



RESEARCH ARTICLE

# Exhaustive Exercise Does Not Affect Humoral Immunity and Protection after Rabies Vaccination in a Mouse Model

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## Abstract

Rabies is one of the most dangerous and widespread zoonosis and is characterized by severe neurological signs and a high case-mortality rate of nearly 100%. Vaccination is the most effective way to prevent rabies in humans and animals. In this study, the relationship between exhaustive exercise and the humoral immune response after immunization with inactivated rabies vaccine was investigated in a mouse model with one-time exhaustive exercise. It was found that compared with the mice with no exercise after vaccination, no significant differences were found in those with exhaustive exercise after vaccination on body-weight changes, virus-neutralizing antibody (VNA) titers, antibody subtypes and survivor ratio after lethal rabies virus (RABV) challenge. This study indicated that exhaustive exercise does not reduce the effects of the rabies inactivated vaccine.

**Keywords** Rabies · Vaccination · Exhaustive exercise · Virus-neutralizing antibody · Protection

## Introduction

Rabies, which is one of the oldest infectious diseases and is caused by the RABV, still poses a threat to public health throughout the world (Zhao *et al.* 2010; WHO 2013; Molanouri Shamsi *et al.* 2017). Over 3 billion humans are threatened by rabies, and there are more than 59,000 deaths every year, most of which occur in developing countries within Asia and Africa (Fooks *et al.* 2014; Chen *et al.* 2015). Vaccination is the most effective way to prevent and control rabies. China, which is the biggest market for

human rabies vaccine consumption, commits a substantial amount of money and personnel into the control of rabies each year (Chen *et al.* 2015).

It has been reported that the functions of several components of both the innate (e.g., natural killer cell activity and neutrophil oxidative burst activity) and adaptive (e.g., T and B cell functions) immune system could be suppressed after exhaustive exercise (Nieman 1997; Steensberg *et al.* 2001; Szymura *et al.* 2016; Liu *et al.* 2017; Zamani *et al.* 2017). Even a single acute bout of exercise is reported to elicit significant changes in the immune system (Walsh *et al.* 2011). Other studies demonstrated that moderate exercise as a behavioral factor could be helpful for controlling sickness, infections and tumors, as well as enhancing the immune response (Nieman *et al.* 1990; Hojman 2017; Molanouri Shamsi *et al.* 2017). However, it is still not clear whether involvement in exhaustive exercise will affect the immune response after rabies vaccination.

In the present study, the relationship between exhaustive exercise (swimming was specified in this study) and the humoral immune response induced by the rabies vaccine was investigated in a mouse model of one-time exhaustive exercise. It was found that there was no significant

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difference on body-weight changes, VNA titers, antibody subtypes and survival ratio between the mock group and the exhaustive group after rabies vaccination.

## Materials and Methods

### Cells, Viruses, Antibodies, and Animals

BSR cells, a cloned cell line derived from BHK-21 cells, were maintained in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Herndon, VA) containing 10% FBS. RABV LBNSE was propagated in BSR cells. LBNSE, an attenuated vaccine strain, was constructed from the SAD-B19 strain through a mutation of the G at amino acid positions 194 and 333 (Rasalingam *et al.* 2005; Wen *et al.* 2011). LBNSE were UV-inactivated with a 254-nm UV light source for 30 min before immunization. The challenge virus CVS-24 was propagated in the brains of suckling mice. A fluorescein isothiocyanate (FITC)-conjugated antibody against RABV N was purchased from Fujirebio (Malvern, PA). The antibodies used for ELISA analyses including HRP-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgM, were all purchased from BioLegend (San Diego, CA). Eight-week-old female ICR mice and 1-day-old suckling Kunming mice were purchased from the Center for Disease Control and Prevention of Hubei Province, China. The mice were housed in a controlled specific pathogen-free environment at room temperature ( $22 \pm 2$  °C) and moderate humidity (45%–55%). All animal experiments were carried out as approved by the Scientific Ethics Committee of Huazhong Agricultural University (permit number HZAUMO-2015-016).

