Vaccine development based on gene-edited parasite of *Neospora caninum* and identification of drug candidates from wild medical plants for control of protozoan infection

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遺伝子編集ネオスポラ原虫を用いたワクチン開発および原虫感染を制御するための野生薬草由来薬剤候補物質の同定

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Abbreviations

C. CCL2 -C-C motif chemokine ligand 2

CCL8 - C-C motif chemokine ligand 8

cDNA - Complementary DNA

CRISPIR /Cas9 - Clustered Regularly Interspaced Short Palindromic Repeats-Cas9

Ct - Cycle threshold value for quantitative PCR analysis

CXCL10 - Cysteine-X-cysteine motif chemokine ligand 10

CXCL9 -Cysteine-X-cysteine motif chemokine ligand 9

D. Dpi - Days post infection

Dpv - Days post vaccination

DMSO - Dimethyl sulfoxide

DMEM - Dulbecco's Modified Eagle Medium

E. EDTA -Ethylenediaminetetraacetic acid

EMEM - Eagle's minimum essential medium

F. IFAT - Immunofluorescence antibody test

IFN-γ - Interferon-gamma

G. GST - Glutathione S-transferase

H. HRP - Horseraddish peroxidase

HFF - Human foreskin fibroblast

I iELISA - Indirect ELISA

IL-10 - Interleukin-10

IL-4 - Interleukin-4

M. M3-NcGRA7 - NcGRA7 entrapped in liposome

MIC - Minimum inhibitory concentration

N. *N. caninum* - *Neospora caninum*

NcGRA7 - *Neospora caninum* dense granule antigen 7

NcGRA7KO - NcGRA7 deficient parasite

NcGRA7-comp - *Neospora caninum* dense granule antigen 7-complemented parasites

NcCyp - *N. caninum* cyclophilin

NcGRA6 - *N. caninum* dense granule antigen 6

NcGRA7 - *N. caninum* dense granule antigen 7

NcGRAs - *N. caninum* dense granule antigens

NcSAG1 - *N. caninum* surface antigen-1

NcSRS2 - SAG1-related sequence 2

NcROP2 - *N. caninum* rhoptry protein 2

NcMIC4 - *N. caninum* microneme protein 4

NcSAG1-GST - NcSAG1 fused with glutathione S-transferase

NcGRA6-GST - NcGRA6 fused with glutathione S-transferase

Nc1-GFP -Green fluorescent protein-expressing Nc1 strain of *N. caninum*

O. OD - Optical density

P. PVM - Parasitophorous vacuole membrane

PV - Parasitophorous vacuole

PCR -Polymerase chain reaction

PBS - Phosphate-buffered saline

PBST - PBS containing 0.05% Tween 20

PBS-SM - PBS containing 3% skimmed milk

P. falciparum - *Plasmodium falciparum*

T. *T. gondii* - *Toxoplasma gondii*

Tgmic1 -*Toxoplasma gondii* microneme antigen 1

Tgmic3 - *Toxoplasma gondii* microneme antigen 3

Th1 -T helper type 1

Th2 - T helper type 2

TNF-α - Tumor necrosis factor-alpha

V. Vero - African green monkey kidney cells

Unit abbreviations

hr : hour

μg : microgram

μL : microliter

mg : milligram

mL : milliliter

% : percentage

General introduction

Neospora caninum infections are associated with endemic and epidemic abortions in cattle, as well as neonatal mortality (Anderson et al., 1997). Infected cows may abort from 3 months of gestation until birth, but most abortions occur at 5–7 months of pregnancy (Almería and López-Gatius, 2013). *N. caninum* infections are naturally transmitted via horizontal or vertical transmissions (Dubey et al., 2007). Vertical transmission is the main route of transmission in cattle. Two forms of vertical transmission were identified: exogenous and endogenous vertical transmission (Trees and Williams, 2005; Williams et al., 2009). Exogenous transplacental transmission requires the ingestion of sporulated oocysts by the intermediate hosts and is associated with epidemic abortion storms in cattle herds (Williams et al., 2009). Endogenous transplacental transmission follows relapse of infection in persistently infected cattle during pregnancy and is the main reason for maintaining the parasite within the populations, resulting in fetal transmission rates as high as 95% (Dubey et al., 2006; Williams et al., 2009; Reichel et al., 2013). The zoonotic potential of neosporosis has not been confirmed yet (Dubey et al., 2007). A commercial inactivated vaccine (Neoguard®, Intervet International B.V., Boxmeer, The Netherlands) was withdrawn from the market, as only moderate protection against abortions was observed in field trials (Romero et al., 2004; Weston et al., 2012).

Various compounds had been evaluated for their efficacy against *N. caninum* tachyzoites in vitro (Lindsay et al. 1996; 1997). Sulphadiazine has some effect in mice (Lindsay and Dubey, 1990) and dogs (Barber and Trees, 1996; Reichel et al., 2014). Treatment with Toltrazuril, an anticoccidial drug, effectively eliminated experimental infection with *N. caninum* tachyzoites in calves (Kritzner et al. 2002).

