Food and Chemical Toxicology 152 (2021) 112239

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

SARS-CoV-2 inactivated vaccine (Vero cells) shows good safety in repeated administration toxicity test of Sprague Dawley rats

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ARTICLE INFO

Handling Editor: Dr. Jose Luis Domingo

Keywords:
COVID-19
The inactivated vaccine
Safety
The repeated administration toxicity

ABSTRACT

The outbreak of COVID-19 has posed a serious threat to global public health. Vaccination may be the most effective way to prevent and control the spread of the virus. The safety of vaccines is the focus of preclinical research, and the repeated dose toxicity test is the key safety test to evaluate the vaccine before clinical trials. The purpose of this study was (i) to observe the toxicity and severity of an inactivated SARS-CoV-2 vaccine (Vero cells) in rodent Sprague Dawley rats after multiple intramuscular injections under the premise of Good Laboratory Practice principles and (ii) to provide a basis for the formulation of a clinical trial scheme. The results showed that all animals in the experimental group were in good condition, no regular changes related to the vaccine were found in the detection of various toxicological indexes, and no noticeable stimulating reaction related to the vaccine was found in the injected local tissues. The neutralizing antibodies in the low- and high-dose vaccine groups began to appear 14 days after the last administration. In the negative control group, no neutralizing antibodies were observed from the administration period to the recovery period. Therefore, the repeated administration toxicity test of the inactivated SARS-CoV-2 vaccine (Vero cells) in Sprague Dawley rats showed no obvious toxic reaction. It was preliminarily confirmed that the vaccine can stimulate production of neutralizing antibodies and is safe in Sprague Dawley rats.

1. Background

The rapid spread of COVID-19 since the outbreak has posed a serious threat to global public health. According to the World Health Organization (WHO) website, on November 17, 2020, the number of confirmed COVID-19 cases reached 39,023,292, and the number of deaths reached 1,099,586. The WHO has named it 2019 novel coronavirus (2019-nCoV) on January 12, 2020, and announced that the COVID-19 epidemic was listed as a "public health emergency of international concern" on January 30, 2020, and at the same time, the disease was officially named coronavirus disease 2019 (COVID-19) on February 11, 2020. 2019-nCoV is a novel coronavirus strain that has never been found in humans before. Middle East respiratory syndrome-related coronavirus (MERSr-CoV) and severe acute respiratory syndrome-related coronavirus (SARSr-CoV) belong to the β-coronavirus family (Xu et al., 2020), and have 88% nucleotide homology with SARS-CoV-2 (Lu et al., 2020).

In August 2020, the latest research results revealed that 2019-nCoV is highly infectious and its transmission is difficult to detect (Hao et al., 2020). Due to these features, cases of 2019-nCoV infection, especially asymptomatic ones, are not easy to find; hence, the virus easily spread globally and in medical institutions. Even if the virus can be completely eliminated from the population, the viral transmission mechanism from

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https://doi.org/10.1016/j.fct.2021.112239
Received 18 January 2021; Received in revised form 2 April 2021; Accepted 17 April 2021
Available online 24 April 2021

Table 1
Grouping and administration.

Group	Male	Female	Name of administration	Dosing volume (mL)	Administration concentration
The negative control group	M001-020	F001-020	0.9% NaCl	0.5	0.9%
The solvent control group	M021-040	F021-040	Menstruum control of inactivated SARS-CoV-2 vaccine	0.5	–
The low-dose vaccine group	M041-060	F041-060	Inactivated SARS-CoV-2 vaccine	0.5	100U/0.5 mL
The high-dose vaccine group	M061-080	F061-080	Inactivated SARS-CoV-2 vaccine	0.5	150U/0.5 mL
The negative control satellite group	M081-085	F081-085	0.9% NaCl	0.5	0.9%
The low-dose vaccine satellite group	M086-090	F086-090	Inactivated SARS-CoV-2 vaccine	0.5	100U/0.5 mL
The high-dose vaccine satellite group	M091-095	F091-095	Inactivated SARS-CoV-2 vaccine	0.5	150U/0.5 mL

Table 2
Blood collection and necropsy.

3 days after the first administration (5♀ and5♂/group)	3 days after the last administration (10♀ and 10♂/group) 14 days after the last administration (5♀ and5♂/group)
Hematology examination	Hematology and coagulation examination $CD4^+/CD8^+$ neutralizing antibody、antinuclear antibody
Serum biochemistry test (Includes electrolyte)	Serum biochemistry test (Includes electrolyte)
Gross necropsy	Histopathological examination (Include organ wet weight、collect pathological specimens)

host to human remains unclear, and there is a risk of re-outbreak or periodic epidemics (Zhao et al., 2020). Vaccination may be the most effective method to prevent and control the epidemic. Since China has published the 2019-nCoV genome sequence, China, the United States, Britain, Canada, Australia, and many vaccine companies have been working on developing a COVID-19 vaccine. A SARS-CoV-2 inactivated vaccine has been developed in Institute of Medical Biology, Chinese Academy of Medical Sciences. The SARS-CoV-2 inactivated vaccine will be used in the healthy population to prevent the spread of COVID-19, so its safety is of great importance and is the focus of pre-clinical research. According to the General Principles for the Technical Review of Pre-clinical Safety Evaluation of Biological Products for Prevention (CDE, 2008), the toxic reactions caused by vaccines mainly include (i) direct damage to the body caused by toxic components, (ii) the immune-related toxicity caused by the induced immune system, and (iii) the toxicity caused by pollutants and residual impurities. Since the vaccine induces the immune system to produce antibodies and/or effector T cells, the most important potential toxicity comes from the toxicity related to the immune system.

Preclinical safety evaluation is one of the important phases in vaccine development. Its goal is to predict the safety of clinical application by detecting the potential toxicity of new vaccines in animals, reduce the risks taken by clinical trial subjects and clinical users, and provide a basis for the formulation of clinical trial protocols (Forster, 2012; Liu and Li, 2018; Sun et al., 2012). The repeated administration toxicity test is a critical safety test for evaluating vaccines before clinical trials. When applying for an investigational new drug permit for the novel coronavirus vaccine, it is necessary to vaccinate at least one related animal species with one more repeat than the planned clinical trial. In view of the rapid spread of SARS-CoV-2 and the wide range of transmission, more risks and challenges may be faced. In order to obtain more comprehensive virus data and understand the cumulative toxicity that may be caused by repeated clinical injections, in this study we performed the repeated administration toxicity test for the SARS-CoV-2 inactivated vaccine in animals and carried out necessary preclinical toxicological studies for clinical trials and marketing of the SARS-CoV-2 inactivated vaccine.

2. Materials and methods

2.1. Vaccines

All SARS-CoV-2 virus strains used in this work were isolated from hospitalized patients including domestic and foreign patients with confirmed COVID-19 in Yunnan Hospital of Infectious Diseases from January to May 2020. A strain with a D614G mutation in the S protein was isolated from a pediatric patient who had returned from the U.S. to their hometown and was identified as being infected with SARS-CoV-2 through clinical diagnosis and q-RT-PCR in March 2020. The virus was proliferated in Vero cells (WHO) and was titrated with a micro-titration assay. Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Corning, NY, USA) containing 5% fetal bovine serum (FCS; HyClone, Logan, USA).

The SARS-CoV-2 inactivated vaccine was developed by the Institute of Medical Biology (IMB), Chinese Academy of Medical Sciences (CAMS). Briefly, the virus strain, named KMS-1 (GenBank No: MT226610.1), was inoculated into Vero cells for production in an environment that met the BSL requirements. The harvested viruses were inactivated by formaldehyde (v:v = 1:4000) for 48 h to lyse the viral membrane, purified via chromatography and concentrated. A second inactivation with beta-propiolactone (v:v = 1:2000) was performed to destroy the viral genomic structure, followed by a second purification and concentration using the same protocol. The vaccine stock was evaluated using various quality indexes, including antigen content, immunogenicity, sterility and residue testing. The viral antigen content was measured via ELISA. The vaccine contained 100 or 150 U of inactivated viral antigen adsorbed to 0.25 mg of Al(OH)3 adjuvant and suspended in 0.5 mL of buffered saline for each dose. The placebo contained only the same amount of Al(OH)3 in buffer.

2.2. Animals

Specific Pathogen Free Sprague Dawley rats, 6–8 weeks old; animal weight range when purchased: female, 150.8–192.7 g; male, 158.7–203.5 g; and the weight variance of the animals in the same batch did not exceed 20% of the average weight. Animals were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd., production license No. SCXK (Jing) 2016-0006. During the test, rats were kept with two animals per cage, with free access to maintenance feed and sterilized water. This protocol has been approved by the Experimental Animal Ethics Committee of institute of Medical Biology, Chinese Academy of Medical Sciences before its implementation (approval No. DWSP202005

Table 3
wt statistics of all animals (MEAN ± SD, g).

Group	Group day (n = 40)	6 days after the first administration (n = 30)	13 days after the first administration (n = 30)	6 days after the second administration (n = 30)	13 days after the second administration (n = 30)	6 days after the third administration (n = 30)	13 days after the third administration (n = 30)	Weight gain percentage (%)	6 days after the last administration (n = 10)	13 days after the last administration (n = 10)	Weight gain percentage (%)
The negative control group	200.3 ± 17.3	249.6 ± 41.7	290.4 ± 60.3	317.0 ± 74.1	341.6 ± 85.7	357.6 ± 94.6	372.9 ± 102.4	84.4 ± 37.0	409.8 ± 118.4	427.6 ± 122.3	106.6 ± 46.1
The solvent control group	198.6 ± 19.9	249.0 ± 39.4	292.1 ± 53.9	320.6 ± 68.6	347.3 ± 80.4	367.5 ± 88.6	385.6 ± 99.5	91.4 ± 33.9	398.1 ± 106.1	413.6 ± 118.9	105.6 ± 40.1
The low-dose vaccine group	198.1 ± 19.4	248.2 ± 39.2	293.0 ± 56.1	321.8 ± 71.0	347.6 ± 81.7	365.9 ± 94.0	382.6 ± 102.9	90.1 ± 36.3	397.5 ± 123.3	414.0 ± 131.0	108.1 ± 50.5
The high-dose vaccine group	200.5 ± 19.1	248.1 ± 40.9	287.8 ± 56.1	315.0 ± 68.9	339.1 ± 84.2	358.0 ± 94.7	379.3 ± 103.9	86.0 ± 35.2	400.5 ± 117.2	419.2 ± 131.8	105.9 ± 47.3

Note：Weight gain percentage = (Current weight - weight before administration)/weight before administration × 100%, ' n ' was the Sprague Dawley rats number of each group.