### Experimental Design

A mouse model of one-time exhaustive exercise was established firstly. Eight-week-old female ICR mice were allowed to adapt to their surroundings for 1 week before being subjected to the experiments, and all of the mice were adapted to water for 3 days before the formal experiment. Then those mice were randomly divided into four groups ( $n = 5$ ) and were subjected to a forced-swimming exercise (an artificial flow was created with a magnetic stirrer) in a round container (diameter: 45 cm and depth: 35 cm) filled with water at  $30 \pm 2$  °C. This device enabled the swimming evaluation of 20 mice simultaneously. It has been demonstrated that the intensity of swimming exercise was significantly raised by interaction among the mice (Iemitsu *et al.* 2004). The water depth was 35 cm so that the mice could not support themselves by touching the bottom with their feet (Mazzardo-Martins *et al.* 2010). The swimming was continuously monitored. GLU, LAC, UREA, CK, SOD and

MDA in the sera were measured at 0, 45, 90 and 120 min by ELISA. After swimming, the mice were dried with a towel and kept warm by an electric heater. The exhaustion standard involved sinking to the bottom of the water for no less than 3 s and the inability to respond to outside stimuli.

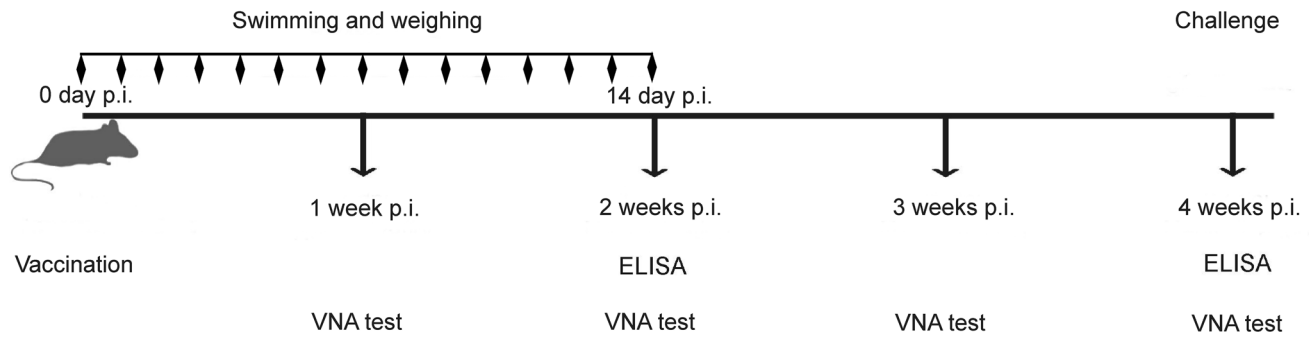
As shown in Fig. 1, the mice immunized with inactivated LBNSE by the intramuscular (i.m.) route immediately followed by 45 or 90 min of swimming or no swimming were designated LBNSE + 45 min swimming (aerobic exercise group), LBNSE + 90 min swimming (exhaustive exercise group) and LBNSE, respectively. The forced swimming was carried out daily for 2 weeks in the swimming groups. Body weight of all mice were weighed daily for 2 weeks. Additionally, the mice in the mock group immunized with the same volume (100  $\mu$ L) of DMEM with 90 min of swimming were designated DMEM. The swimming was conducted at  $30 \pm 2$  °C water, and the mice body weights were measured daily for 2 weeks. VNA titers and antibody subclasses in the sera were evaluated every week. After 4 weeks post immunization (wpi), the mice were challenged with 50 times of the mouse intracranial median lethal dose (MICLD<sub>50</sub>) of CVS-24 by the intracranial (i.c.) route under conditions of isoflurane anesthesia. The insulin syringe with 29G  $\times$  1/2" guage (0.33 mm  $\times$  12.7 mm) was used when i.c. challenge and the volume of inoculum was 30  $\mu$ L. All the mice were then observed for 2 weeks for clinical signs of rabies. The mice that appeared moribund or that lost more than 30% of their body-weight were humanely euthanized with aether.

### Measurement of of GLU, LAC, UREA, CK, SOD and MDA in the Sera

Blood samples (100  $\mu$ L) were collected and mixed with an equal volume of 7.5% EDTA-Na<sub>2</sub> at 0, 45, 90 and 120 min to analyze the level of the important indicators of sports fatigue [glucose (GLU), lactic acid (LAC) and urea (UREA)] and the indicators of oxidative tissue damage [creatinine kinase (CK), superoxide dismutase (SOD) and malondialdehyde (MDA)]. After centrifugation for 10 min at 10,000  $\times$ g, the supernatant was recovered. The GLU, LAC, UREA and CK were determined using a IDEXX biochemical analyzer, the SOD and the MDA were analyzed by the Hydroxylamine method and colorimetry, respectively, according to the manufacture's (Nanjing Jiancheng Bioengineering Institute) instruction.