Multiple crude extracts have been screened for their in vitro and/or in vivo anti-malarial activities. Active anti-plasmodial components have been successfully characterized from some plant extracts, following the pharmaceutical industry for drug development (Batista et al., 2009; Oliveira et al., 2009; Kaur et al., 2009).

This study aimed to evaluate the effect of a gene-edited parasites line (NcGRA7KO) as a live attenuated vaccine against *N. caninum* infection with identification of the role of NcGRA7 in vertical transmission of *N. caninum* in mice, and identification of the efficacy of wild medicinal plant extracts in the treatment of different protozoan infections.

Chapter 1

Role of dense granule antigen 7 in vertical transmission of *Neospora caninum* in C57BL/6 mice infected during early pregnancy

1-1. Introduction

Neospora caninum, an obligate intracellular apicomplexan parasite, causes abortion in infected livestock. The parasite can be horizontally transmitted through ingestion of oocysts shed in the faeces of definitive hosts, but vertical transmission is considered its main infection route in cattle (Dubey et al., 2007). *Neospora caninum* causes encephalomyelitis and myelitis in dogs (Bjerkås et al., 1984), and abortion in both dairy and beef livestock at 5–6 months of pregnancy (Dubey and Schares, 2006). Furthermore, bovine fetuses can die *in utero* and then be resorbed, mummified, autolyzed or stillborn, or born alive with or without clinical signs and possibly persistently infected (Dubey and Schares, 2006). Transplacental transmission of *N. caninum* can occur over several generations, which explains the global persistence of this disease (Schares et al., 1998). In the USA, South America, Switzerland, New Zealand, and the European Union, neosporosis is considered the main cause of bovine abortions (Hemphill and Gottstein, 2000; Moore, 2004; Dubey and Lindsay, 2006; Dubey et al., 2007;). Although antibodies to *N. caninum* have been detected in humans (Tranas et al., 1999; Dubey et al., 2007), including immunocompromised patients infected with the immunodeficiency virus (Lobato et al., 2006), and two umbilical cord blood samples were positive for *N. caninum* infection in pregnant women (Duarte et al., 2020), unlike *Toxoplasma gondii*, the zoonotic potential of *N. caninum* has not been confirmed so far. In Spain, a study was carried out where 600 DNA samples from humans with clinical signs compatible with toxoplasmosis but with negative PCR results for both *T. gondii* and *N. caninum* were analyzed (Calero-Bernal et al., 2019).

Dense granule organelles are present in all apicomplexan parasites, and these granules play a role in the host-parasite relationship. After parasite invasion, the dense granule proteins secreted from these organelles are released into the parasite parasitophorous vacuole (PV) and its membrane (PVM) where they perform roles in nutrient uptake and waste excretion in infected host cells (Cesbro-Delauw et al.,

1994). Among the 18 tested *N. caninum* dense granule antigens (NcGRAs), we previously found that NcGRA7 plays a role in regulating *N. caninum* pathogenesis and host immune response modulation in mice (Nishikawa et al., 2018).

NcGRA7 has been reported to be a marker of primo-infection, recrudescence, and reinfection in serum samples from herds associated with abortion and/or vertical transmission (Aguado-Martínez et al., 2008). In addition, the production of anti-NcGRA7 antibodies was correlated with the virulence in pregnant and non-pregnant mice. High levels of the antibodies were developed in the mice inoculated with high virulence isolates compared to those inoculated with low-to-moderate virulence *Neospora* isolates (Jimenez-Ruiz et al., 2013). NcGRA7, a highly immunogenic antigen required during the initial development of the intracellular parasite, also performs an important role during the initial invasion of the parasite into host cells (Soltani et al., 2013). NcGRA7 antigen has also been used in combination with a plasmid-containing adjuvant for establishing immune resistance in mice infected with *N. caninum* (Jenkins et al., 2004). Vaccination with recombinant NcGRA7 encapsulated alone or combined with recombinant NcSAG4 resulted in slight protection against challenge infection with Nc1 isolate in non-pregnant mice, but it elicits a strong humoral and cellular immune response (Aguado-Martínez et al., 2009). Additionally, immunization before pregnancy with NcGRA7 entrapped in oligomannose-coated liposomes resulted in increased offspring survival and decreased infection rates in the brains of dam mice (Nishikawa et al., 2009). However, little is known about the role of NcGRA7 during pregnancy. Therefore, my main goal was to evaluate the role of NcGRA7 in the vertical transmission of *N. caninum* using a pregnant mouse model.

1-2. Materials and methods

Ethics statement

This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan (permit numbers 20-27,

21-36). To minimize animal suffering, all surgical operations, blood collection, and cervical dislocation were performed under isoflurane anesthesia.