005). The use of animals conforms to the 3R (replace, reduce, refine) principle.

2.3. Animal grouping and administration

190 Sprague Dawley rats (half ♂ and half ♀) were randomly divided into four main test groups and three satellite groups. The main test groups included the following: a negative control group (normal saline), a solvent control group (SARS-CoV-2 inactivated vaccine blank control, 0 U in 0.5 mL/dose), a low-dose vaccine group (SARS-CoV-2 inactivated vaccine, 100 U in 0.5 mL/dose), and a high-dose vaccine group (SARS-CoV-2 inactivated vaccine, 150 U in 0.5 mL/dose), with 20 male and 20 female rats per group. The satellite groups, which were only prepared for blood collection for detection of neutralizing antibody, included a negative control satellite group, a low-dose vaccine satellite group, and a high-dose vaccine satellite group, with 5 male and 5 female rats per group. All satellite groups were administrated by vaccines or normal saline corresponding to the main test groups. A total volume of 0.5 mL (0.25 mL on both sides of the hind limb) was used per vaccination. The dosage of vaccine was selected according to the General Principles for the Technical Review of Pre-clinical Safety Evaluation of Biological Products for Prevention (CDE, 2008), which also conformed to WHO guidelines on the nonclinical evaluation of vaccine (WHO, 2014). All vaccines were administered intramuscularly by 1 mL aseptic injection syringe (Shaanxi Longkangxin Medical Instrument Co., Ltd). Referring to above guiding principles, animals received at least one more vaccination than clinically prescribed. Exposure intervals should generally be determined according to the immune response of animals. Because animals generally reach stable antibody formation after 2–3 weeks of inoculation, long-term toxicity tests generally use exposure intervals of 2–3 weeks. Referring to the above principles, the SARS-CoV-2 inactivated vaccine (Vero cells) was administered once each on days 0, 14, 28, and 42, for a total of four administrations, and the interval was 2 weeks, followed by a recovery period after the last administration of 2 weeks (14 days). Therefore, rats received four bilateral vaccinations Specific grouping information is shown in Table 1.

2.4. Clinical indicators

All indicators describe below was adopted to comply with recommendations contained in General Principles for the Technical Review of Pre-clinical Safety Evaluation of Biological Products for Prevention (CDE, 2008).

2.4.1. General clinical observations

Observations were carried out three times on days of administration: once in the morning before administration, once 30–60 min after administration, and once in the afternoon. Observation was conducted twice daily on the non-administration days: once in the morning and once in the afternoon.

2.4.2. wt and food intake test

During the experiment, body weight and food intake were measured once a week with a YP20001 balance (Sartorius Scientific Instruments (Beijing) Co., Ltd). Weight gain was calculated as follows:

$$weight\,gain\,percentage = \frac{current\,weight - weight\,before\,administration}{weight\,before\,administration} \times 100\%$$

Each cage animal was given enough feed every week. The amount of food intake per rat per day was calculated as follows:

$$food intake = \frac{amount\,given\,per\,week - remaining\,amount\,per\,week}{number\,of\,animals}$$

Table 4
Food intake statistics of all animals (MEAN ± SD, g).

Group	13 days after the first administration (n = 30)	6 days after the second administration (n = 30)	13 days after the second administration (n = 30)	6 days after the third administration (n = 30)	13 days after the third administration (n = 30)	13 days after the last administration (n = 10)
The negative control group	167.4 ± 39.5	167.8 ± 36.7	170.4 ± 34.9	165.6 ± 33.3	167.2 ± 30.2	180.8 ± 37.2
The solvent control group	172.4 ± 34.4	168.1 ± 33.3	172.3 ± 32.1	172.5 ± 34.2	170.6 ± 33.9	162.8 ± 38.5
The low-dose vaccine group	171.4 ± 36.4	174.3 ± 36.6	172.9 ± 37.8	167.8 ± 36.7	165.6 ± 33.7	173.4 ± 40.9
The high-dose vaccine group	169.5 ± 35.2	172.8 ± 43.6	166.8 ± 37.1	165.9 ± 38.0	175.4 ± 38.0	177.6 ± 48.7

Note: 'n' was Sprague Dawley rats number of each group.

2.4.3. Eye and urine routine examination

At 2 and 13 days after the last administration, the animals were examined with a YZ11 ophthalmoscope (66 Vision Technology Co., Ltd., China) to check whether the optic nerve disc, macula, venules/arterioles on the macula, and retinal venules/arterioles were abnormal. At 2 and 13 days after the last administration, urine of animals was collected for analysis with a UA-66 urine automabic analyzer (Mindray Medical International Ltd., China) after 4 h of fasting.

2.4.4. Hematology examination

At 3 days after the first administration, 3 and 14 days after the last administration, about 1.5 mL of blood was collected from the abdominal vein of rats in the experimental group (EDTA-2K anticoagulation). The samples were centrifuged at 3000 rpm for 10 min, and the plasma was collected and blood cells were counted by a 950-hematology analyzer (Drew Scientific Company, USA).

2.4.5. Serum biochemistry

At 3 days after the first administration, 3 and 14 days after the last administration, about 3 mL of blood was collected from the abdominal vein of rats in the experimental group. The blood samples were centrifuged at 3000 rpm for 10 min to separate the serum. About 100 μL of serum was taken 3 and 14 days after the last administration for antinuclear antibody (ANA) detection. The rest of the serum was detected by an Olympus AU400 automatic biochemical analyzer (Olympus Corporation, Japan).

2.4.6. Coagulation examination

At 3 and 14 days after the last administration, about 2 mL of blood was collected from the abdominal vein of rats in the main experimental group (9:1 sodium citrate anticoagulant) and centrifuged at 3000 rpm for 10 min, and plasma was detected by a CA500 automatic coagulation analyzer (Sysmex Co., Ltd, Japan).

2.4.7. $CD4^+$ T cell and $CD8^+$ T cell determination

At 3 and 14 days after the last administration, about 100 μL of blood added with EDTA-2K anticoagulant was taken as the sample for flow cytometry. Cells stained with CD3-PE (201412), CD4-FITC(201505), CD8a-PerCP(201712, all purchased from Biolegend Inc., USA) at room temperature for 15–30min followed by OpitiLyse C (A11895, Beckman Coulter Inc., USA) lysing solution at room temperature for 2–5min. Cells were then added with phosphate-buffered saline (PBS) and blended by vortex for testing by Cytoflex flow cytometry (Beckman Coulter Inc., USA). The proportions of $CD3^+$ $CD4^+$ cells and $CD3^+$ $CD8^+$ cells in T lymphocytes were analyzed by Cytexpert software, and the ratios of $CD3^+$ $CD4^+$ cells and $CD3^+$ $CD8^+$ cells were calculated.

2.4.8. Antinuclear antibody detection

About 100 μL of the remaining serum, which had been biochemically separated from the animals, was dissected 3 days after the last administration and tested by double-antigen enzyme-linked immunosorbent assay (ELISA) (Jiang Lai Bio Co., Ltd., China).

2.4.9. Neutralizing antibody detection

About 0.5 mL of venous blood was taken from the tail vein of animals in the satellite group before the first administration and 14 days after each administration. The blood was separated by centrifugation for 15 min at 3000 rpm without anticoagulant. The neutralization test was used to evaluate the efficacy of virus neutralization.

2.5. Gross anatomy

At 3 days after the first administration, autopsy was performed on the main experimental group by intraperitoneally injecting 2% pentobarbital sodium at a dose of 100 mg/kg and then dissecting the animals for visual observation. The specific number of dissected animals is shown in Table 2.

2.6. Pathological examination

At 3 and 14 days after the last administration, the animals in the main experimental group were anesthetized with 2% sodium pentobarbital at a dose of 100 mg/kg, sacrificed, and dissected. The wet weights of brain, heart, liver, thymus, spleen, lung, kidney, adrenal gland, testis, epididymis, ovary, and uterus were measured, and the organ coefficient was calculated as follows: $organ\ coefficient = \frac{organ\ wet\ weight}{body\ weight} \times 100\%$. All organs were observed according to the guiding principles, including the sublingual gland, submandibular gland, parotid gland, spinal cord (neck, chest, and lumbar segment), pituitary gland, trachea, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, pancreas, aorta, skin (abdomen), breast (female), vagina, fallopian tube, bladder, sciatic nerve, sternum, femur, thyroid, muscle (skeletal muscle), distal lymph nodes, eyes, Haugh's glands, adrenal glands, brain (brain, cerebellum, brainstem), prostate, seminal vesicle (male), thyroid (including parathyroid), the administration site and all abnormal tissues (CDE, 2008). The eyes were fixed with eye fixator, and other organs were fixed with neutral formalin solution. Paraffin-embedded sections were made and hematoxylin eosin staining was used for microscopic examination. The terms and criteria of pathological diagnosis were mainly based on the International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) (Berridge et al., 2016; Brändli-Baiocco et al., 2018; Mann et al., 2012; Renne et al., 2009; Thoolen et al., 2010).

Table 5
Statistical data of urine routine examination of all animals.