### VNA Test

VNA titers were measured by an OIE prescribed method, fluorescent-antibody virus neutralization (FAVN) test, described elsewhere (Cliquet *et al.* 1998). Briefly, 50  $\mu$ L volumes of serial threefold dilutions of test sera and



**Fig. 1** Schematic of the experimental design. Eight-week-old female ICR mice ( $n = 15$ ) were immunized by the i.m. route with  $10^7$  FFU of LBNSE or mock-immunized with DMEM. The blood samples were collected and separated sera was used to test VNA by FAVN and the

immunoglobulin subclass antibody by ELISA at each time point as described in the text. At 4 wpi, the mice were challenged by the i.c. route with 50 MICLD<sub>50</sub> of CVS-24.

standard serum were prepared on 96-well microplates in 150  $\mu$ L volumes. Each sample was added to four adjacent wells. A 50  $\mu$ L volume of a rabies challenge virus (CVS-11) suspension, containing 50–200 fluorescent focus units (FFU), was added to each well. The microplates were then incubated at 37 °C for 1 h in an incubator with 5% CO<sub>2</sub>. Following incubation, 50  $\mu$ L of the cell suspension, containing  $2 \times 10^4$  BSR cells, was added to each well, and the microplates were incubated at 34 °C in an incubator with 5% CO<sub>2</sub> for 60 h. Then the plates were fixed with 80% ice-cold acetone at 20 °C for 30 min and air dried. Cells were stained with FITC-conjugated antibodies against RABV N for 45 min at 37 °C. After staining, cells were washed three times with phosphate-buffered saline (PBS). The results were assessed by using an Olympus IX51 fluorescence microscope. The neutralizing antibody titers were calculated by Spearman-Kärber formula. VNA titers were expressed in international units per milliliter by comparison with the titer of a reference serum (obtained from the National Institute for Biological Standards and Control, Herts, United Kingdom) included in each test.

### Evaluation of RABV-Specific Antibody Subtypes and IgG Subclasses by ELISA

ELISA plates were coated with coating buffer (5 mmol/L Na<sub>2</sub>CO<sub>3</sub>, pH 9.6) containing 500 ng/well of purified RABV overnight at 4 °C. The plates were then washed three times in PBS and blocked in 5% low-fat milk-PBS for 2 h at 37 °C. The sera were diluted 30 times in 100  $\mu$ L PBS, and then added to the RABV G-containing wells. The samples were incubated for 2 h at 37 °C. After incubation, the plates were washed three times with PBS, and then 100  $\mu$ L of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:1000), IgG1 (1:1500), IgG2a (1:1500), IgG2b (1:2000), IgG3 (1:1500) or IgM (1:2000) (Boster, Wuhan, China) was added to each well, respectively. The

plates were incubated at 37 °C for 45 min, and then washed three times with PBS-Tween. Tetra-methyl-benzidine (TMB) substrate was then prepared and added to each well according to the instructions of the manufacturer (Boster, Wuhan, China). The plates were incubated for 30 min at 37 °C in the dark, and the reaction was stopped by adding 2 M H<sub>2</sub>SO<sub>4</sub>. Optical density was read at 450 nm using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA).

### Statistical Analysis

All of the data were analyzed with GraphPad Prism software (GraphPad Software, Inc., CA). An unpaired two-tailed *t* test was used to determine the statistical significance of the contents of the GLU, LAC, UREA, CK, SOD and MDA, and the VNA titers. For the results of survivorship after the challenge, Kaplan-Meier survival curves were analyzed by the log-rank test. For all of the tests, the following notations were used to indicate the significant difference: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

