT50, LISPOT sVNT	(40)
MVA-S(3Pbeta)	MVA-based vaccine	C57BL/6 mice	1×10^7 PFUs of MVA-S(3P) or MVA-S(3Pbeta) by the IN route	ICS assay	(41)

ACE 2, Angiotensin-converting enzyme 2; PRNT, plaque reduction neutralization test; Ig, immunoglobulin; cVNT, conventional virus neutralization test; sVNT, surrogate virus neutralization test; pVNT, pseudovirus-based neutralization titers; IgA, Immunoglobulin A; IL, Interleukin; Th2, T helper cells; IFN-γ ELISPOT, interferon-γ enzyme-linked immunospot; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; VAERD, vaccine-associated enhanced respiratory disease; IHC, immunohistochemical; LNP-mRNA, lipid nanoparticle-containing messenger RNA; PCR, polymerase chain reaction; CBC, complete blood count; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; CRP, c-reactive protein; NZW, new Zealand white; COVID-19, coronavirus disease; SARS-CoV-2, severe acute respiratory syndrome–related coronavirus 2; TCID 50, median tissue culture infectious dose; mVNT, micro neutralization test; VSV, vesicular stomatitis virus; WBC, white blood cells; ALT, alanine aminotransferase; AST, aspartate transaminase; LDH, lactate dehydrogenase.

Table 2. Comparative Overview of Vaccines Evaluated for Protective Efficacy in Animal Models.

Vaccine Candidate	Vaccine Platform	Animal Model	Antigen & Dosage	Evaluated factors	Reference
BBIBP-CorV	Inactivated SARS-CoV-2 Vaccine candidate	Rhesus macaques *(Macaca mulatta)*	2, 4, 8 µg/ inoculation	Virus titers in throat and anal swabs Lung histopathology VNT	(10)
NRC-Vacc	Inactivated SARS-CoV-2 Vaccine candidate	Syrian hamsters	6 and 15 µg (300 µL)	Nasal swab for virus detection Histopathological evaluation	(15)
BIV1-Cov Iran	Inactivated SARS-CoV-2 Vaccine candidate	Rhesus macaques *(Macaca mulatta)*	3 and 5 µg	IgG1, IgG2a, IFN-γ, TNF-α, IL-4, IL-6, IL-10 CD4, CD8, CD20 Glucose, Urea, Creatinine, Total protein, Albumin, CRP, ALT, AST, LDH, Hematological indices Histopathological evaluation	(27)
BBV154	Replication-defective chimpanzee adenovirus (ChAd)-vectored vaccine	Young (9-11 weeks) or aged (28-36 weeks) Syrian Hamsters	$5 \times 10^{9\text{-}11}$ VP	IgG1, IgG2a, IgG2a/IgG1	(28)
PTX-COVID19-B	mRNA vaccine	Balb/C mice; C57BL/6 mice; Syrian hamsters	Balb/C mice: 1, 4, and 20 µg; C57BL/6 mic: 1, 4, and 20 µg: Syrian hamsters: 20 µg (100 µL)	Determination of infectious SARS-CoV-2 titer, Lung histopathology Lung virus titers TCID50	(29)
PastoCoAd	Recombinant adenovirus type 5 (rAd5) containing the full-length spike protein (rAd5-S)	Syrian hamsters	First-dose rAd5-S (low, 5×10^7 VPs), Second-dose rAd5 RBD-N (high; 10^8 VPs)	Clinical symptoms, Total IgG, IgG1 and IgG2a IFN-γ, TNF-α, IL-2, IL-6 Hematological and Biochemical parameters	(31)

Table 2. Continuing...