Mice

C57BL/6 female and male mice, 8–10 weeks old, were obtained from Clea Japan (Tokyo, Japan). The animals were housed under specific-pathogen-free conditions in the animal facility of the National Research Center for Protozoan Diseases at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. These animals were treated and used according to the Guiding Principles for the Care and Use of Research Animals published by the Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. The animals were kept under standard laboratory conditions and fed with commercial food and water *ad libitum* on a 12/12-h light/dark cycle at 21°C under 40% relative humidity.

Parasites and cell cultures

Neospora caninum Nc1 (parental strain), the NcGRA7-deficient parasite (NcGRA7KO) generated by the clustered regularly interspaced short palindromic repeats (CRISPR)-associated gene 9 (CRISPR/CAS9) system (Nishikawa et al., 2018) were maintained in African green monkey kidney epithelial cells (Vero cells). Parasites were cultured in Eagle's minimum essential medium (Sigma, St. Louis, MO) supplemented with 8% heat-inactivated fetal bovine serum (Nichirei Biosciences, Tokyo, Japan), 100 U/mL penicillin, and 10 mg/mL streptomycin. At 72 hours after the infection, parasites and host cell debris were washed with cold phosphate-buffered saline (PBS) to exclude the extracellular parasites and the final pellet resuspended in cold PBS was passed through a 27-gauge needle and a 5.0-μm-pore size filter (Millipore, Bedford, MA). After washing the parasites with PBS by centrifugation at 1,500 × *g* for 10 min, the parasites were refiltered, and their numbers were hemocytometrically counted for each experiment according to the previous study (Nishikawa et al., 2018).

Experimental design

In our previous study using non-pregnant C57BL/6 mice (Abe et al., 2015; Fereig et al., 2019), 10^6 *N. caninum* tachyzoites of Nc1 strain were intraperitoneally injected into the mice for observation of mouse survival rates, bodyweight, and clinical scores and analyses of immune response. In addition, NcGRA7KO showed reduced virulence in non-pregnant C57BL/6 mice (Nishikawa et al., 2018). Therefore, we used 10^6 *N. caninum* tachyzoites of Nc1 strain in addition to 10^5 tachyzoites for intraperitoneal inoculation into pregnant C57BL/6 mice in this study (Fig. S1).

Eight mice were used for each group of each trial in the mating procedures. Estrus synchronization was initiated in a group (8 mice /cage) by the Whitten effect (Whitten, 1956; 1958; Marsden et al., 1964). Male and female mice were mated for 14 h (one female with one male /cage) after 18:00 pm. Mating procedures were done on 3 successive days and females were checked for vaginal plugs or swelling the next morning at 8:00 am after each mating day. Mice with positive vaginal plugs were considered at day 0.5 of pregnancy. Eight mice were mated for each group. The mice without vaginal plugs were excluded from the experiment and their bodyweight was observed to ensure that they were non-pregnant. Pregnancies were further confirmed by recording an increased bodyweight percentage in each of the estimated pregnant dam mice until day 10 post-mating. The number of pregnant mice used was shown in Fig. S1, Tables 2 and 3.

To compare parasite virulence in the mice based on survival rate and clinical score of dams, number of offspring, and the daily survival of the offspring, pregnant C57BL/6 mice were intraperitoneally inoculated with different doses of *N. caninum* tachyzoites (trial 1: 10^6 tachyzoites/mouse, Nc1, and NcGRA7KO, trial 2: 10^5 tachyzoites/mouse, Nc1, NcGRA7KO) or RPMI-1640 medium (negative control) on day 3.5 of pregnancy. The daily bodyweight measurement of each dam mouse including RPMI-1640 medium-injected mice (negative control), as recorded from -2 to 13 days post-infection (dpi) with Nc1 or NcGRA7KO, was compared with the weight of the same mouse on the first day of measurement before infection. Clinical scores were assigned based on hunching, piloerection, warm-seeking behavior, ptosis, sunken eyes, ataxia, latency of movement, flaccidity, touch reflexes, the skin and eye reflexes, and lying on belly. The scores varied from 0 (no signs) to 10 (all signs) (Hermes et al., 2008). Clinical scores of dam mice were estimated by recording the clinical signs

manifested in each mouse from −2 to 13 dpi. To exclude the pregnancy parturition effects and avoid the disturbances of mice, only recording of the number of dams and offspring was performed from 14 dpi (correspond to day 17.5 of pregnancy) to the day 30 post-partum.

For pathological and mRNA expression analyses, experiment trial 3 was performed. Pregnant C57BL/6 mice were intraperitoneally inoculated with 10^6 *N. caninum* tachyzoites on day 3.5 of pregnancy and then postmortem examination was performed at day 13.5 of pregnancy (10 dpi). The fertility rates were as follows, negative control (5/6, 83.3%), Nc1-infected (3/5, 60.0%), NcGRA7KO-infected (4/5, 80.0%). To analyze mRNA expression, spleen (right half) and pooled placenta samples were collected for each dam. For the pathological analysis, placental tissues from each pregnant dam were collected. For determination of the parasite burden, spleen (left half), placental tissue, and whole fetal bodies were collected from each pregnant dam. The number of dams for above experiments was as follows, negative control, n = 5; Nc1-infected, n = 3; NcGRA7KO-infected, n = 4.