## Results

### The Establishment of the Mouse Model of One-Time Exhaustive Exercise

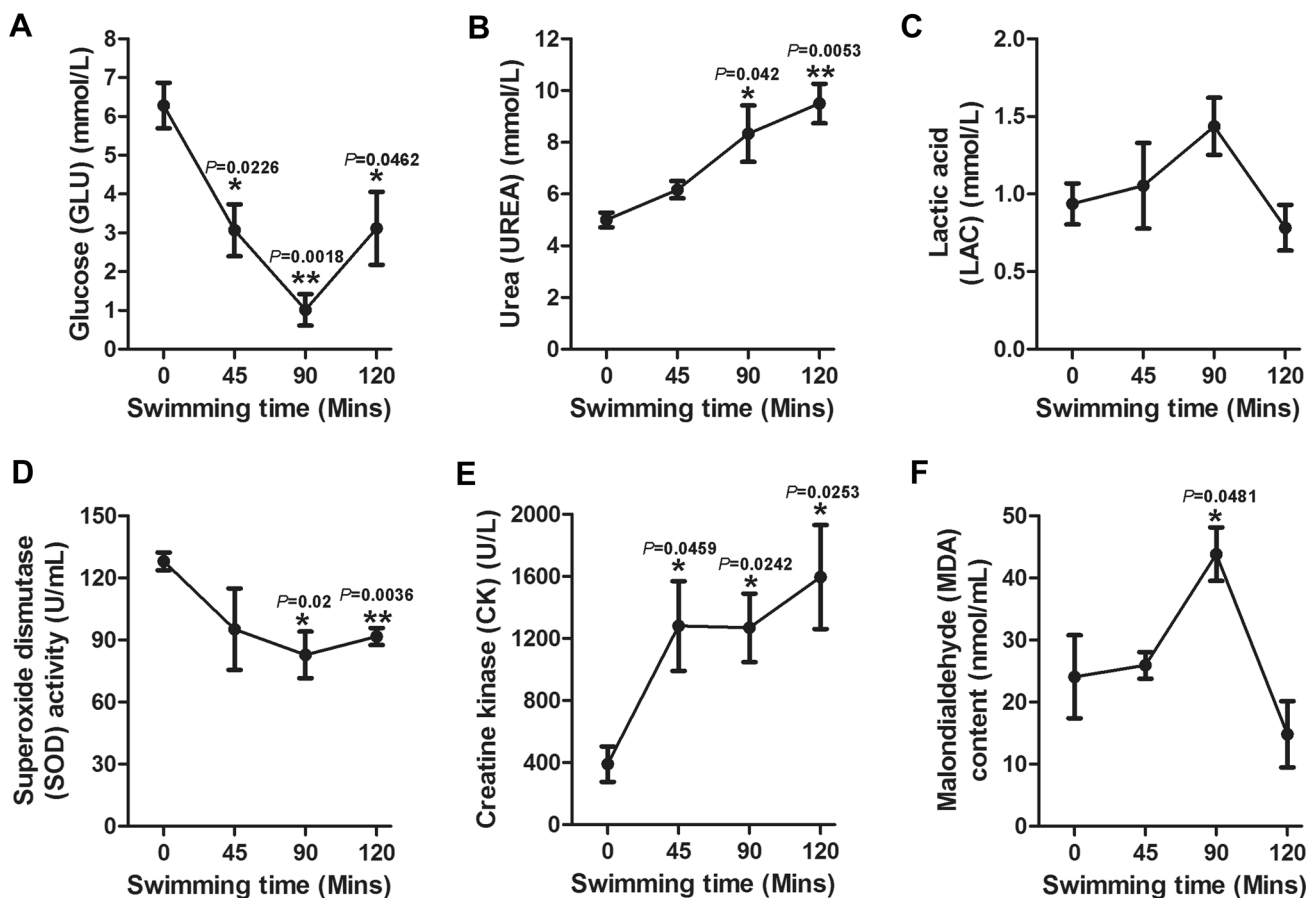
ICR mice were used to create an animal model of one-time exhaustive exercise. All of the mice were adapted to water for 3 days before the formal experiment. The adaptation was aimed to reduce the water-induced stress without promoting physiological alterations in relation to the formal experiment (Contarteze *et al.* 2008). The adaptation consisted of keeping the mice in shallow water at  $30 \pm 2$  °C (Harri and Kuusela 1986) for 7 days and

60 min/day. All of the mice were immediately euthanized with aether after exhaustive exercise.