RAZI-COV PARS	Recombinant Spike protein	Syrian hamsters	1µg (low), 2µg (middle), 3 µg (high)	VNT Histopathology Oropharyngeal swabs Granzyme B cVNT TCID50 Lung histopathology, Viral RNA estimation Post-challenge VNT Clinical signs	(32)
Osvid-19®	Inactivated SARS-CoV-2 Vaccine candidate	Rhesus macaques *(Macaca mulatta)*	5 µg purified inactivated Ag (500 µL)	Lung CT-Scan Modified-SARS-CoV2 specific IgG IFN-γ, TNF-α, IL-1 β, IL-10, IL-6 Hematological and Biochemical parameters Modified-SARS-Cov2 Neutralizing Ab test Histopathological evaluations	(32)
COVAX-19 vaccine	Recombinant spike protein vaccine	Ferret *(Mustela putorius furo)*	12.5, 25 or 50 µg	Clinical signs Lung histopathology Virus titers in anal swabs Ferret spike RBD-binding IgG Bronchoalveolar lavage and Swab S-specific and RBD-specific	(38)
Spikevax Or mRNA-1273	mRNA vaccine	Syrian hamsters Rhesus macaques *(Macaca mulatta)*	Syrian hamsters: 1, 5, 25 µg; Rhesus macaques *(Macaca mulatta):* 2.5, 10, 30, 100 µg	IgA and IgG CD8 T-cell, CD4 T-cells IL-2, IFN-γ, TNF-α Post-challenge VNT Lung histopathology	(42)
Nanocovax	Protein Subunit Vaccine	Syrian hamsters	25, 50, 75, 100 µg	Signs of morbidity Lung virus titers Pseudovirus neutralization assay	(35)
MRT5500	mRNA vaccine	Syrian hamsters	0.15, 1.5, 4.5, 13.5 µg	Lung histopathology Quantification of SARS-CoV-2 subgenomic RNA (sgRNA) in lungs and nares	(36)
SOmicron-6P	Omicron-specific mRNA vaccine	Syrian hamsters	10, 25, 50 µg of mRNA	S-specific IgG VNT50 Neutralization assay Lungs and nasal turbinates for virus RNA detection Lung histopathology	(43)
SpikoGen	Advax-CpG55.2-adjuvanted SARS-CoV-2 spike protein vaccine	Balb/C mice	1 or 5 µg rSp alone or mixed with either 1 mg Advax-SM adjuvant	Serum IgG titers VNT Lung weight and histopathology Lung virus titers	(38)
MVA-SARS-2-S	COVID-19 candidate vector vaccine	Balb/C mice	10^7 or 10^8 PFU recombinant MVA-SARS-2-S	RBD-Specific IgG, Lung histopathology IFN-γ, TNF-α RNA in lung tissue sVNT PRNT50 ELISPOT for IFN-γ–producing cells	(40)

Table 2. Continuing...

MVA-S(3Pbeta)	MVA-based vaccine	K18-hACE2 mice	1×10^7 PFUs of MVA-S(3P) or MVA-S(3Pbeta) by IM	IL-6, IL-12b, CCL2, CCL12, IFNb1, TNF-α and CXCL10 Plaque assay on lung and BAL samples Lung histopathology IgG, IgG1, IgG2c and IgG3 TCID50	(41)
BNT162b2	mRNA vaccine	Rhesus macaques *(Macaca mulatta)*	100 µg/ dose	Immunity response (Humeral and Cellular), cytokines, sVNT, BALF evaluation, Nasal and Rectal Swab, X-Ray and CT-Scan	(44)
AfriVac 2121 (Wuhan)	mRNA	Syrian hamsters	1.5 or 5 µg	RT-qPCR Lung histopathology Serum IgG titers TCID50 assay IFN-γ, TNF,IL-6, IL-10, IL-1β and CXCL-10, CCL-5 in lungs sVNT Nasal washes	(45)
RQ3013 mRNA	mRNA	Rhesus macaques Syrian hamsters	240 µg/dose and 60 µg/dose	PVNT Serum IgG titers PRINT Elispot assay RT-qPCR Lung histopathology and immunohistochemistry TNF-α, IFN-γ, IL-2, IL-4, IL-5, and IL-6	(46)

ACE 2, Angiotensin-converting enzyme 2; PRNT, plaque reduction neutralization test; Ig, immunoglobulin; cVNT, conventional virus neutralization test; sVNT, surrogate virus neutralization test; pVNT, pseudovirus-based neutralization titers; IgA, Immunoglobulin A; IL, Interleukin; Th2, T helper cells; IFN-γ ELISPOT, interferon-γ enzyme-linked immunospot; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; VAERD, vaccine-associated enhanced respiratory disease; IHC, immunohistochemical; LNP-mRNA, lipid nanoparticle-containing messenger RNA; PCR, polymerase chain reaction; CBC, complete blood count; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; CRP, c-reactive protein; NZW, new Zealand white; COVID-19, coronavirus disease; SARS-CoV-2, severe acute respiratory syndrome–related coronavirus 2; TCID 50, median tissue culture infectious dose; mVNT, micro neutralization test; VSV, vesicular stomatitis virus; WBC, white blood cells; ALT, alanine aminotransferase; AST, aspartate transaminase; LDH, lactate dehydrogenase.

safety evaluations (49-51).