DNA isolation and quantitative PCR (qPCR) to determine *N. caninum* distribution

Parasite burdens were quantified in the brain, placenta, spleen and whole fetus bodies. DNA was extracted from the brain of dams and offspring from trials 1, 2 and 3, and from dam placenta and spleen, and whole fetus bodies from trial 3 as follows: each tissue or organ was thawed in 10 volumes of extraction buffer (0.1 M Tris-HCl [pH 9.0], 1% SDS, 0.1 M NaCl, 1 mM EDTA) and 20 μg/ml of proteinase K at 50°C. The DNA was purified by phenol-chloroform extraction and ethanol precipitation. Parasite DNA was then amplified with the following *N. caninum* Nc5 gene-specific primers: forward 5′-ACT GGA GGC ACG CTG AAC AC-3′ and reverse 5′-AAC AAT GCT TCG CAA GAG GAA-3′ (Nishimura et al., 2015). Amplification, data acquisition, and data analysis were performed on the ABI Prism 7900HT sequence detection system (Applied Biosystems), and the cycle threshold (Ct) values were calculated as described previously (Nishimura et al., 2015). Standard curves were constructed using 10-fold serial dilutions of *N. caninum* DNA extracted from 10^5 parasites; thus, each curve ranged from 0.01 to 10,000 parasites. Parasite numbers were calculated from the standard curve.

Real-time reverse transcriptase (RT)-PCR analysis of chemokine expression

Total RNA was extracted from cells or homogenized tissues using TRI Reagent (Sigma-Aldrich). RNA was reverse transcribed with a Prime Script II First Strand cDNA synthesis kit (TaKaRa Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. The cDNA recovered was amplified using RT-PCR with PowerUp SYBR green master mix (Thermo Fisher Scientific, Inc., Waltham, MA) and 500 nM of gene-specific primers in a 10 μl reaction volume according to the manufacturer's protocol. The target molecules were interleukin-10 (IL-10), interleukin-4 (IL-4), interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF-α), C-C motif chemokine ligand 2 (CCL2), C-C motif chemokine ligand 8 (CCL8), cysteine-X-cysteine motif chemokine ligand 9 (CXCL9) and cysteine-X-cysteine motif chemokine ligand 10 (CXCL10). Gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the internal control by Ref Finder (Xie et al., 2011), and gene expression was normalized against this control. The primer sequences of the target genes are shown in Table 1. The relative mRNA levels were calculated using the fold change Ct method.

Histopathological analysis

Placental tissue was fixed in neutral-buffered formalin and processed using routine methods. Serial sections (4 μm each) made from paraffin-embedded tissues were stained with hematoxylin and eosin. Immunohistochemistry was performed using the following procedures (Ramos-Vara et al., 2008). Deparaffinized sections were incubated with polyclonal antibodies: rabbit anti-*N. caninum* dense granule antigen 6 (NcGRA6), rabbit anti-NcSAG1, rabbit anti-CD3 (Abcam, Cambridge, UK) for T cell marker, and rabbit anti- ionized calcium binding adapter protein 1 **(**Iba1) (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) for macrophage marker at 4˚C for overnight. Antigen retrieval was performed at 98˚C for 45 min with Immunosaver (Nisshin EM Co., Ltd, Tokyo, Japan). Non-specific reactions and endogenous peroxidases were blocked by blocking solution (10 mM PBS pH7.4 with 8% skim milk and 3% tween 20 (Santa Cruz Biotechnology, inc., Dallas, TX)) and 10% H_2O_2 methanol. Signals were visualized on the Envision system (Agilent, Santa Clara, CA, USA) using 3, 3'-diaminobendidin. To characterize the inflammatory cells and evaluate the inflammatory response, we manually scan the slides and select an inflammatory region. CD3 and Iba1-positive cells were counted

on three aleatory fields (×400; total 0.711mm^2 in each) in three placental zones (decidua, junctional zone, and labyrinth).

Statistical analysis

GraphPad Prism 6.0 software and its updated version 8.3.4 (GraphPad Software Inc., La Jolla, CA, USA) was used. Data represent the mean ± SD. Statistical analyses were performed using Student's t-test, Mann–Whitney U test, and one-way or two-way analysis of variance (ANOVA) followed by the Tukey-Kramer hoc test for group comparisons. Survival rates, vertical transmission rates, and statistical comparisons were assessed using a χ^2 test. The levels of statistical significance are shown as asterisks and defined in each figure legend together with the name of the statistical test that was used. *P* value of < 0.05 was considered statistically significant.