Group	Test time	Number of animals	WBC	NIT	URO	PRO	PH	BLD	SG	KET	BIL	GLU	Vc
The negative control group	2 days after the last administration	20	±:5/20 -:12/20 3+:2/20 2+:1/20	-:14/20 +:6/22	Norm:19/20 3+:1/20	3+:6/20 -:1/20 1+:2/20 2 + 11/20	6.5:2/20 9.0:2/20 8.5:7/20 7.0:3/20 8.0:2/20 7.5:3/20 6.0:1/20	-:12/20 2+:1/20 1+:6/20 3+:1/20	1.030:4/20 1.020:8/20 1.025:7/20 1.015:1/20	±:5/20 -:2/20 1+:6/20 2+:7/20	1+:4/20 2+:13/20 3+:2/20 -:1/20	-:19/20 3+:1/20	1+:3/20 2+:2/20 -:15/20
The solvent control group			±:1/20 3+:9/20 -:10/20	+:10/20 -:10/20	Norm:18/20 3+:2/20	1+:4/20 2+:5/20 3+:7/20 ±: 4/20	6.5:2/20 6.0:3/20 7.5:6/20 7.0:4/20 8.0:3/20 8.5:2/20	-:11/20 1+:6/20 2+:2/20 3+:1/20	1.025:4/20 1.020:4/20 1.030:11/20 1.010:1/20	1+:4/20 -:4/20 ±: 3/20 2+:8/20 3+:1/20	-:6/20 2+:4/20 1+:6/20 3+:4/20	-:19/20 2+:1/20	1+:3/20 2+:3/20 -:14/20
The low-dose vaccine group			±:2/20 2+:2/20 3+:6/20 -:10/20	+:14/20 -:6/20	Norm:15/20 3+:5/20	3+:7/20 1+:4/20 2+:8/20 -:1/20	6.0:2/20 6.5:4/20 7.0:7/20 7.5:2/20 8.0:4/20 8.5:1/20	1+:6/20 -:8/20 2+:1/20 3+:5/20	1.030:5/20 1.025:7/20 1.010:1/20 1.020:7/20	±:8/20 -:1/20 1+:9/20 2+:2/20	-:6/20 3+:1/20 1+:8/20 2+:5/20	-:20/20	-:20/20
The high-dose vaccine group			±:6/20 -:6/20 3+:8/20	-:12/20 +:8/20	Norm:16/20 3+:4/20	1+:4/20 -:1/20 2+:7/20 3+:8/20	6.5:3/20 6.0:4/20 7.0:4/20 7.5:1/20 8.0:3/20 8.5:4/20 9.0:1/20	2+:1/20 -:6/20 1+:10/20 3+:3/20	1.025:5/20 1.020:5/20 1.030:7/20 1.015:3/20	±:5/20 -:2/20 1+:11/20 2+:2/20	2+:6/20 -:2/20 3+:9/20 1+:3/20	-:20/20	-:17/20 1+:2/20 2+:1/20
Group	Test time	Number of animals	WBC	NIT	URO	PRO	PH	BLD	SG	KET	BIL	GLU	Vc
The negative control group	13 days after the last administration	10	3+:3/10 ±:1/10 -:5/10 2+:1/20	+:4/10 -:6/10	Norm:8/10 3+:2/10	1+:3/10 2+:1/10 3+: 6/10	7.0:1/10 8.0:3/10 8.5:4/10 9.0:2/10	1+:2/10 -:6/10 2+:2/10	1.025:5/10 1.015:1/10 1.030:3/10 1.020:1/10	-:1/10 1+:4/10 ±: 2/10 2+:3/10	1+:4/10 2+:2/10 3+:4/10	-:10/10	-:6/10 1+:2/10 2+:2/20
The solvent control group			-:5/10 ±:4/10	+:4/10 -:6/10	Norm:9/10 3+:1/20	3+:3/10 2+:3/10	7.5:1/10 7.0:1/10 8.0:4/10	2+:2/10 -:6/10	1.030:2/10 1.025:4/10	±:2/10 -:1/10	1+:2/10 -:2/10	-:10/10	-:10/10

(*continued on next page*)

Table 5 (*continued*)

Group	Test time	Number of animals	WBC	NIT	URO	PRO	PH	BLD	SG	KET	BIL	GLU	Vc
			3+:1/20			±:1/10 1+:3/10	8.5:3/10 9.0:1/10	1+:2/10	1.020:4/10	1+:4/10 2+:3/10	2+:3/10 3+:3/10		
The low-dose vaccine group			±:5/10 -:4/10 3+:1/10	+:2/10 -:8/10	Norm:8/10 3+:1/10 1+:1/10	3+:4/10 2+:2/10 1+:1/10 ± : 1/10 -:2/10	6.5:2/10 8.5:5/10 8.0:3/10	2+:3/10 1+:5/10 -:2/10	1.020:2/10 1.015:1/10 1.030:4/10 1.025:3/10	1+:2/10 -:3/10 2+:3/10 3+:2/10	-:3/10 3+:6/10 2+:1/10	-:10/10	-:8/10 1+:2/10
The high-dose vaccine group			-:2/10 ±:3/10 3+:5/10	-:5/10 +:5/10	Norm:8/10 3+:2/10	±:2/10 1+:4/10 3+:1/10 2+:3/10	7.5:1/10 7.0:1/10 8.0:2/10 8.5:4/10 9.0:2/10	2+:3/10 1+:2/10 -:5/10	1.020:8/10 1.015:1/10 1.025:1/10	±:5/10 -:3/10 1+:2/10	-:2/10 3+:1/10 2+:3/10 1+:4/10	-:10/10	-:10/10

2.7. Statistical analysis

SPSS 21.0 was used for statistical analysis, and the *t*-test was used for comparison between the two groups. $P < 0.05$ was considered to indicate a statistical difference. Data, including body weight, food intake, organ weight and organ coefficients, hematological indexes, lymphocyte subsets, are expressed as mean ± SD. If the paired organs of an animal were missing on one side, the organ weight and organ coefficient of the animal were not used in the calculation of the average. Statistical and biological significance were considered in the analysis of results. The above data were analyzed according to the following process: firstly, Levene's test was conducted for the data homogeneity test. If the data are uniform ($P > 0.05$), then one-way ANOVA is conducted; if the ANOVA difference is significant ($P < 0.05$), Dunnett's multiple comparison (parametric method) is performed. If Levene's test results were significant ($P < 0.05$), the Kruskal–Wallis nonparametric test was performed. If the Kruskal–Wallis nonparametric test results were significant ($P < 0.05$), the Mann–Whitney *U* test was used for pairwise comparison. In addition, for the semiquantitative ordered data results of urine examination, the Ridit test of multiple groups was used. If the results were significant ($P < 0.05$), the Ridit test of two groups was used for further statistics. For the observation of clinical symptoms, no statistics were conducted because there were no abnormal symptoms related to the test product, and no statistical tests were performed in the pathological examination.

3. Results

3.1. The clinical symptoms

The results of clinical symptoms showed that during the trial, the rats in each group were in good condition and exhibited normal activity. After receiving multiple repeated administrations, no obvious abnormalities were seen at the injection site, such as redness, swelling, bruising, induration, suppuration, fester, and other irritation related to the vaccine.

3.2. Body weight and food intake

During the administration and recovery period, the body weight of rats in all groups showed an increasing trend, and there were no statistically significant changes in body weight and body weight growth rates in the solvent control group, the low-dose vaccine group, and the high-dose vaccine group compared with the negative control group ($P > 0.05$), as shown in Table 3. During the whole experimental period, the food intake of male rats was significantly higher than that of female rats, but there was no difference between groups ($P > 0.05$), as shown in Table 4. It was concluded that the test product had no significant effect on the weight gain of the animals.

3.3. Urine routine examination

At 2 and 13 days after the last administration of the SARS-CoV-2 inactivated vaccine, compared with the negative control group, there was no statistically significant difference in urine biochemical indexes ($P > 0.05$) (Table 5). At 2 days after the last administration, although occult blood (BLD) was not only found in an animal, there was no statistical difference between the groups, and it had no toxic significance.

3.4. Hematology and coagulation examination

At 3 days after the first administration of the SARS-CoV-2 inactivated vaccine, there were no statistically significant differences in various hematological indicators compared with the negative control group ($P > 0.05$). At 3 days after the last administration, white blood cell (WBC) and lymphocyte (LYM) were slightly increased in one rat in the negative control group, solvent control group, and low-dose vaccine group, but the difference was not statistically significant ($P > 0.05$). There were no statistically significant differences between the other dose groups and the negative control group ($P > 0.05$). At 14 days after administration withdrawal, there was no statistically significant difference between the other dose groups and the negative control group ($P > 0.05$), as shown in Table 6. There was no statistically significant change ($P > 0.05$) in coagulation indexes of rats in each group (Table 7). Thus, no toxicological changes were observed in the hematological and coagulation indexes of the main test group during the experiment.

3.5. Serum biochemistry and electrolyte examination

At 3 days after the first administration of the SARS-CoV-2 inactivated

Table 6
Statistical data of hematology examination in all animals (MEAN ± SD).