In toxicology studies, it is imperative to document the species, sex, age, and group size of the animals used and the source from where the animals were acquired. Detailing regarding animal husbandry, such as housing, feeding, management, and the health status of animals, must be meticulously recorded. Outbred animals are generally recommended for use in toxicology studies. The health status of the animals must be thoroughly assessed both before and during the study by veterinarians according to established veterinary medical practice guidelines (51). For the safety assessment, using a species sensitive to the vaccine antigen, the pathogenic, or the toxin being studied is crucial. Additionally, the selected animal species for the toxicology study must be capable of mounting an immune response to the vaccine antigen (50).

Typically, a single relevant animal species is sufficient for toxicity studies. However, in cases where

the protection mechanism induced by newly developed vaccines is not well understood, or there are species/strain-specific variations in responses to the product's pharmacodynamics, it becomes essential to employ multiple species. This approach is crucial to accurately characterizing the outcome and ensuring the toxicity profile is well-defined across different biological systems. The choice of animal model influences the group sizes in toxicity studies. For smaller animal models like rats and mice, approximately ten animals per sex are recommended in each group. Conversely, for larger animal models, e.g., monkeys, it is suggested that a minimum of three adult animals per sex be used in each group. The optimal mean age at the study's onset is 6-8 weeks for rodents and 3 - 4 months for rabbits (52).

The toxicity study must be performed using a maximum dose of a candidate vaccine capable of eliciting an appropriate immune response in the animal model. Detailed dose-response evaluation and determining a lethal dose are not required for vaccine toxicology studies. However, pilot dose-response studies can be conducted to identify the dose that produces the maximum antibody response in the selected animal model. It is advised that the highest dose proposed for utilize in the clinical trial be assessed during the preclinical studies (53). There may be instances where the volume of a human dose exceeds the capacity for a single injection site, necessitating administration at multiple sites. Additionally, animal dosage must be calculated on a mg/kg basis in cases involving newly developed vaccine formulations to induce an immune response. Under such circumstances, the scaling factor between humans and animals must be justified (17, 47, 50). The vaccine doses can be administered intermittently, potentially reducing the interval compared to the clinically recommended interval. The dosing interval for preclinical toxicology studies might be determined based on the vaccinated animal's primary and secondary antibody responses, as informed by previous pharmacodynamics studies (9, 26, 47).

The route of administration in animals must resemble that proposed for the clinical trial. In safety studies, if toxic effects are detected following a specific path of administration, for example, intranasal or oral, other toxicity studies with a diverse way of administration might be beneficial in considering the aspects of the product toxicity (14, 53). The study design includes a negative control group to establish a reference point level, an active control group (e.g., vaccine formulation agent without the antigen or adjuvant), and the treatment group with both antigen and formulated vaccine. An additional treatment group should be considered for sacrifice and assessment during late-phase scarification to estimate reversible and delayed adverse effects observed during treatment (11, 20, 26).

The area of study should assess local inflammatory responses, the impact on regional lymph nodes, systemic toxicity, and the overall functioning of the immune system. It is essential to conduct daily clinical observations, along with monitoring body weight gain and food intake on a weekly basis. Therefore, it is recommended to measure body weight and food consumption during the initial week of management (15). Additionally, hematology analysis, such as different white blood cell counts, red blood cell count, albumin/globulin ratio, electrolytes, and serum biochemistries, must be measured 1-3 days after the first and last dose administration and at the end of the recovery period. For new vaccine development, assessing coagulation parameters, urine samples, serum immunoglobulin classes, and acute-phase proteins may also be necessary. Data should be collected during treatment, 1-2 weeks or more after treatment, and after the last dose to determine the persistence or reversibility of observed adverse effects (15, 54, 55).