1-3. Results

Parasite virulence in pregnant mouse model under a higher infection dose

During the infections with 1×10^6 tachyzoites of either parasite, we observed that the bodyweight gains in the infected groups did not show significant differences with respect to the uninfected group (negative control) (Fig. S2). However, the clinical scores of the infected dams were significantly higher than those of the uninfected animals (negative control). The clinical manifestations observed were hunching, piloerection, warm-seeking behavior, and sunken eyes (Fig. S2). All dams survived during the pregnancy until 15-16 dpi (Fig. 1A). The survival rate of the NcGRA7KO-infected dams (60%, 3/5) was lower than that of the Nc-1-infected dams (100%, 4/4) and negative control (100%, 5/5), but no significant difference was found (Fig. 1A). The fertility rates were as follows, negative control (5/6, 83.3%), Nc1-infected (4/5, 80.0%), NcGRA7KO-infected (5/5, 100.0%) (Table 2). The number of parasites in the brain also showed no significant difference between the Nc1- and NcGRA7KO-infected dams (Fig. 1B). There was no significant difference either in the mean number of offspring per litter among the experimental groups (Table 2). Although infection with *N. caninum* decreased survival in the offspring compared with the negative control mice, the survival rates of the offspring from the NcGRA7KO-infected dams (28.0%) were significantly higher than those from the Nc1-infected dams

(7.7%) (Fig. 1C, Table 2). The number of parasites in the brains of the offspring of the NcGRA7KO-infected dams was significantly lower than in the brains of the offspring of the Nc-1-infected dams (Fig. 1D). The proportion of vertical transmission was calculated retrospectively in both infected groups (Table 2). Parasite DNA was detected in 20/21 (95.2%) in the brains of offspring from Nc1-infected dams. However, a significantly lower percentage of positivity was seen in the brains of offspring from NcGRA7KO-infected dams (14/21, 66.7%) (Table 2). Additionally, parasite DNA was detected during acute death of offspring from Nc1-infected dams compared with those from NcGRA7KO-infected dams (Fig. 1E).

Parasite virulence in pregnant mouse model under a lower infection dose

To confirm the virulence of NcGRA7KO, trial 2 experiment under a lower infection dose was conducted. The bodyweight gains in the infected groups did not show significant differences with respect to the uninfected group (negative control) (Fig. S3). However, the clinical scores of both infected dams were significantly higher than those of the uninfected animals (negative control). The clinical manifestations observed were hunching, piloerection, warm-seeking behavior, and sunken eyes (Fig. S3). Survival of all dams was seen during the pregnancy until 15-16 dpi (Fig. 2A). The survival rate of the NcGRA7KO-infected dams (80%, 4/5) was lower than that of the Nc-1-infected dams (100%, 5/5) and negative control (100%, 4/4), but no significant difference was found (Fig. 2A). The fertility rates were as follows, negative control (4/6, 66.6%), Nc1-infected (5/6, 83.3%), NcGRA7KO-infected (5/7, 71.4%) (Table 3). The number of parasites in the brain also showed no significant difference between the Nc1- and NcGRA7KO-infected dams (Fig. 2B). Furthermore, there was no significant difference either in the mean number of offsprings per litter among the experimental groups (Table 3). In contrast, the offspring survival rates for the NcGRA7KO-infected dams (50.0%) were significantly higher than those for the Nc1-infected dams (19.4%) (Fig. 2C, Table 3). Parasite numbers in the brain tissues of the offspring from the NcGRA7KO infected dams were significantly lower than those from the Nc-1-infected dams (Fig. 2D). The proportion of vertical transmission was monitored in both infected groups (Table 3). The percentage of the parasite DNA positive in the brain of offspring from

NcGRA7KO-infected dams (14/20, 70.0%) was significantly lower than that from Nc1-infected mice (36/36, 100.0%). As shown in Fig. 2E, parasite DNA was detected in the offspring from the Nc-1-infected dams. Together with the results shown in Fig. 1, NcGRA7KO parasite numbers in the brains of the offspring were significantly lower than those for Nc1, indicating lower vertical transmission of NcGRA7KO.

Histopathological and qPCR analyses of parasite numbers in placentas and fetuses

To estimate the tissue damage caused by *N. caninum* infection, histopathological analysis of the placentas on day 13.5 of pregnancy (10 dpi with 1×10^6 tachyzoites) was performed as trial 3. As we observed in trial 1, the bodyweight gains in the infected groups did not show significant differences with respect to the uninfected group (negative control) (Fig. S4). On the other hand, the clinical scores of the infected dams were significantly higher than those of the uninfected animals (negative control) (Fig. S4). The fertility rates were as follows, negative control (5/6, 83.3%), Nc1-infected (3/5, 60.0%), NcGRA7KO-infected (4/5, 80.0%). In Nc1- and NcGRA7KO-infected dams, mild edema, and mononuclear cell infiltration were observed in the decidua and junctional zone. In decidua, T lymphocyte-rich inflammation was more severe in NcGRA7KO- than Nc1-infected groups (Fig. 3B). Hemorrhage and necrosis were not observed in the placenta. There was only one case where a NcGRA7KO-infected mouse had observable *N. caninum* tachyzoites in the trophoblast with focal vasculitis and neutrophil infiltration (Fig. S5; Table S1). T lymphocyte and macrophage numbers were higher in the decidua and junctional zones of the Nc1- and NcGRA7KO-infected groups than in the negative control mice (Figs. 3B, C, and 4), unlike in the labyrinths (Figs. 4 and S6). Furthermore, the number of T lymphocytes in the NcGRA7KO-infected dams was significantly higher in the deciduas and junctional zones than that in the Nc1-infected animals (Fig. 4, Table S1).