Group	Test time	Number of animals	WBC × 109/L	RBC × 1012/L	Hb × g/L	HCT %	MCV fL	MCH Pg	MCHC × g/L	PLT × 109/L	NE# × 109/L	LY# × 109/L	EO# × 109/L	MO# × 109/L	BA# × 109/L
The negative control group	3 days after the first administration	10	4.55 ± 1.11	6.30 ± 0.39	164.5 ± 9.0	35.9 ± 2.7	56.9 ± 3.3	26.1 ± 1.1	459.3 ± 16.1	779.6 ± 104.1	1.58 ± 0.42	2.61 ± 0.68	0.03 ± 0.03	0.34 ± 0.10	0.01 ± 0.02
The solvent control group			4.73 ± 1.93	6.16 ± 0.48	165.2 ± 10.5	36.1 ± 2.6	58.7 ± 1.2	26.9 ± 1.0	458.0 ± 13.9	773.3 ± 82.3	1.74 ± 1.22	2.66 ± 0.75	0.03 ± 0.02	0.29 ± 0.14	0.01 ± 0.03
The low-dose vaccine group			5.36 ± 1.82	6.56 ± 0.37	174.3 ± 8.1	37.7 ± 2.1	57.5 ± 2.4	26.6 ± 1.2	462.8 ± 14.1	849.4 ± 110.3	1.68 ± 0.83	3.20 ± 0.98	0.04 ± 0.08	0.43 ± 0.13	0.01 ± 0.03
The high-dose vaccine group			5.10 ± 1.93	6.55 ± 0.65	170.3 ± 13.2	37.1 ± 2.2	56.9 ± 4.0	26.1 ± 1.6	459.0 ± 17.1	838.7 ± 95.2	1.80 ± 1.01	2.82 ± 0.74	0.02 ± 0.04	0.44 ± 0.26	0.01 ± 0.02
The negative control group	3 days after the last administration	20	5.31 ± 2.66	7.69 ± 0.76	185.4 ± 18.3	44.9 ± 3.9	58.5 ± 2.0	24.1 ± 1.3	412.8 ± 14.7	783.4 ± 106.5	1.32 ± 0.81	3.70 ± 1.91	0.05 ± 0.06	0.23 ± 0.09	0.01 ± 0.03
The solvent control group			4.86 ± 2.85	7.42 ± 0.75	181.2 ± 22.9	44.5 ± 5.0	60.0 ± 2.4	24.4 ± 1.1	406.4 ± 9.2	760.0 ± 162.6	1.27 ± 1.05	3.32 ± 1.92	0.04 ± 0.03	0.24 ± 0.13	0.01 ± 0.02
The low-dose vaccine group			5.27 ± 2.38	7.54 ± 1.10	185.5 ± 30.9	45.0 ± 6.8	59.8 ± 2.6	24.6 ± 1.5	411.2 ± 13.7	758.1 ± 207.3	1.50 ± 1.05	3.46 ± 1.52	0.04 ± 0.04	0.24 ± 0.08	0.01 ± 0.02
The high-dose vaccine group			4.80 ± 1.97	7.39 ± 0.64	178.6 ± 12.5	43.4 ± 3.4	58.8 ± 3.2	24.2 ± 1.4	412.2 ± 14.6	807.5 ± 134.6	1.08 ± 0.50	3.46 ± 1.58	0.04 ± 0.03	0.21 ± 0.11	0.00 ± 0.01
The negative control group	14 days after the last administration	10	5.45 ± 1.71	7.28 ± 0.71	176.0 ± 9.2	39.3 ± 2.8	54.2 ± 3.0	24.3 ± 1.6	448.5 ± 14.5	817.1 ± 111.2	1.27 ± 0.45	3.90 ± 1.44	0.03 ± 0.02	0.24 ± 0.11	0.01 ± 0.03
The solvent control group			5.07 ± 1.99	7.25 ± 0.69	168.5 ± 12.1	38.0 ± 3.5	52.5 ± 3.3	23.3 ± 1.3	444.0 ± 17.6	817.3 ± 110.5	1.15 ± 0.41	3.71 ± 1.63	0.03 ± 0.02	0.19 ± 0.09	0.00 ± 0.00
The low-dose vaccine group			5.56 ± 2.07	7.55 ± 0.59	174.0 ± 5.3	40.0 ± 2.1	53.2 ± 2.7	23.1 ± 1.6	435.4 ± 20.0	850.0 ± 198.0	1.13 ± 0.36	4.20 ± 1.72	0.03 ± 0.02	0.20 ± 0.06	0.00 ± 0.00
The high-dose vaccine group			5.03 ± 2.50	7.31 ± 0.60	177.1 ± 11.9	39.0 ± 2.3	53.5 ± 2.3	24.3 ± 1.1	453.8 ± 12.8	845.1 ± 87.1	1.18 ± 0.66	3.57 ± 1.71	0.03 ± 0.01	0.25 ± 0.17	0.00 ± 0.00

Note:WBC,White blood cell; RBC,Red blood cell; Hb,Hemoglobin; HCT,Red blood cell specific volume; MCV,Mean corpuscular volume; MCH,Mean corpsular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; RDW, Red blood cell volume distribution width; PLT,Platelet; MPV,Mean platelet volume; NE, Neutrophils; NE%,Neutrophils%; LY,lymphocyte; LY%,lymphocyte%; EO,Eosinophils; EO%,Eosinophils%; MO,Monocytes; MO%,Monocytes%; BA,Basophils.

vaccine, the alkaline phosphatase (ALP) value of the solvent control group was increased compared with the negative control group, and the difference was statistically significant ($P < 0.05$). There were no statistically significant differences between the other dose groups and the negative control group ($P > 0.05$). At 3 and 14 days after administration withdrawal, there was no statistically significant difference in serum biochemical indexes between each group and the negative control group ($P > 0.05$), as shown in Table 8. During the study, the electrolyte including potassium (K+), sodium (Na+), chloride (Cl-), calcium (Ca+), had no significant difference between groups (Table 9).

3.6. Immune function-related indicators

ANA levels were determined by double antigen ELISA after repeated administration of the SARS-CoV-2 inactivated vaccine. The results showed that the serum ANA was negative in the negative control group, solvent control group, and low- and high-dose groups during the experiment, as shown in Table 10. $CD4^+$ and $CD8^+$ T cell counts, as determined by flow cytometry, at different time points (3 and 14 days after the last administration) are shown in Table 10 and Fig. 1. Compared with the negative control group, there was no significant difference in the proportion of peripheral blood $CD4^+$ and $CD8^+$ T cells in the solvent control group and the low- and high-dose groups. Furthermore, the CD4+/CD8+ cell ratio of each group showed no statistical difference with the negative control group. The neutralizing antibodies in all groups were negative before grouping. At 14 days after the first administration, the neutralizing antibodies in the low-dose vaccine satellite group and the high-dose vaccine satellite group began to appear, with average titers of 1:5.2 and 1:17.6, respectively; at 14 days after the second administration, the levels of neutralizing antibodies of the two groups gradually increased, with average titers of 1:80 and 1:108.8, respectively; and at 14 days after the third administration,

Table 7
Statistical data of coagulation examination in all animals (MEAN ± SD).

Group	Test time	Number of animals	Indices of blood coagulation function		
			PT (Second)	APTT (Second)	Fbg (Second)
The negative control group	3 days after the last administration	20	13.3 ± 0.4	13.9 ± 2.0	12.2 ± 2.0
The solvent control group			13.4 ± 0.5	14.7 ± 1.6	11.7 ± 1.8
The low-dose vaccine group			13.5 ± 0.7	14.1 ± 2.1	11.6 ± 3.5
The high-dose vaccine group			13.6 ± 0.8	14.5 ± 2.1	11.3 ± 1.2
The negative control group	14 days after the last administration	10	13.4 ± 0.5	14.9 ± 1.6	11.9 ± 1.7
The solvent control group			13.2 ± 0.6	13.4 ± 3.1	11.6 ± 3.4
The low-dose vaccine group			13.4 ± 0.8	14.6 ± 3.4	11.9 ± 1.4
The high-dose vaccine group			13.3 ± 0.8	14.2 ± 2.1	12.2 ± 3.5

Note: PT, Prothrombin time; APTT, Activated partial thromboplastin time; Fbg, fibrinogen.

the levels of neutralizing antibodies of the two groups continued to rise, with average titers of 1:128 and 1:128, respectively. The neutralizing antibody levels of the two groups remained at 1:128 until 14 days after the fourth administration, as shown in Table 11 and Fig. 2.

3.7. Eye exam

During the trial, no abnormal changes were found in the eyelid, conjunctiva, sclera, cornea, iris, pupil, lens, optic disc, macula, arterioles, retinal arterioles, vitreous body, and fundus of the rats in the test group.

3.8. Pathological examination

3.8.1. Gross necropsy

Rats were dissected 3 days after the first administration and 3 and 14 days after the last administration. There were no obvious abnormalities in the apparent pathology of each group of animals. The skin, subcutaneous tissue, and muscle of all animals in all autopsies were examined at the original injection site, and no irritant reaction related to the SARS-CoV-2 inactivated vaccine was found.

3.8.2. Organ weight

During the test period, compared with the negative control group in the same period, there was no statistically significant difference in the weight and relative weight of all organs in the test group ($P > 0.05$), as shown in Table 12, indicating that this vaccine does not affect the weight of organs.

3.8.3. Histopathological examination

At 3 days after the last dose, a small number of inflammatory cells were found in the myocardium of two animals in the low-dose vaccine group, 14 days after the last dose, and one animal in the solvent control group, one in the low-dose group, and one in the high-dose vaccine group had the same pathological symptoms (Fig. 3A). At 3 days after the last dose, there were two small foci of inflammatory necrosis in the liver parenchyma and sink area, mainly including lymphocytes and emptying hepatocytes. This pathological symptom also appeared 14 days after the last dose in two animals in the negative control group and the solvent control group (Fig. 3B); these two animals also developed glomerular atrophy or increased glomerular capillaries (Fig. 3C), but its structure was clear and complete, and there was no obvious structural cell necrosis. At 3 days after the last dose, pulmonary pathology showed that one or two animals in each group showed local pulmonary interstitial thickening, alveolar epithelial cell proliferation, shedding and varying degrees of vascular and alveolar septal capillary congestion, a small amount of inflammatory cell infiltration, or thickened alveolar septa in the vascular lumen and perivascular and interstitial lung (Fig. 3D and E). In the negative control group, two animals showed a large amount of exudate and a small amount of monocytes and local glomerular atrophy with increased glomerular capillaries (Fig. 3C).

Even though some minor lesion was observed in each group, there no significant group correlation for injuries. Most pathological results of rats in each group, no matter at 3 or 14 days after the last administration, were normal. Fig. 4 showed that there were no obvious histopathological changes in main organs including lung, liver, kidney and heart in each group. Meanwhile, muscles at injection site were also pathologically examined. No obvious pathological changes were found in each group as well (Fig. 5).

4. Discussions

Vaccination is the most effective and economical means to prevent and control many infectious diseases (Iwasaki and Omer, 2020; Leidner et al., 2019). Because vaccines are mainly used for immunization of healthy people, the safety requirements of vaccines should be much higher than those of therapeutic drugs. Pre-clinical safety evaluation is one of the important phases in the development of new vaccines. However, due to the sudden outbreak, the pre-clinical safety evaluation of the SARS-CoV-2 inactivated vaccine remains to be conducted. How to evaluate the safety of the vaccine is also under continuous exploration.

It is generally considered that the vaccination dose is small and the adverse reactions are mild. According to the WHO guidelines on vaccine toxicology studies, no matter what animal model is used, it is recommended to give at least the dose that one person would receive (WHO, 2005, 2014). The General Principles for the Technical Review of Pre-clinical Safety Evaluation of Biological Products for Prevention also indicate that the vaccine dose should, in general, enable the vaccine to achieve the best immune response in animals, and that long-term toxicity tests can be conducted directly using the high dose (per person) proposed in clinical trials (CDE, 2008). In the current study, the candidate dose of the SARS-CoV-2 inactivated vaccine for clinical trial, 100 U/person and 150 U/person, proposed by the results of previous immunogenicity and protection studies was respectively used as low dose and high dose.

In the pre-clinical safety evaluation of vaccine products, a single animal species should be used to study the toxicity of repeated administration (CDE, 2008). The Sprague Dawley rats used in this study are the most commonly used rodents in vaccines general toxicity studies (Mancebo et al., 2012; Sifontes-Rodríguez et al., 2009; Stokes et al., 2020). The background information of this family of animals is abundant, and the supply of animals is sufficient. The candidate vaccine was planned to apply for adults over 18 years old. Therefore, the choice of Sprague Dawley rats on 5–6 weeks, which reached sexual maturity, were more reasonable in repeated dose toxicity study. In neutralizing

Table 8
Statistical data of serum biochemistry of all animals (MEAN ± SD).