Histopathological evaluations of tissues should give attention to the immune organs responses such as the spleen, lymph nodes, thymus, bone marrow, and Peyer’s patches, as well as vital organs such as brain, kidneys, lungs, heart, liver, male and female reproductive organs, and the vaccine injection site (56). The scope of tissue to be inspected depends on the vaccine, the available knowledge, and the insights gained from previous preclinical and clinical testing. Comprehensive tissue examination will be necessary for the innovative vaccines lacking preclinical and clinical experience. Therefore, the list of tissues to be examined must be well-defined after consultation with the relevant National Regulatory Authority (11, 14, 57).

Single-dose toxicity studies (Acute toxicity studies). When a pharmaceutical agent is prescribed in one or more doses over a short period, not exceeding 24 hours, it is defined as acute toxicity. Single-dose studies provide valuable data that describe the association between administered doses and systemic or

local toxicity. The primary purposes include evaluating the initial maximum tolerable dose with no adverse effect, doses causing life-threatening toxicity, safety, and binding reactions (47).

Acute toxicity studies in animals are typically conducted using two routes of substance administration (1). The route proposed for humans and (2), where possible, intravenous administration. If intravenous dosing is recommended for humans, evaluating this route in animals provides adequate information (58, 59). Research must be performed in at least two mammalian species, one of which should be a non-rodent species. However, under certain circumstances and contingent upon validated safety data, studies may be limited to a single species. In instances where acute toxicity studies in animals are provided, it is essential to conduct toxicity assessments to evaluate dose-response relationships and pharmacokinetics (14, 60). In single-dose toxicity studies, animals should be monitored for at least 14 days post-vaccine administration. Any mortalities, clinical symptoms, duration of signs, and reversibility of toxicity should be documented. Gross necropsies and histopathological evaluations should be performed on all studied animals, including those purposefully sacrificed and those that die during or after the study (7, 14).

Repeated dose toxicity studies. The foremost aim of repeated-dose toxicity studies is to recognize the adverse toxicological effects resulting from administering repeated doses of a substance for a specified period up to the anticipated lifespan of the test species, which can range from 3 weeks to 2 years in various animal studies. Furthermore, repeated dose toxicity should be carried out in two mammalian species: rodent and non-rodent (7, 14).

For repeated dose toxicity assessment, the duration of investigation should span at least 28 days, while immunotoxicology assessments must be undertaken between 14 days and three months. Studies must include equal numbers of both sexes (female and male animals). The size of treatment groups should be adequately large, particularly for interim scarifications and recovery phases. Evaluated factors include changes in clinical symptoms, physiology, growth or life span, biochemistry, behavior, and gross pathological alterations in various organs (7, 26, 61). The repeated-dose toxicity studies provide insights into several aspects, including general toxicity, toxicity to specific target organs, dose-response relationships, responses to toxic metabolites from the body, and information on delayed/reversibility/irreversibility responses (20, 61).

In specific vaccine repeated-dose toxicity studies, three doses are administered: 1) the lowest dose that yields appropriate pharmacodynamics effects; 2) the highest dose levels designed to induce toxicity without causing death; and 3) an intermediate dose. This approach demonstrates any dose-related response and establishes a no-observed-adverse-effect level (NOAEL) at the lowest dose or Lowest Observed Adverse Effect Level (LOAEL). The units of NOAEL or LOAEL include mg/kg/BW/day or ppm. For inhalation studies, the unit can be mg/L/6h/day (62-64).

Developmental and reproductive performance toxicity (DART) studies. COVID-19 protective vaccines during pregnancy, in women of childbearing potential, and breastfeeding women are crucial considerations in pandemic conditions. Thus, the FDA recommends that clinical sponsors conduct DART animal studies before enrolling pregnant and breastfeeding women in clinical trials. Vaccine manufacturers may submit data from prior DART studies utilizing similar platform technologies in certain situations. After review by the national FDA, if these data are deemed scientifically robust, they can be used in clinical trials (64-66).

Local tolerance studies. It can be directed as part of the repeated dose toxicity study or as a separate study. Tolerance should be determined at the site in contact with the vaccine antigen, including the location of administration, and also at those sites that might be accidentally exposed, such as the eye. Both gross macroscopic evaluations and histopathological assessments of the muscles, skins of injected areas, and local draining lymph nodes are necessary for regional tolerance assessment. Local tolerance data from toxicity studies may be submitted to expedite progression to the first-in-human (FIH) clinical trials with COVID-19 vaccine candidates (67, 68).