To evaluate when the mice reach the exhaustive condition after acute swimming, the sera were collected at the indicated time points (0, 45, 90 and 120 min) and immediately evaluated by the biochemical analyzer, hydroxylamine method or colorimetry method. It was found that the concentrations of UREA, CK and MDA significantly increased at 90 min compared with their initial value (Fig. 2B, 2E, 2F, respectively). The LAC and MDA in the sera attained a peak value (1.44 mmol/L and 43.83 nmol/mL, respectively, Fig. 2C, 2F) at 90 min, and the concentrations of UREA and CK consistently increased over time (Fig. 2B, 2E, respectively). However, as depicted in Fig. 2A and 2D, the content of GLU and SOD in the sera significantly decreased at 90 min compared with their initial value. We also found that the first mouse showed exhaustion signs after 82 min. Taken together in this study, 90 min of swimming was considered exhaustive exercise.

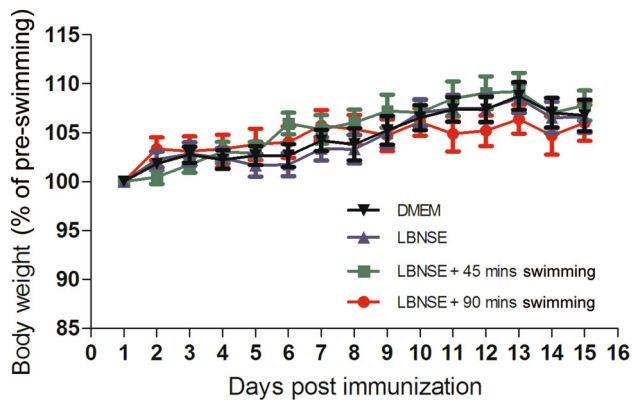
### Body-Weight Changes of Mice Under the Exercises of Different Intensity

Prior to checking the effect of exhaustive exercise on the humoral immune response, the body-weight changes and the level of fatigue of mice under conditions of different exercise intensity were monitored. Very few mice sunk to the bottom of the water within 90 min in the first 5 days, all other mice in DMEM + 90 min swimming group and LBNSE + 90 min swimming group showed inability to respond to outside stimuli after 90 min swimming. As depicted in Fig. 3, no significant difference was observed in mice among any of the groups; this outcome indicated that the mice could rapidly recover after exhaustive exercise daily for 2 weeks.



**Fig. 2** Concentrations of GLU, UREA, LAC, SOD, CK and MDA in mice sera during swimming. The sera were collected to test UREA, CK, GLU and LAC by a biochemical analyzer, and analysis of SOD and MDA was conducted by the hydroxylamine and colorimetry method, respectively, at the indicated time points (0, 45, 90 and

120 min). The results are presented as the mean  $\pm$  SE (n = 5). Asterisks (\* $P$  < 0.05; \*\* $P$  < 0.01; \*\*\* $P$  < 0.001) indicate significant differences among the different time points as analyzed by the unpaired  $t$  test.