Group	Test time	Number of animals	TBIL umol/L	TP g/L	ALB g/L	GLO g/L	ALT U/L	AST U/L	ALP U/L	GGT U/L	CK U/L	BUN mmol/L	CREA umol/L	CHOL mmol/L	TG mmol/L	GLU mmol/L
The negative control group	3 days after the first administration	10	0.70 ± 0.32	54.3 ± 3.8	30.5 ± 2.1	23.8 ± 1.8	33.8 ± 5.0	100.5 ± 23.3	239.5 ± 74.4	0.50 ± 0.24	162.3 ± 81.6	4.9 ± 0.8	30.1 ± 2.3	1.62 ± 0.34	0.53 ± 0.22	8.1 ± 0.7
The solvent control group			0.86 ± 0.43	52.6 ± 2.2	29.6 ± 1.3	23.1 ± 1.1	36.5 ± 6.6	102.5 ± 23.6	334.9 ± 75.7*	0.50 ± 0.19	179.4 ± 117.4	4.3 ± 0.9	27.7 ± 2.2	1.68 ± 0.31	0.62 ± 0.30	7.5 ± 0.7
The low-dose vaccine group			0.81 ± 0.46	52.9 ± 2.0	30.2 ± 1.2	22.7 ± 1.2	38.9 ± 6.6	94.2 ± 18.3	302.1 ± 95.8	0.54 ± 0.25	149.8 ± 74.4	4.9 ± 1.0	28.7 ± 2.3	1.70 ± 0.41	0.54 ± 0.26	7.9 ± 0.8
The high-dose vaccine group			0.78 ± 0.47	55.2 ± 3.4	31.0 ± 2.2	24.2 ± 1.4	34.6 ± 11.3	95.9 ± 17.3	260.2 ± 141.6	0.60 ± 0.28	192.7 ± 86.3	4.9 ± 1.2	29.9 ± 2.8	1.54 ± 0.38	0.43 ± 0.11	8.2 ± 1.0
The negative control group	3 days after the last administration	20	1.92 ± 0.55	60.7 ± 6.0	31.9 ± 4.2	28.8 ± 2.1	34.9 ± 6.4	110.7 ± 25.8	114.6 ± 38.6	0.73 ± 0.16	257.4 ± 166.5	5.7 ± 0.7	41.6 ± 6.0	1.33 ± 0.38	0.59 ± 0.44	8.3 ± 1.2
The solvent control group			1.58 ± 0.62	60.7 ± 4.8	31.7 ± 3.8	29.0 ± 1.6	44.3 ± 50.4	113.0 ± 59.1	124.6 ± 46.8	0.63 ± 0.17	249.7 ± 148.9	5.5 ± 0.7	42.2 ± 7.0	1.54 ± 0.23	0.60 ± 0.28	9.6 ± 2.8
The low-dose vaccine group			1.77 ± 0.47	60.5 ± 4.4	30.7 ± 3.0	29.8 ± 2.0	34.9 ± 5.6	94.5 ± 17.2	119.5 ± 42.3	0.65 ± 0.20	207.2 ± 141.9	5.6 ± 1.0	42.6 ± 7.5	1.45 ± 0.32	0.49 ± 0.21	9.0 ± 1.2
The high-dose vaccine group			1.97 ± 0.86	60.9 ± 5.3	31.2 ± 4.1	29.7 ± 1.9	35.9 ± 12.2	116.6 ± 67.5	122.5 ± 35.3	0.64 ± 0.13	238.5 ± 160.0	5.6 ± 0.8	43.8 ± 7.8	1.39 ± 0.21	0.44 ± 0.19	9.2 ± 2.4
The negative control group	14 days after the last administration	10	1.90 ± 0.47	59.7 ± 4.2	32.0 ± 3.8	27.7 ± 1.9	40.5 ± 7.3	104.5 ± 26.5	104.0 ± 32.1	0.58 ± 0.26	196.1 ± 103.5	4.9 ± 0.6	48.4 ± 5.1	1.46 ± 0.22	0.40 ± 0.08	9.2 ± 1.5
The solvent control group			1.82 ± 0.51	60.0 ± 7.1	31.9 ± 5.6	28.1 ± 2.1	45.0 ± 10.5	119.7 ± 51.8	106.0 ± 37.1	0.59 ± 0.12	200.2 ± 90.0	5.3 ± 0.5	43.6 ± 6.1	1.57 ± 0.35	0.61 ± 0.29	9.8 ± 1.3
The low-dose vaccine group			1.81 ± 0.39	61.6 ± 6.4	32.3 ± 4.7	29.3 ± 1.9	47.7 ± 8.7	107.9 ± 19.2	99.8 ± 37.5	0.65 ± 0.15	207.5 ± 97.2	4.7 ± 0.4	42.9 ± 5.6	1.56 ± 0.41	0.53 ± 0.28	9.3 ± 2.1
The high-dose vaccine group			1.95 ± 0.60	65.7 ± 8.5	34.5 ± 6.4	31.2 ± 2.9	46.5 ± 17.6	124.3 ± 82.2	110.1 ± 53.7	0.48 ± 0.10	378.7 ± 312.1	5.0 ± 0.8	41.5 ± 5.1	1.62 ± 0.37	0.75 ± 0.56	9.5 ± 1.0

Note: TP,Total bilirubin; ALB,Albumin; GLO,Globulin; A/G,Albumin/Globulin; ALT,Alanine amiotransferase ; AST,Aspartate aminotransferase; ALP,Alkaline phosphatase; GGT,γ-glutamyl transpeptadase; CK,Creatine kinase; BUN,Blood urea nitrogen; CREA, Creatinine; CHOL, Cholesterol ; TG,Triglyceride; GLU,Glucose; TBIL, Total bilirubin, "*"Compared with Saline control group,$p < 0.05$.

Table 9
Statistical data of electrolyte of all animals (MEAN ± SD).

Group	Test time	Number of animals	K+ mmol/L	Na+ mmol/L	Cl- mmol/L	Ca+ mmol/L
The negative control group	3 days after the first administration	10	5.31 ± 0.85	141.13 ± 1.62	100.34 ± 1.46	1.45 ± 0.06
The solvent control group			5.43 ± 1.01	141.03 ± 1.46	100.08 ± 3.05	1.44 ± 0.04
The low-dose vaccine group			5.15 ± 0.66	141.28 ± 0.85	101.52 ± 1.48	1.42 ± 0.06
The high-dose vaccine group			5.36 ± 1.04	141.02 ± 0.86	99.06 ± 2.39	1.43 ± 0.09
The negative control group	3 days after the last administration	20	4.62 ± 0.80	141.14 ± 1.11	99.72 ± 2.19	1.41 ± 0.05
The solvent control group			4.90 ± 2.29	140.89 ± 2.00	99.97 ± 2.17	1.42 ± 0.06
The low-dose vaccine group			4.91 ± 1.17	140.96 ± 1.23	99.21 ± 1.85	1.45 ± 0.07
The high-dose vaccine group			4.99 ± 1.10	140.79 ± 1.28	100.39 ± 2.11	1.42 ± 0.05
The negative control group	14 days after the last administration	10	4.81 ± 0.58	141.36 ± 0.85	102.20 ± 3.68	1.42 ± 0.06
The solvent control group			4.83 ± 0.52	141.50 ± 1.13	101.96 ± 2.91	1.43 ± 0.07
The low-dose vaccine group			4.78 ± 1.09	142.23 ± 1.01	104.04 ± 2.25	1.44 ± 0.06
The high-dose vaccine group			4.75 ± 0.74	141.21 ± 1.00	102.31 ± 2.38	1.46 ± 0.07

Note: K+:Potassium; Na+:Sodium; Cl-:Chloride; Ca+:Calcium.

Table 10
T lymphocyte subset distribution and antinuclear antibody of rats (MEAN ± SD).

Group	Test time	Number of animals	CD4+ (%)	CD8+(%)	CD4+/CD8+	ANA
The negative control group	3 days after the last administration	20	35.42 ± 3.62	16.35 ± 3.67	2.29 ± 0.64	0.10 ± 0.01
The solvent control group			37.10 ± 4.14	16.47 ± 4.07	2.39 ± 0.63	0.10 ± 0.02
The low-dose vaccine group			36.27 ± 6.98	16.27 ± 2.73	2.29 ± 0.58	0.09 ± 0.01
The high-dose vaccine group			35.17 ± 5.18	14.85 ± 3.57	2.49 ± 0.60	0.10 ± 0.01
The negative control group	14 days after the last administration	10	29.63 ± 6.96	17.44 ± 2.76	1.72 ± 0.47	0.10 ± 0.01
The solvent control group			35.26 ± 3.84	17.39 ± 3.05	2.09 ± 0.42	0.10 ± 0.01
The low-dose vaccine group			32.90 ± 6.90	16.51 ± 1.91	1.99 ± 0.34	0.10 ± 0.00
The high-dose vaccine group			34.14 ± 4.22	17.66 ± 3.13	1.99 ± 0.40	0.09 ± 0.00

Note:CD4+,CD4+ positive T cell; $CD8^+$,CD8+ positive T cell; CD4+/CD8+,CD4+ positive T cell/CD8+ positive T cell; ANA,Antinuclear antibody.