Genotoxicity (In-vivo and in-vitro). Genotoxicity tests include in vitro and in vivo tests designed to identify compounds that cause genetic damage through various mechanisms. These tests identify DNA damage, gene mutations, chromosomal mutations, inherited effects, the process of malignancy or tumor genesis, and genetic deviations. Compounds

that produce such types of damage are considered carcinogens or mutagens in humans. While the association between some chemicals and their carcinogenesis effects has been established in humans, drawing a direct connection to heritable diseases remains challenging. Thus, for some pharmaceutical products, genotoxic consequences could suggest potential hereditary effects, and subsequently, the results can be used to predict carcinogenicity. Moreover, developed genotoxicity tests provide sufficient information to interpret carcinogenicity studies (68, 69).

In new drug development, the registration of pharmaceuticals needs a comprehensive assessment of their genotoxic potential. Previous studies have shown that many compounds found mutagenic in specific tests, like the Ames test or the bacterial reverse mutation test, also exhibited carcinogen effects when tested on rodents. On the other hand, in vitro mammalian tests are more sensitive in detecting rodent carcinogens; however, in some cases, they yield a higher incidence of positive results that are not correlated with rodent carcinogenicity. Furthermore, various approaches remain valid for evaluating vaccines, as no single test can identify all genotoxic mechanisms pertinent to tumorigenesis. Therefore, it is recommended to assess mutagenicity using the bacterial reverse gene mutation test, which has proven effective in detecting appropriate genetic variations associated with rodent genotoxicity and human carcinogens. Genotoxicity assessments should also encompass evaluations in mammalian cells and in vivo models. Various in vitro protocols utilizing mammalian cells are commonly employed, including the metaphase chromosome aberration assay, the micronucleus assay, and the mouse lymphoma assay (MLA) (70-72).

Therefore, these assays are considered appropriate methods and can be recommended for measuring chromosomal damage in combination with other genotoxicity tests. In vivo tests are preferred because some agents show mutagenic effects in vivo but not in vitro, and factors such as absorption, distribution, metabolism, and excretion can interact with the results (72).

Carcinogenicity. Carcinogenicity studies in animals are necessary to determine the tumorigenic potential of pharmaceutical agents and address any concerns resulting from laboratory research, animal toxicology studies, and unpredicted findings in clinical trials. If a pharmaceutical is used continuously for at least six months, carcinogenicity studies should be undertaken. Pharmaceutical agents or vaccines administered intermittently or for a short period do not require carcinogenicity studies unless specific concerns exist (73, 74).

Experiments for preclinical studies in COVID-19 Vaccine production. In recent years, due to outbreaks of infectious diseases, new platforms for vaccine production have gained significant attention for their quality control assessments, preclinical studies, and clinical trials. This was evident when the WHO Emergency Use Listing Procedure (EUL) approved several vaccines at the onset of the COVID-19 outbreak, including BNT162b2/COMIRNATY, Covishield, mRNA-1273/Spikevax, and Ad26.COV2.S. The recent outbreak compelled companies and authorized organizations to expedite procedures to assess the suitability of novel health products during public health emergencies. A list of immunogenicity and protection studies performed on animals receiving WHO-approved COVID-19 vaccines is mentioned in Tables 1 and 2. The objective is to make drugs, vaccines, and diagnostics tools available as rapidly as possible to address the crisis while adhering to stringent safety, efficacy, and quality criteria. The assessment balances the threat posed by the emergency with the benefits of using the product, considering any potential risks. Inactivated and recombinant vaccines are technically well-developed in Iran. They can be produced and pass clinical trials with fewer regulatory barriers to licensing due to appropriate preclinical studies. Nevertheless, in the context of exploring the potential commercialization and clinical implementation of vaccines intended for human use, this review article highlights, to the best of our understanding, some challenges that arise:

Novelty of vaccines platforms. The preclinical evaluation of COVID-19 vaccines has been pivotal in assessing their efficacy and safety before clinical trials. Various novel vaccine candidates, including mRNA and DNA-based formulations, have demonstrated promising immunogenicity results in animal models, indicating their potential for further human safety evaluation. Some newly developed vaccines, such as mRNA vaccines, require modifications and additional assessments to ensure they possess the ap-

propriate protective properties. As mentioned in the introduction of the current article, existing preclinical studies for vaccines will need to be modified to ensure they meet the necessary criteria for obtaining licenses for human use.