To determine whether parasites were present in the placentas or fetuses, parasite loads were estimated by qPCR. Although parasite DNA was detected in the placentas of both experimental groups, no significant difference in the parasite numbers was found between Nc1 and NcGRA7KO parasites

(Fig. 5A). The parasite DNA was not detected in the spleen of the dams (data not shown). Moreover, parasite DNA was not detectable in the whole bodies of fetuses from most of the infected dams (Fig. 5B).

Proinflammatory marker expression in the spleen and placenta

To examine the inflammatory responses during the pregnancy, we analyzed mRNA expression, which we have previously found to be regulated by *N. caninum* infection [13], in both placenta and spleen tissues on day 13.5 of pregnancy (10 dpi) (Fig. 6). In the spleen, TNF-α expression was lowered by *N. caninum* infection, and CCL8 expression was raised in the NcGRA7KO-infected mice, as compared with negative control mice (Fig. 6A). The expression levels of CCL2, CCL8 and CXCL9 were higher in the placentas from the NcGRA7KO-infected dams than in the placentas from Nc1-infected animals (Fig. 6B). Under our experimental conditions, changes in the expression of IL-4, IL-10, IFN-γ and CXCL-10 were not seen (Fig. S7).

1-4. Discussion

Neospora caninum has one of the highest known vertical transmission rates of parasites and its transplacental transmission results in the persistent transfer of parasites from infected dams to their fetuses during gestation (Dubey et al., 2006). Vertical transmission has been previously evaluated by many studies in dairy cows (Bergeron et al., 2000; Davison et al., 2001), dogs (Barber and trees, 1998), and mice such as Swiss-Webster mice (Cole et al., 1995), Qs out-bred mice (Miller et al., 2005), BALB/c mice (Nishikawa et al., 2001; Omata et al., 2004) and C57BL/6 mice (Ramamoorthy et al., 2007a). In this study, the route infection (intraperitoneal), and the use of animal strain (C57BL/6) of my experimental model was based on a previous study (Ramamoorthy et al., 2007a), while the doses of inoculation, time of vertical transmission monitored and the parasite lines used were different (Ramamoorthy et al., 2007a). The reported study (Liddel et al., 1999) evaluated the vertical transmission in the fetuses at days 18-20 of gestation using one parasite strain (Nc1), while in this study

in trials 1 and 2 the vertical transmission of the offspring was determined until day 30 post-partum, using two parasite lines, Nc1 and NcGRA7KO. These studies prove that the vertical transmission varies according to the use of the mouse strain and the parasite strain.

In my study, 95.2%-100.0% PCR positive offspring were observed after the intraperitoneal infection with Nc1 strain at the early period of pregnancy (day 3.5), resulting in the successful vertical transmission in C57BL/6 mice in both high and low doses of the infection. However, the vertical transmission rate of NcGRA7KO in C57BL/6 mice was 66.7%-70.0%. Moreover, the fertility rates of uninfected dams (negative control), dams infected with Nc1, dams infected with NcGRA7KO were 66.6%-83.3%, 80.0%-83.3%, 71.4%-100.0%, and the mean numbers of offspring obtained were 5.0-6.4, 6.5-7.2, and 4.4-5.0, respectively. Previous study using C57BL/6 mice (Ramamoorthy et al., 2007a) showed the vertical transmission rate in the mice infected with 5×10^6 tachyzoites of Nc1 strain at day 12-14 of gestation were 100%. Moreover, the fertility rates and mean number of fetuses of the uninfected controls and mice infected during pregnancy were 71.4% and 8.2, 57.1% and 8.0, respectively, while there was no significant difference between the uninfected and infected mice. Together, the infection of C57BL/6 mice with Nc1 strain induced vertical transmission, but did not affect the fertility rate and litter size. Therefore, these results indicated C57BL/6 mice can be used for the study of the vertical transmission of *N. caninum* and vertical transmission of NcGRA7KO was reduced compared with Nc1.