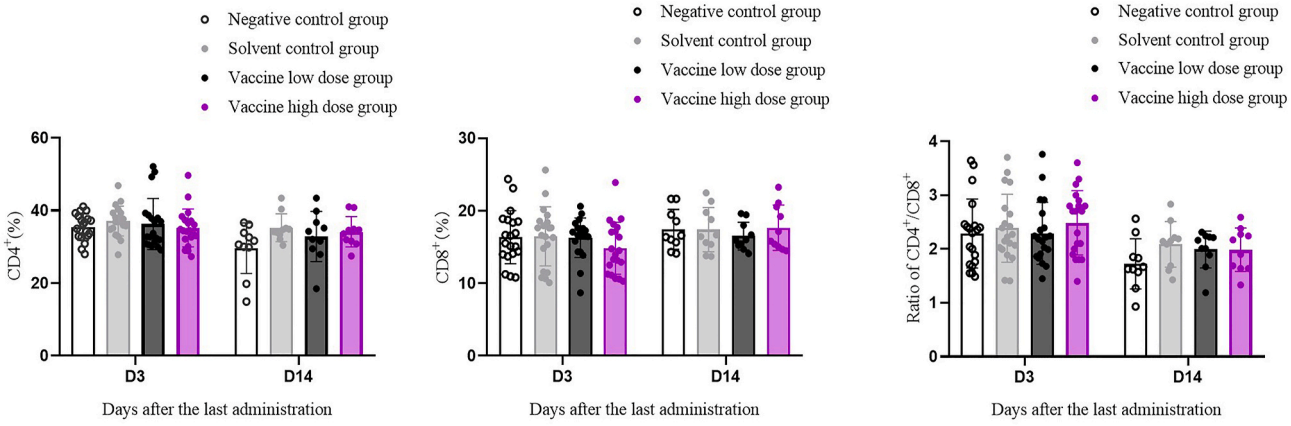

Fig. 1. The results of the $CD4^+$ and $CD8^+$ T cell counts. Compared with the negative control group, there was no significant difference in the proportion of peripheral blood $CD4^+$ and $CD8^+$ T cells in the solvent control group and the low- and high-dose groups. Furthermore, the $CD4^+/CD8^+$ cell ratio of each group showed no statistical difference with the negative control group.

antibody testing experiment, both low-dose vaccine group and the high-dose vaccine group could be detected at 14 days after the first administration. By 14 days after the second vaccination, the neutralizing antibodies were positive in all vaccinated groups. From 14 days after the second dose to 14 days after the third dose, the levels of neutralizing antibodies of these two groups continued to rise, and this trend remained until 14 days after the fourth administration, showing that the SARS-CoV-2 inactivated vaccine (Vero cells) could stimulate Sprague Dawley rats to produce a humoral immune response, and the antibody is maintained at a high level for at least 2 weeks. Therefore, the dose set in the repeated administration toxicity study produced a good immune response in Sprague Dawley rats. These results conformed to further support the applicability of the animal species in the repeated administration toxicity study.

For the purpose that providing sufficient evidence of toxicity might induced by proposed dose of SARS-Cov-2 inactivated vaccine, as many indicators as possible were tested in the repeated dose toxicity study. One of the potential toxicity of vaccines comes from immune system-related toxicity, including hypersensitivity reactions and autoimmunity. Autoimmunity is the main research content of immunotoxicology, especially for adjuvanted vaccines (Agmon-Levin et al., 2009; Batista--Duharte et al., 2014). So far, only a few autoimmune diseases caused by vaccine immunization have been reported, such as Guillain–Barré syndrome, caused by influenza vaccination in the United States (Schonberger et al., 1979). However, the detailed mechanism is still not fully understood. From the perspective of strict regulation, product-related immunotoxicological evaluation should be carried out during the safety evaluation of new vaccines before clinical trials. Serum autoantibodies have been widely used for the detection of specific autoimmune diseases (Descotes, 2000; Verdier et al., 1997), although the association between autoantibody levels and the development and severity of autoimmune diseases is not fully established. In this study, no

Table 11
Statistical data of neutralizing antibody detection in all animals.

Group	Before administration			14 days after the first administration	14 days after the second administration	14 days after the third administration	14 days after the last administration
	Test number	Animal number	Determination of neutralizing antibody results	Determination of neutralizing antibody results	Determination of neutralizing antibody results	Determination of neutralizing antibody results	Determination of neutralizing antibody results
The negative control satellite group	F081	1717	Neg	Neg	Neg	Neg	Neg
	F082	1589	Neg	Neg	Neg	Neg	Neg
	F083	1704	Neg	Neg	Neg	Neg	Neg
	F084	1783	Neg	Neg	Neg	Neg	Neg
	F085	1775	Neg	Neg	Neg	Neg	Neg
	M081	1809	Neg	Neg	Neg	Neg	Neg
	M082	1891	Neg	Neg	Neg	Neg	Neg
	M083	1876	Neg	Neg	Neg	Neg	Neg
	M084	1858	Neg	Neg	Neg	Neg	Neg
	M085	1879	Neg	Neg	Neg	Neg	Neg
The low-dose vaccine satellite group	F086	1762	Neg	1:4	1:64	1:128	1:128
	F087	1770	Neg	1:4	1:32	1:128	1:128
	F088	1735	Neg	1:4	1:64	1:128	1:128
	F089	1781	Neg	Neg	1:64	1:128	1:128
	F090	1702	Neg	1:8	1:128	1:128	1:128
	M086	1825	Neg	1:4	1:128	1:128	1:128
	M087	1794	Neg	1:16	1:64	1:128	1:128
	M088	1870	Neg	1:4	1:128	1:128	1:128
	M089	1844	Neg	1:4	1:64	1:128	1:128
	M090	1817	Neg	1:4	1:64	1:128	1:128
The high-dose vaccine satellite group	F091	1760	Neg	1:16	1:128	1:128	1:128
	F092	1729	Neg	1:4	1:64	1:128	1:128
	F093	1732	Neg	1:8	1:128	1:128	1:128
	F094	1753	Neg	1:4	1:128	1:128	1:128
	F095	1766	Neg	1:8	1:64	1:128	1:128
	M091	1854	Neg	1:16	1:128	1:128	1:128
	M092	1873	Neg	1:8	1:64	1:128	1:128
	M093	1833	Neg	1:64	1:128	1:128	1:128
	M094	1885	Neg	1:16	1:128	1:128	1:128
	M095	1818	Neg	1:32	1:128	1:128	1:128

Note：if the neutralizing antibody titer is lower than 1:4, it is judged as “negative”, and if it is higher than or equal to 1:4, it is judged as positive. Neg,Negative.

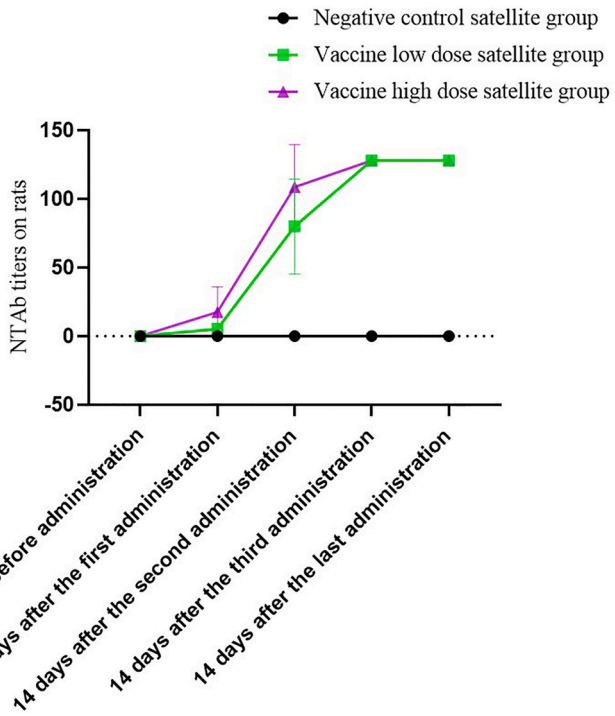

Fig. 2. The results of the neutralizing antibodies. The neutralizing antibodies in all groups were negative before grouping. At 14 days after the first administration, the neutralizing antibodies in the low-dose vaccine satellite group and the high-dose vaccine satellite group began to appear, with average titers of 1:5.2 and 1:17.6, respectively; at 14 days after the second administration, the levels of neutralizing antibodies of the two groups gradually increased, with average titers of 1:80 and 1:108.8, respectively; and at 14 days after the third administration, the levels of neutralizing antibodies of the two groups continued to rise, with average titers of 1:128 and 1:128, respectively. The neutralizing antibody levels of the two groups remained at 1:128 until 14 days after the fourth administration.

Table 12
Statistical data of organ wet weight in all animals (MEAN ± SD) .

Group	Test time	Number of animals	Body weight (g)	Brain		Heart		Liver		Kidney		Adrenal		Thymus	
				Absolute value	Relative weight	Absolute value	Relative weight	Absolute value	Relative weight	Absolute value	Relative weight	Absolute value	Relative weight	Absolute value	Relative weight
				(g)	(g/100 g)	(g)	(g/100 g)	(g)	(g/100 g)	(g)	(g/100 g)	(g)	(g/100 g)	(g)	(g/100 g)
The negative control group	3 days after the last administration	20	348.1 ± 95.7	2.04 ± 0.17	0.62 ± 0.15	1.10 ± 0.28	0.32 ± 0.03	10.05 ± 2.59	2.92 ± 0.35	2.31 ± 0.58	0.67 ± 0.06	0.07 ± 0.02	0.02 ± 0.01	0.47 ± 0.15	0.14 ± 0.03
The solvent control group			376.6 ± 98.0	2.05 ± 0.14	0.58 ± 0.14	1.21 ± 0.28	0.33 ± 0.04	11.55 ± 3.45	3.07 ± 0.38	2.57 ± 0.70	0.68 ± 0.06	0.16 ± 0.33	0.05 ± 0.12	0.50 ± 0.12	0.14 ± 0.04
The low-dose vaccine group			369.2 ± 97.3	2.04 ± 0.21	0.58 ± 0.12	1.18 ± 0.29	0.32 ± 0.04	10.94 ± 3.47	2.96 ± 0.38	2.51 ± 0.76	0.68 ± 0.07	0.07 ± 0.02	0.02 ± 0.01	0.49 ± 0.12	0.14 ± 0.03
The high-dose vaccine group			364.0 ± 97.4	2.03 ± 0.17	0.59 ± 0.14	1.19 ± 0.27	0.33 ± 0.03	10.97 ± 2.88	3.03 ± 0.28	2.40 ± 0.63	0.66 ± 0.07	0.07 ± 0.01	0.02 ± 0.01	0.47 ± 0.12	0.13 ± 0.03