Recently developed mRNA AfriVac 2121 (Wuhan) vaccine elicited a protective immune response in hamsters comparable to Moderna's mRNA-1273, demonstrating robust humoral responses against the ancestral B.1 strain of SARS-CoV-2 (45). The broad-spectrum RQ3013 mRNA vaccine showed high antibody titers against multiple variants, including Omicron, and protected mice and nonhuman primates (46). Conversely, a novel DNA vaccine, pVAX1/S2-6EHGFP, was developed targeting conserved epitopes of SARS-CoV-2. It induced significant humoral and cellular immune responses in BALB/c mice, including neutralizing antibodies against the Omicron variant (75). The MVA-S(3P-beta) vaccine candidate, designed to target the beta variant, demonstrated complete protection in transgenic mice against lethal challenges, eliciting strong immune responses (41). Consequently, innovative platforms necessitate additional preclinical investigations compared to conventional platforms before initiating clinical trials.

In vivo efficacy and safety. Validation of vaccines' in vivo efficacy and safety is needed. These vaccines might have protective effects, biodistribution, and tolerance that differ from those seen with traditional platforms. While these preclinical studies show encouraging results, challenges such as vaccine hesitancy and the emergence of new variants continue to pose significant hurdles in the global vaccination effort.

New developed delivery system. Developed approved vaccines such as Pastocovac®, BNT162b2, and Ad26.COV2.S have typically novel delivery vehicles, which can have varied effects within the body. Consequently, delivery vehicles should be evaluated separately and as part of the formulated vaccine to assess potential side effects on target tissues. However, there have not been enough studies to determine the best vaccine delivery vehicle combining high efficacy and low complications in an appropriate animal model.

Future directions. Preclinical studies are crucial in vaccine development and pivotal for gaining FDA approval and licensing. Before vaccines proceed to clinical trials, these studies assess various factors, including safety, efficacy, protective effects, and toxicology. Therefore, understanding the specific pathways requires a comprehensive and multidisciplinary approach to prevent complications during human trials. Throughout this review and based on our experience with preclinical studies of various vaccine platforms produced in the SARS-Cov2 outbreak, we have explored several guidelines, suggesting directions for future research in this domain. For future studies in this area, we propose the following suggestions:

1: Evaluate the effects of additive excipients on the efficacy and safety of vaccines.

2: Assess the potential immunomodulatory effects of selected antigens and delivery vehicles.

3: Test formulated vaccines against specific pathogens using animal models to measure their protective efficacy.

4: Investigate the influence of sex and age on the efficacy of vaccines.

5: Evaluate the potential impacts of concomitant diseases and environmental factors, such as minerals and vitamins, on vaccine efficacy.

CONCLUSION

In conclusion, this review delivers an inclusive overview of the existing knowledge on Preclinical studies on COVID-19 candid vaccines with various platforms, highlighting immunogenicity, protective effects, and toxicology studies. Preclinical testing is a prerequisite for conducting clinical trials involving human subjects. The primary objective of these preclinical trials is to assess the potential toxicity and efficacy of novel therapeutic drugs or vaccines through the use of human cell cultures and various animal models before their administration in human participants. Typically, comprehensive preclinical testing is essential to gather sufficient data that can reliably demonstrate the safety of a new vaccine, including an acceptable dosage, as well as its potential efficacy, toxicity, and pharmacokinetic characteristics. Furthermore, preclinical trials provide an opportunity for companies, researchers, and regulatory bodies to simulate potential interactions between the vaccine and its targets.

For instance, the development of new vaccines,

such as those aimed at combating COVID-19, necessitates rigorous preclinical testing as an integral part of the broader research and development process for vaccine production. Generally, these preclinical evaluations must be completed before a vaccine can advance to clinical trials, with insights derived from the ongoing COVID-19 pandemic informing current practices. Subsequently, the vaccine undergoes regulatory scrutiny and approval by the National Food and Drug Administration, followed by the establishment of manufacturing processes and quality control measures for both the vaccine and its adjuvants. This discussion outlines the critical steps leading to the approval of preclinical tests for clinical trials and the associated processes involved.

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