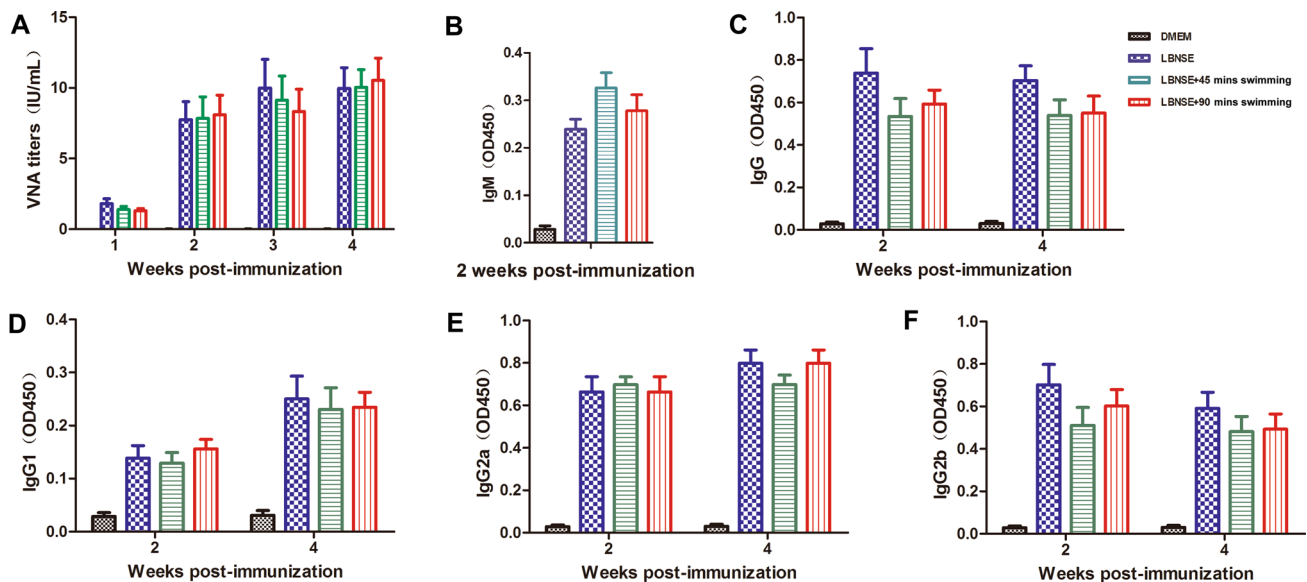
**Fig. 3** The body-weight change of immunized mice with a different intensity of exercise. Eight-week-old female ICR mice ( $n = 15$ ) were immunized by the i.m. route with  $10^7$  FFU of LBNSE or mock immunized with the same volume of DMEM and trained to swim for 45 or 90 min during the first 2 weeks after immunization. The body-weights were measured daily and the curves for the body-weight changes were depicted accordingly.

### VNA Induction and RABV-Specific Antibody Isotype Identification in Immunized Mice with Exhaustive Exercise

To determine whether exhaustive exercise could affect the humoral immune responses after rabies vaccination, the mice were immunized with  $10^7$  FFU of LBNSE or the same volume of DMEM, and this immunization was followed by 45 or 90 min of swimming or no swimming in the different experimental groups as described in the

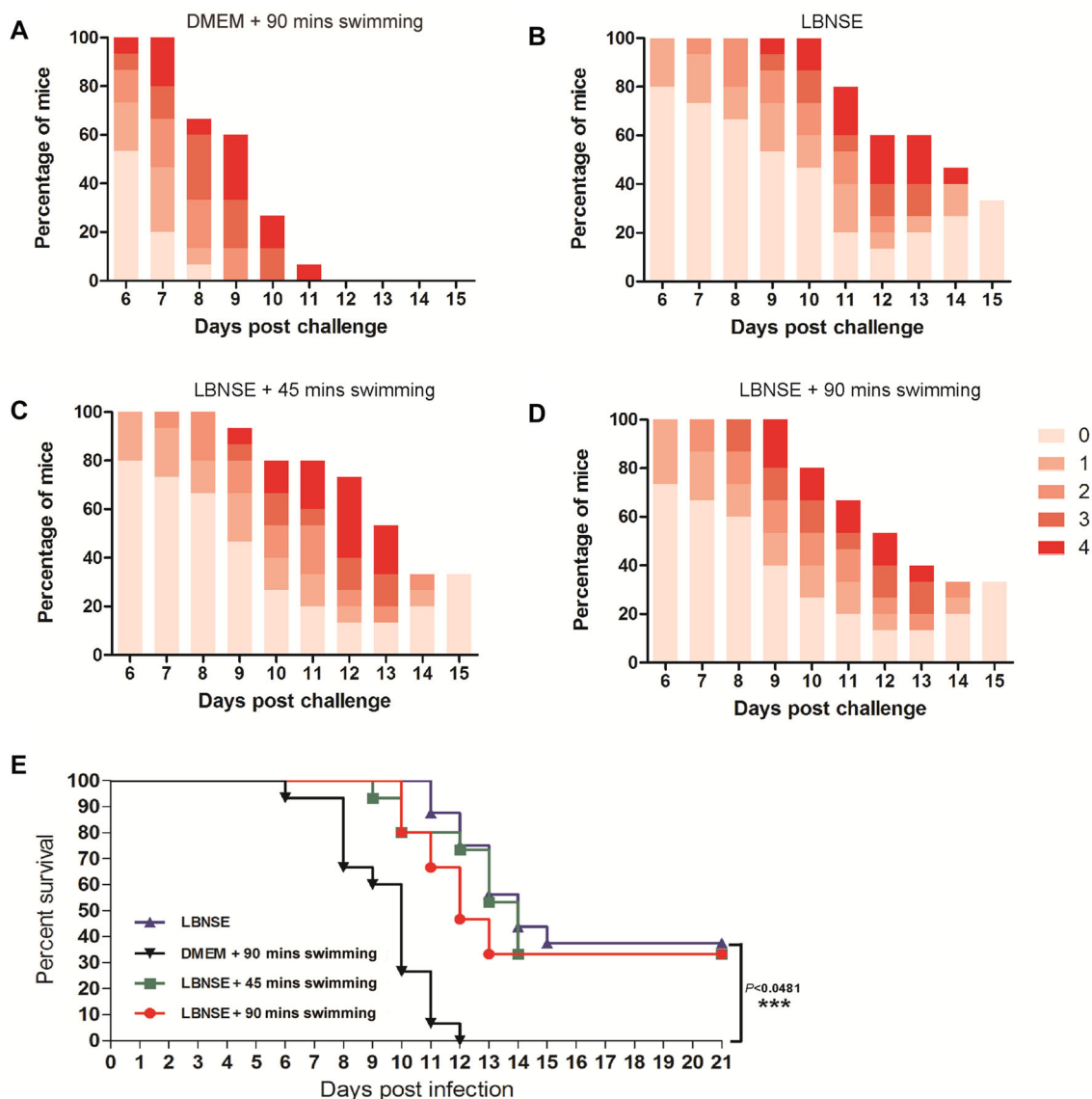
“Experimental Design.” The blood samples were collected weekly from 1 to 4 wpi, and the sera were used for the VNA titration via FAVN tests and the testing of antibody subtypes. As shown in Fig. 4A, no significant difference of the VNA titers was observed among any the LBNSE immunized mice. These data indicate that exhaustive exercise does not affect the VNA production after rabies vaccination in mice.