On the basis of the mouse model of vertical transmission, vertical transmission prevention has been reported in BALB/c mice (Nishikawa et al., 2001) and C57BL/6 mice (Ramamoorthy et al., 2007b). Type 1 and type 2 immune responses are involved in both vertical transmission and abortion after *N. caninum* infection (Quinn et al., 2002). A study illustrated that modulation of a Th2 cytokine can reduce the frequency of transplacental transmission of *N. caninum*. The reduction in the transplacental transmission was associated with lower levels of maternal mRNA expression of IL-4 and elevated levels of IFN-γ production (Long and Baszler, 2000). The type 1 immune response is associated with protection against intracellular *N. caninum* infection, and its downregulation during pregnancy may

trigger transplacental transmission (Williams et al., 2000). However, the mechanism of abortion and vertical transmission caused by *N. caninum* infection is poorly understood. Studies on the relationship between the infection and the inflammatory response occurring during pregnancy are vital if we are able to understand the local immune response at the site of the infection. IFN-γ and TNF-α are reportedly associated with the fetal loss caused by *N. caninum* infection-related placental damage (Long and Baszler, 1996; Williams et al., 2000; Kano et al., 2005). Another hypothesis is that induction of the T helper type 1 (Th1) immune response at the maternal level and modulation to a T helper type 2 (Th2) immune response can prevent vertical transmission of *N. caninum* in mice (Long and Baszler, 2000). In fact, the immune response to *N. caninum* is typically associated with IFN-γ and $CD4^+$ T cells (Williams et al., 2000; Nishikawa et al., 2001; Staska et al., 2005; Rosbottom et al., 2011). It has been reported that the placental pro-inflammatory response against *N. caninum* infection causes placental damage and fetal death via the destruction of fetal trophoblast cells and the effects of cytotoxic T cells and pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-2 (Dubey et al., 2006; Williams et al., 2000; Nishikawa et al., 2001). Elevated Th1 and Th2 cytokine expression have also been reported to occur after *N. caninum* infection (Khan et al.,1997; Eperon et al., 1999; Quinn et al., 2004; Kano et al., 2005). In the present study, the *N. caninum* Nc1 strain and NcGRA7KO enhanced mRNA expression of IFN-γ in the placenta on day 13.5 of pregnancy (10 dpi) while there was no significant difference among the experimental groups including negative control mice. Therefore, *N. caninum* infection might trigger the placental pro-inflammatory response.

The Th2 immune response is characterized by IL-4 production. IL-4 has been reported to be related to susceptibility to *N. caninum* infection (Long et al., 1998; Baszler et al., 1999). IL-4 may aid *N. caninum* replication at the maternal-fetal junction (Quinn et al., 2004; Innes et al., 2005) and lead to high levels of vertical transmission (López-Pérez et al., 2006; 2008; 2010). Moreover, increased IL-4 expression might reduce the harmful effects of the pro-inflammatory immune response to maintain pregnancy (López-Pérez et al., 2010). In the present study, no statistically significant difference in placental IL-4 expression levels was found between the infected and uninfected dams on day 13.5 of pregnancy (10 dpi), suggesting that the Th2 immune response in the host may not contribute to vertical transmission under the present experimental conditions.

Several *N. caninum* dense granule proteins such as NcGRA7 play a role in the expression and production of cytokines, chemokines, and chemokine receptors through the activation of NF-$_{K}$B (nuclear factor kappa-light-chain-enhancer of activated B cells), NFAT (nuclear factor of activated T-cells), and CAMP/PKA (cyclic adenosine monophosphate dependent protein kinase) signals, and therefore NcGRA7KO parasites appear to reduce parasite virulence in immunocompetent and immunocompromised non-pregnant mice, as evidenced by lower parasite burdens and mild brain lesions (Nishikawa et al., 2018). Unexpectedly, in the present study, the expression levels of CCL2, CCL8, and CXCL9 were higher in the placentas from the NcGRA7KO-infected dams than in the placentas from Nc1-infected animals. This suggests that NcGRA7 has a different activity in the host under non-pregnant and pregnant conditions. An unknown factor produced by the placenta might interact with NcGRA7, resulting in inhibition of chemokine expression. Alternatively, a gene downregulated by NcGRA7 may predominantly affect pathogenesis in the placenta. In fact, several genes that are involved in the cell cycle, RAS signaling, apoptosis, cell differentiation, and metabolism are downregulated by NcGRA7 in macrophages (Nishikawa et al., 2018). Therefore, loss of NcGRA7 may enhance chemokine expression in the placenta, resulting in the immune reaction for prevention of vertical transmission of NcGRA7KO parasites. But, further studies are needed for confirmation.