Group	Test time	Number of animals	Spleen Absolute value (g)	Relative weight (g/100 g)	Lung Absolute value (g)	Relative weight (g/100 g)	Testis Absolute value (g)	Relative weight (g/100 g)	Epididymis Absolute value (g)	Relative weight (g/100 g)	Ovary Absolute value (g)	Relative weight (g/100 g)	Uterus Absolute value (g)	Relative weight (g/100 g)
The negative control group	3 days after the last administration	20	0.70 ± 0.17	0.21 ± 0.04	1.67 ± 0.33	0.50 ± 0.09	3.29 ± 0.29	0.12 ± 0.26	1.54 ± 0.34	0.06 ± 0.14	0.10 ± 0.03	0.04 ± 0.01	0.61 ± 0.12	0.24 ± 0.05
The solvent control group			0.74 ± 0.17	0.20 ± 0.03	1.77 ± 0.35	0.48 ± 0.05	3.28 ± 0.40	0.70 ± 0.11	1.41 ± 0.29	0.30 ± 0.06	0.12 ± 0.04	0.01 ± 0.02	0.68 ± 0.26	0.24 ± 0.09
The low-dose vaccine group			0.74 ± 0.16	0.20 ± 0.03	1.78 ± 0.33	0.49 ± 0.07	3.61 ± 0.36	0.79 ± 0.06	1.66 ± 0.32	0.37 ± 0.08	0.10 ± 0.01	0.04 ± 0.01	0.71 ± 0.27	0.26 ± 0.11
The high-dose vaccine group			0.76 ± 0.24	0.21 ± 0.03	1.72 ± 0.55	0.48 ± 0.07	3.38 ± 0.37	0.74 ± 0.06	1.49 ± 0.19	0.33 ± 0.05	0.10 ± 0.02	0.04 ± 0.01	0.59 ± 0.21	0.22 ± 0.08

Group	Test time	Number of animals	Body weight (g)	Brain Absolute value (g)	Relative weight (g/100 g)	Heart Absolute value (g)	Relative weight (g/100 g)	Liver Absolute value (g)	Relative weight (g/100 g)	Kidney Absolute value (g)	Relative weight (g/100 g)	Adrenal Absolute value (g)	Relative weight (g/100 g)	Thymus Absolute value (g)	Relative weight (g/100 g)
The negative control group	14 days after the last administration	10	412.3 ± 115.3	2.11 ± 0.13	0.55 ± 0.14	1.34 ± 0.42	0.32 ± 0.04	11.27 ± 3.08	2.75 ± 0.22	2.56 ± 0.70	0.63 ± 0.06	0.08 ± 0.02	0.02 ± 0.01	0.46 ± 0.14	0.11 ± 0.03
The solvent control group			396.3 ± 110.5	2.05 ± 0.18	0.55 ± 0.13	1.24 ± 0.35	0.31 ± 0.04	11.66 ± 3.30	2.94 ± 0.18	2.56 ± 0.69	0.65 ± 0.05	0.07 ± 0.01	0.02 ± 0.01	0.46 ± 0.13	0.12 ± 0.04
The low-dose vaccine group			393.0 ± 122.6	2.09 ± 0.17	0.58 ± 0.16	1.32 ± 0.38	0.34 ± 0.03	11.06 ± 2.96	2.86 ± 0.28	2.59 ± 0.78	0.66 ± 0.06	0.08 ± 0.02	0.02 ± 0.01	0.48 ± 0.10	0.13 ± 0.03
The high-dose vaccine group			404.0 ± 122.4	2.12 ± 0.10	0.57 ± 0.16	1.27 ± 0.30	0.32 ± 0.05	12.44 ± 3.99	3.10 ± 0.39	2.64 ± 0.66	0.67 ± 0.08	0.08 ± 0.01	0.02 ± 0.01	0.56 ± 0.19	0.14 ± 0.03

Group	Test time	Number of animals	Spleen Absolute value	Relative weight	Lung Absolute value	Relative weight	Testis Absolute value	Relative weight	Epididymis Absolute value	Relative weight	Ovary Absolute value	Relative weight	Uterus Absolute value	Relative weight

(*continued on next page*)

Table 12 (*continued*)

Group	Test time	Number of animals	Body weight (g)	Brain		Heart		Liver		Kidney		Adrenal		Thymus	
				Absolute value	Relative weight	Absolute value	Relative weight	Absolute value	Relative weight	Absolute value	Relative weight	Absolute value	Relative weight	Absolute value	Relative weight
			(g)	(g/100 g)	(g)	(g/100 g)	(g)	(g)	(g/100 g)	(g)	(g/100 g)	(g)	(g/100 g)	(g)	(g/100 g)
The negative control group	14 days after the last administration	10	0.74 ± 0.14	0.18 ± 0.03	1.84 ± 0.37	0.46 ± 0.07	3.48 ± 0.21	0.68 ± 0.06	1.66 ± 0.27	0.32 ± 0.06	0.14 ± 0.03	0.04 ± 0.01	0.80 ± 0.28	0.26 ± 0.10	
The solvent control group			0.75 ± 0.24	0.19 ± 0.02	1.72 ± 0.39	0.44 ± 0.05	3.28 ± 0.28	0.66 ± 0.07	1.56 ± 0.23	0.31 ± 0.05	0.10 ± 0.02	0.03 ± 0.01	0.61 ± 0.11	0.21 ± 0.04	
The low-dose vaccine group			0.75 ± 0.25	0.19 ± 0.02	1.74 ± 0.34	0.46 ± 0.09	3.64 ± 0.38	0.72 ± 0.03	1.51 ± 0.48	0.30 ± 0.11	0.10 ± 0.02	0.04 ± 0.01	0.77 ± 0.24	0.28 ± 0.08	
The high-dose vaccine group			0.78 ± 0.26	0.19 ± 0.02	1.79 ± 0.47	0.45 ± 0.07	3.49 ± 0.33	0.69 ± 0.03	1.48 ± 0.30	0.29 ± 0.07	0.09 ± 0.02	0.03 ± 0.01	0.76 ± 0.18	0.26 ± 0.06	

Note：Relative weight=(Absolute value)/(Body weight) × 100.

antinuclear antibodies were detected in rats after repeated immunization with the SARS-CoV-2 inactivated vaccine. These results indicate that the experimental vaccine might have a low risk of causing an autoimmune response. Meanwhile, the results of the present study showed that after repeated inoculations with SARS-CoV-2 inactivated vaccine (Vero cells) in rats, no specific affected organs were found. In addition, histopathological examination of the animals' immune organs including bone marrow, spleen, thymus, inguinal lymph nodes, mesenteric lymph nodes and tonsils, showed no significant changes, and repeated administration did not cause a local reflux lymph node stress response at the injection site, which further indicated that the SARS-CoV-2 inactivated vaccine have no obvious immunotoxicity in Sprague Dawley rats (Fig. S2).

$CD4^+$ and $CD8^+$ T cells respectively represent helper T cells and cytotoxic T cells, play a key role in antiviral immunity (Yang et al., 2018), the level of cellular immunity is another important consideration. In this study, the detection of rat $CD4^+$ and $CD8^+$ T cells was carried out, and the ratio of both kinds of cells had not changed significantly (Fig. S1). At the same time, immune cells (including lymphocytes, mononuclear phagocytes, neutrophils, basophils, eosinophils, and platelets) in the blood did not increase or decrease after the first dose or the last dose or during the recovery period. It indicated that the vaccine had no obvious effect on lymphocytes and there was no infectious antigen in inactivated vaccines, which would attack antigen presenting cells to produce cytotoxic T cells ($CD8^+$ T cells) (Yang et al., 2018).

Although there was a difference in serum ALP levels between the negative control group and the solvent control group 3 days after the first administration of the vaccine ($P < 0.05$), it returned to the normal value 3 and 14 days after the last administration, and there was no dose–response relationship. ALP was one of indicators related with liver function, especially serum ALP which was hepatic origin. The increase of ALP usually associated with liver lesions. However, other indexes related to liver function, including γ-glutamyl transpeptidase (GGT), total bilirubin (TBIL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and albumin (ALB), were normal at 3 days after first administration. Only one indicator change was not clinically significant. Meanwhile, the level of ALP in this study was normal comparing with other Sprague Dawley rats in toxic studies (Mancebo et al., 2012; Mary L.A. Giknis, 2006; Sifontes-Rodríguez et al., 2009). No abnormality was found in pathological examination of solvent control group at 3 days after first administration as well. Therefore, it suggested that the inactivated SARS-CoV-2 vaccine has no obvious hepatotoxicity in Sprague Dawley rats. At 3 and 14 days after the last administration, the results of urine routine examination showed that occult blood appeared in all groups, and the index was on the high side. In the comparative analysis with the negative control group, considering that the change of the index was not related to the vaccine, the 4-h urine content is the most ideal in the determination of various indicators of urine. Because the amount of urine is greatly affected by the amount of drinking water, especially for experimental animals, the retention process of collecting 4-h urine is relatively complex. Single measurements of the concentrations of substances in random urine samples give an incomplete view of the real situation. There was no statistically significant difference in other urine indicators between the two groups. In addition, urea nitrogen (BUN) and creatinine (CREA) levels were normal. Although a small number of animals (5/120) showed glomerular atrophy, only a small number of local changes were not obvious, and the incidence rate was low. In this study, the occurrence of renal lesions was not associated with certain doses or groups. We speculate that the lesions were spontaneous changes in the kidney and were due to accidental events unrelated to the vaccine. We conclude that the inactivated SARS-CoV-2 vaccine has no nephrotoxic effects in Sprague Dawley rats.