In addition, the antibody subtypes (IgM and IgG) and IgG subclasses (IgG1, IgG2a and IgG2b) in sera at 2 and 4 wpi were quantified by ELISA. As shown in Fig. 4B, 4C, no significant difference was observed at 2 or 4 wpi for the total IgM and IgG. It was reported that acute exercise may affect immune cell activity through muscle contraction-induced release of immune regulatory cytokines (Catoire and Kersten 2015). Many of these cytokines are also recognized for their role in regulating immune cells. IgG1 is associated with the stimulation of Th2-type immune responses that can regulate the humoral immune responses (Ozaki *et al.* 2002). IgG2a and IgG2b, stimulated during Th1-type immune responses, were suggested to be the main form of immunoglobulin induced by rabies vaccines (Singh and O’Hagan 1999). As shown in Fig. 4D–F, no significant difference were detected in mice immunized with LBNSE for IgG1, IgG2a and IgG2b levels at both 2 and 4 wpi. These results suggest that exhaustive exercise does not affect the RABV-specific antibody subtypes or IgG subclasses after rabies vaccination in mice.



**Fig. 4** Exhaustive exercise after rabies immunization does not affect the VNA titers and antibody subtype production in mice. Eight-week-old female ICR mice ( $n = 15$ ) were i.m. immunized with  $10^7$  FFU of LBNSE or mock immunized with the same volume of DMEM. The mice of the exercise groups were trained to swim for 45 or 90 min

during the first 2 weeks after immunization. At the indicated time points post immunization, the sera were collected to determine the VNA titers (A). Optical density (OD) values of total IgM (B), IgG (C), IgG1 (D), IgG2a (E), and IgG2b (F) against RABV were then determined by ELISA.



**Fig. 5** Protection of immunized mice with exhaustive exercise after challenge. After 4 weeks post vaccination, the immunized mice were i.c. challenged with 50 MICLD<sub>50</sub> CVS-24 and observed daily for 2 weeks. The number of survivors and clinical signs in each group was recorded. The clinical score (A–D) was monitored and calculated

according to the following clinical signs: 0, normal mouse; 1, disorder movement; 2, ruffled fur; 3, trembling and shaking; 4, paralysis; and 5, dead. The survival ratio (E) was analyzed according to the numbers of mice.

### Protection of Immunized Mice with Exhaustive Exercise after a Lethal Challenge

To investigate whether the protection rate would be affected by exhaustive exercise after rabies vaccination, the mice were challenged by i.c. with 50 MICLD<sub>50</sub> of CVS-24 at 4 wpi. The clinical symptoms of rabies were scored and the numbers of deaths were recorded daily for 2 weeks after the challenge. As shown in Fig. 5A, severe clinical signs were observed in the mice of the mock group (DMEM + 90 min swimming) as early as 6 days post challenge. In addition, all of the mice in the mock group

succumbed to rabies within 12 days. Furthermore, the death started at 11, 9, and 10 days post challenge for the mice in the LBNSE group, the LBNSE + 45 min swimming group and the LBNSE + 90 min swimming group, respectively (Fig. 5B–5D). The difference of the protection rate between the LBNSE group (37.5%) and the LBNSE + 90 min swimming group (33%) was not significant. Whereas, there was a very significant difference ( $P < 0.001$ ) in protection rate between mock group and LBNSE group (Fig. 5E). These results are consistent with the VNA titers induced in the immunized mice.

Collectively, the exhaustive exercise after rabies vaccination did not affect the immune responses of the immunized mice.

## Discussion

The most important reason why people abuse the rabies vaccine is because of fears of the protective effect of the rabies vaccine. There is no doubt that a healthy diet, a good lifestyle and adequate rest benefit human immunity. Moderate and regular exercise has been demonstrated to play a key role in increasing resistance to common infectious diseases and in reducing the risk of developing diseases (Baynard *et al.* 2012; Potenza *et al.* 2016). However, exhaustive exercise is known to induce a situation of oxidative stress, fatigue and tissue injury (Malaguti *et al.* 2009). There is no clear evidence that exhaustive exercise after immunization would affect the effectiveness of the rabies vaccine.

Several studies have reported that a mouse or rat model revealed similarities in the adaptations to exercise in relation to those observed in humans. As an innate ability of rodents, swimming exercise presents advantages over treadmill running (Alexandre *et al.* 2001; Seo *et al.* 2014). Previous studies suggest that long-term strenuous exercise could cause less GLU, more LAC and more UREA in the sera (Terada *et al.* 2001). Therefore, the level of GLU, LAC or UREA in the sera correlates with the intensity of the exercise. CK, MDA and SOD are important tissue damage markers (Jia *et al.* 2017). In our study, the changes in these indicators (GL, LAC, UREA, CK, MDA and SOD) are consistent with previous studies. The exhaustive signs were gradually observed after 82 min-swimming. Together, these data illustrate that the construction of the mouse model for one-time exhaustive exercise was successful in this study.

T cells play a very important role in controlling virus infection. Nevertheless, mice lacking CD8<sup>+</sup> T cells showed no significant difference in the development of clinical signs after RABV strain CVS-F3 infection by comparison with intact counterparts with the same genetic background (Hooper *et al.* 1998). In other words, the humoral immune response rather than the cellular immune response is crucial for the control of rabies. Therefore, in this study, the effects of exhaustive exercise on T cell responses were not evaluated.

In this study, we proved for the first time that exhaustive exercise does not affect antibody responses and protection after rabies immunization. In addition, our research may be more informative to evaluate the effects of exhaustive exercise on the immune responses prior to rabies

immunization and we will continually focus on this in our future study.

In summary, exhaustive exercise after rabies vaccination did not affect the VNA titers, antibody subtypes and survivorship after the RABV challenge in mice. These findings provide a new understanding of the relationship between exhaustive exercise and the effectiveness of the rabies vaccine.

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**Author Contributions** ZFF, LZ, MZ, DYT conceived and designed the experiments. LX, MRL, YJ Z, JCR, JP, JLS performed the experiments. DYT, LZ, MZ analyzed the data. LX, DYT, MZ wrote the manuscript and prepared the Figures. ZFF, LZ, MZ, DYT checked and finalized the manuscript. All authors read and approved the final manuscript.

## Compliance with Ethics Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Animal and Human Rights Statement** Animal experiments in this study were approved by the Scientific Ethics Committee of Huazhong Agricultural University (permit number HZAUMO-2016-047).

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