Histopathology examination of heart, liver, lung, and kidney at 3 and 14 days after the last administration showed some mild inflammatory symptoms, but its tissue structure was complete, clear, and no large area

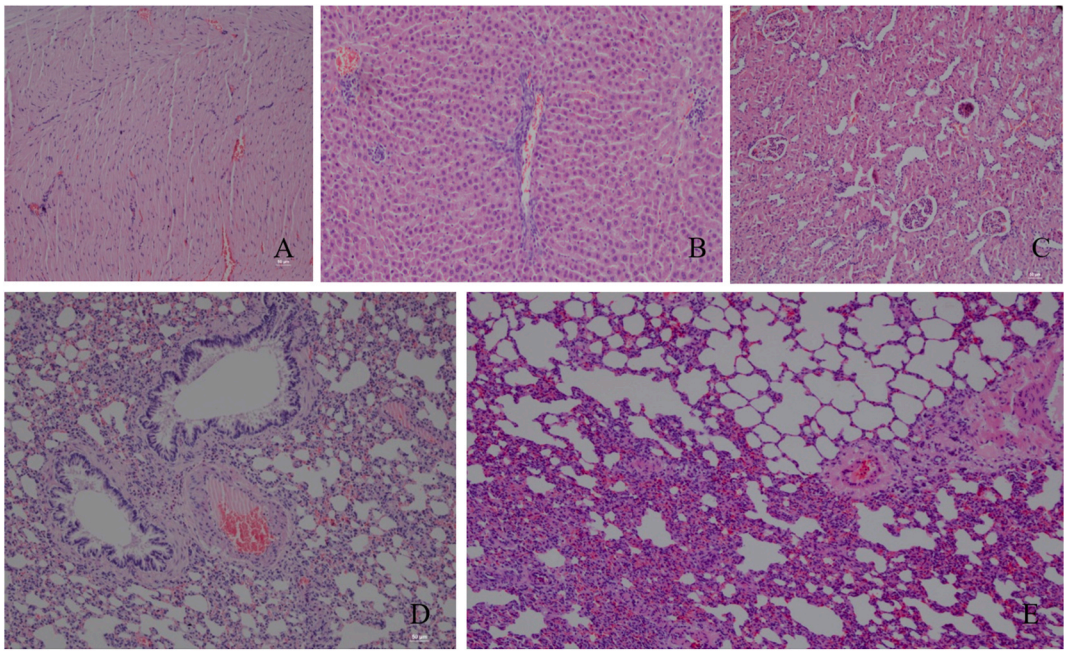

Fig. 3. The results of the histopathological examination. A: At 3 days after the last dose, a small number of inflammatory cells were found in the myocardium of two animals in the low-dose vaccine group, 14 days after the last dose, and one animal in the solvent control group, one in the low-dose group, and one in the high-dose vaccine group had the same pathological symptoms; B: At 3 days after the last dose, there were two small foci of inflammatory necrosis in the liver parenchyma and sink area, mainly including lymphocytes and emptying hepatocytes. This pathological symptom also appeared 14 days after the last dose in two animals in the negative control group and the solvent control group; C: Two animals in the negative control group also developed glomerular atrophy or increased glomerular capillaries, but its structure was clear and complete, and there was no obvious structural cell necrosis. Also in the negative control group, two animals showed a large amount of exudate and a small amount of monocytes and local glomerular atrophy with increased glomerular capillaries; D,E: At 3 days after the last dose, pulmonary pathology showed that one or two animals in each group showed local pulmonary interstitial thickening, alveolar epithelial cell proliferation, shedding and varying degrees of vascular and alveolar septal capillary congestion, a small amount of inflammatory cell infiltration, or thickened alveolar septa in the vascular lumen and perivascular and interstitial lung.

of cell injury was observed. These lesions did not have significant temporal differences and group distribution specificity, and there was no significant gender difference, nor was it a specific change of a certain type of disease. In compilation of histopathological findings in 4-26-week old Sprague Dawley rats compiled by Charles River Laboratory, the incidence of pathological changes in heart, liver, kidney and lung was higher than other organs no matter in male or female rats. All rats reported in this compilation were from 34 toxicity studies conducted in different laboratories (Mary L.A. Giknis, 2012). Also, it has been shown that Sprague Dawley rats often develop spontaneous diseases with chronic inflammation as the main lesion, including chronic interstitial pneumonia or liver and cardiac focal necrosis (Noto et al., 1998; Weber et al., 2011). Spontaneous renal lesions in rats are dominated by tubular lesions, including dilation of renal tubules, protein cast, tubular atrophy, pigmentation, and calcium deposition. The incidence increases with age and may be associated with many factors such as nutrition (Chandra and Frith, 1994; Hannerz et al., 1989). The indicators related to relevant organs function in the same batch were normal. Therefore, the above lesions were probably spontaneous or occasional lesions in Sprague Dawley rats, and some of them may be physical injuries caused by the operation processes. They have no toxic pathological significance, and no dose–effect-related lesions and target organs have been found.

The development of SARS-CoV-2 vaccine was still ongoing. A SARS-CoV-2 inactivated vaccine developed by Wuhan Institute of Biological Products Co. Ltd evaluated safety and immunogenicity of vaccine on seven different species of animals. Sprague Dawley rats were used in this study for repeated dose study as well. After different doses administration and 2-week recovery, there was no obvious systemic toxicity observed in rats (Wang et al., 2020). Similar to the study above, repeated administration of high-dose (1.5 person dosages) SARS-CoV-2 inactivated vaccine did not cause damage to the animals' immune system, and no obvious dose-response histopathological changes were seen in various tissues and organs. Most importantly, in both the low-dose vaccine group (1 person dosage) and the high-dose vaccine group (1.5person dosages), no serious pathological changes and abnormal clinical indicators, predicting systemic toxicity, were found. There was no obvious dose-response change where found in SARS-CoV-2 viral target organ: the lungs and bronchi. The vaccine was preliminarily confirmed to have good safety, providing an experimental basis for further clinical trials and a reference framework for related follow-up research.

Authors' contributions

Kaili Ma and Qihan Li conceived the idea and designed the experiment. Zhangqiong Huang, Qinfang Jiang, Yixuan Wang, Jinling Yang and Tingfu Du performed the main experiments, Zhangqiong Huang analyzed the data and wrote the main manuscript. Hongkun Yi, Cong Li, Yun Li, Zhengcun Wu, Shengtao Fan, Yun Liao, Ying Zhang, Lichun Wang, Guorun Jiang, Donghong Tang, Yousong Ye, Chenyun Wang, Zheli Li, Zhisai Li and Caixing Zhang participated in this work. Kaili Ma and Qihan Li revised the manuscript. We also thank all participants involved in this work. All authors reviewed the manuscript.

CRediT authorship contribution statement

Zhangqiong Huang: Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Qinfang Jiang:** Methodology, Writing – review & editing. **Yixuan Wang:** Methodology, Writing – review & editing. **Jinling Yang:** Methodology, Writing – review & editing. **Tingfu Du:** Methodology, Writing – review & editing. **Hongkun Yi:** Writing – original draft, Writing – review & editing. **Cong Li:** Writing

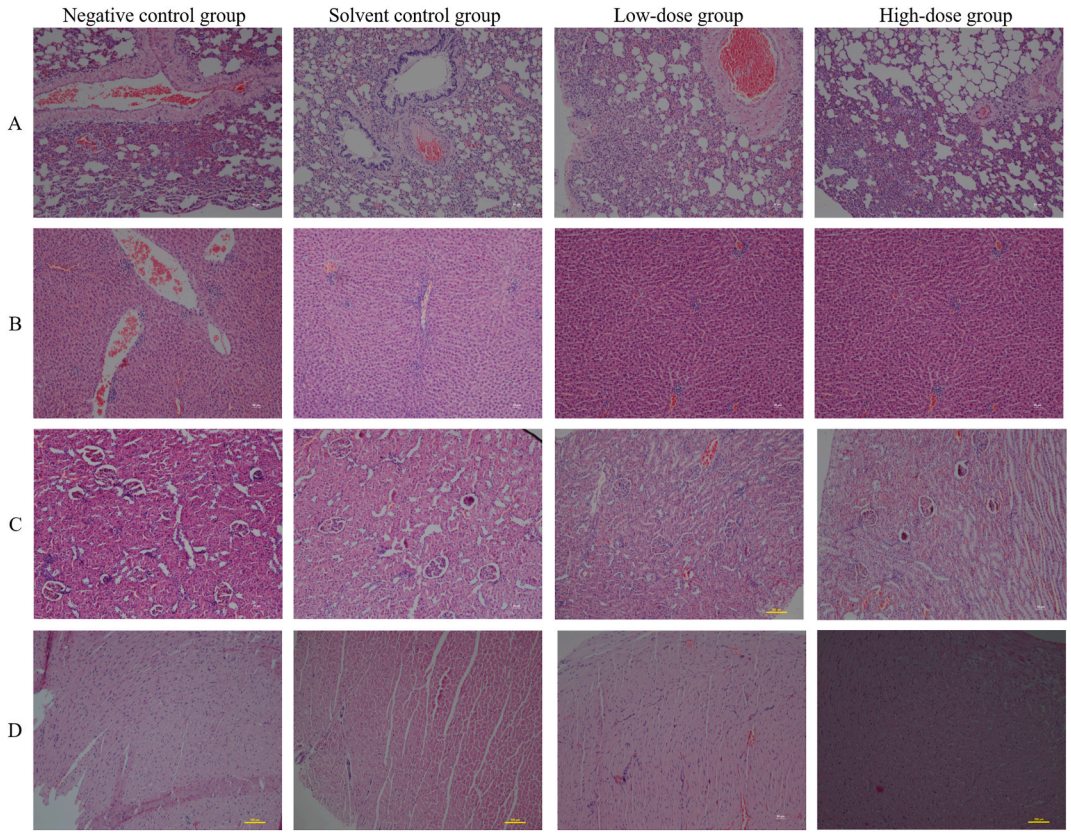

Fig. 4. Comparison of histopathological examination of each group. A. Histopathological examination of lung in each group. B. Histopathological examination of liver in each group. C. Histopathological examination of kidney in each group. D. Histopathological examination of heart in each group. There was no obvious pathological changes in these main organs.

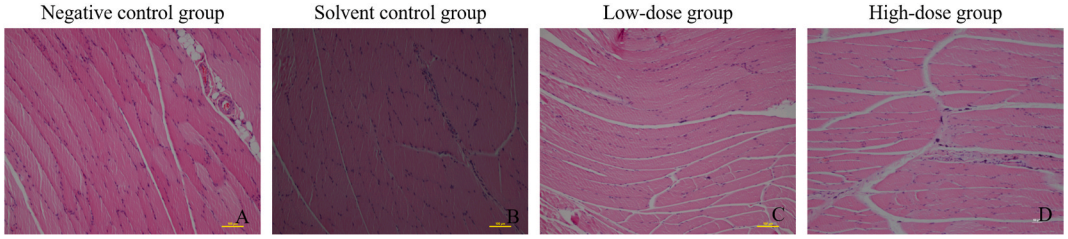

Fig. 5. Pathological results of injection sites muscle from each group. A. The injection sites muscle from negative control group administrated with saline. B. The injection sites muscle from solvent control group administrated with SARS-CoV-2 inactivated vaccine blank control. C. The injection sites muscle from low-dose group administrated with 100U/dose SARS-CoV-2 inactivated vaccine. D. The injection sites muscle from high-dose group administrated with 150U/dose SARS-CoV-2 inactivated vaccine. There was no significant lesion in each group at the injection sites.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the National Key R&D Program of China (2020YFC0849700), the Program of Chinese Academy of Medicine Science (2020HY320001) and the Major Science and Technology Special Projects of Yunnan Province (202003AC100009).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

org/10.1016/j.fct.2021.112239.

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