Neospora caninum secretes molecules that initiate monocyte migration to the site of infection to enhance parasite invasion and multiplication (Mineo et al., 2010). Moreover, *N. caninum* cyclophilin (NcCyp) causes CCR5 (cysteine–cysteine chemokine receptor 5)-dependent migration of murine and bovine cells and is consistently detected in tachyzoites distributed within or around brain lesions (Kameyama et al., 2012) Because mouse placenta reaches to full maturity at 12.5 day of pregnancy (Elmore et al., 2022), the mice were sacrificed at 13.5 day of pregnancy. Both Nc1 and NcGRA7KO parasites were not detected in whole fetus bodies at 13.5 day of pregnancy (10 dpi), suggesting that vertical transmission may occur after this period. Moreover, both Nc1 and NcGRA7KO parasites were detected in the placenta at similar levels on day 13.5 of pregnancy (10 dpi). However, the histopathology analysis reported here reveals that more inflammatory cell infiltration was seen in the placentas from the NcGRA7KO-infected dams than in those from Nc1-infected dams. These results are consistent with the upregulated mRNA chemokine expression levels that we observed in the placenta. This immune

reaction might affect parasite viability in the placenta, resulting in decreased vertical transmission of NcGRA7KO parasites.

The placenta of mice and humans is hemochorial type (Furukawa et al., 2014). Among the placenta of mammals, the layered structure of mice and humans that exists between the blood on the maternal side and the blood on the fetal side is simple. The infection into fetus is established when the pathogen crossed the three layers of trophoblastic cells and two layers of fetal vascular endothelial cells from the maternal side. On the other hand, the placenta of cattle and dogs is epitheliochorial and endotheliochorial type, respectively (Furukawa et al., 2014). To establish the vertical transmission, pathogens must pass three layers consisting of maternal vascular endothelial cells, trophoblastic cells, and fetal vascular endothelial cells in dogs, and four layers consisting of maternal vascular endothelial cells, endometrial epithelium, trophoblastic cells, and fetal vascular endothelial cells in cattle. Compared with the placenta structure of dogs and cattle, it might be easily infected in mice. However, the molecular mechanism for crossing the placental barrier by pathogens including *N. caninum* should be elucidated in future studies.

In conclusion, to our knowledge, my study is the first to evaluate the role of an *N. caninum* dense granule antigen in pregnant mice. Our findings show that NcGRA7 might play a role in the vertical transmission of *N. caninum*. Further studies are vital for understanding the role played by NcGRA7 in the host immune response related to the regulation of vertical transmission of *N. caninum* in mice.

1-5. Summary

Neosporosis is a parasitic disease affecting the health of dogs and cattle worldwide. It is caused by *Neospora caninum*, an obligate intracellular apicomplexan parasite. Dogs are its definitive host, it mostly infects livestock animals, especially cattle that acts as intermediate host. It is necessary to have well-established models of abortion and vertical transmission in experimental animals, in order to determine basic control measures for the *N. caninum* infection. I evaluated the role of *N. caninum* dense granule antigen 7 (NcGRA7) in the vertical transmission of *N. caninum* using the C57BL/6 pregnant mouse model. I inoculated mice on day 3.5 of pregnancy with parental Nc-1 or NcGRA7-deficient

parasites (NcGRA7KO). Post-mortem analyses were performed on day 30 after birth and the surviving pups were kept until day 30 postpartum. The number of parasites in the brain tissues of offspring from NcGRA7KO-infected dams was significantly lower than that of the Nc-1-infected dams under two infection doses (**1** × 10^6 and 1 × 10^5 tachyzoites/mouse). The vertical transmission rates in the NcGRA7KO-infected group were significantly lower than those of the Nc1-infected group. To understand the mechanism by which the lack of NcGRA7 decreases the vertical transmission, pregnant mice were sacrificed on day 13.5 of pregnancy (10 days after infection), although parasite DNA was detected in the placentas, no significant difference was found between the two parasite lines. Histopathological analysis revealed a greater inflammatory response in the placentas from NcGRA7KO-infected dams than in those from the parental strain. This finding correlates with upregulated chemokine mRNA expression for CCL2, CCL8, and CXCL9 in the placentas from the NcGRA7KO-infected mice. In conclusion, these results suggest that loss of NcGRA7 triggers an inflammatory response in the placenta, resulting in decreased vertical transmission of *N. caninum*.

Table 1 Primers used in this study

Primer	**Sequence (5′-3′)**	**Use**
Nc5 gene-specific	5′-ACT GGA GGC ACG CTG AAC AC-3′	Quantitative PCR for measuring the parasite numbers according to the detection of *N. caninum* control DNA
	5′-AAC AAT GCT TCG CAA GAG GAA-3′	
Mouse GAPDH	5′-TGT GTC CGT CGT GGA TCT GA-3′	Internal control gene (housekeeping gene) for real-time RT-PCR analysis
	5′-CCT GCT TCA CCA CCT TCT TGA T-3′	
Mouse IL-10	5′-CCT GGT AGA AGT GAT GCC CC-3′	Real-time PCR of mouse IL-10 mRNA expression
	5′- TCC TTG ATT TCT GGG CCA TG-3′	
Mouse IFN-γ	5′-GCC ATC AGC AAC AAC ATA AGC GTC-3′	Real-time PCR of mouse IFN-γ mRNA expression
	5′-CCA CTC GGA TGA GCT CAT TGA ATG-3′	
Mouse IL-4	5′-CAC GGA TGC GAC AAA AAT CA-3′	Real-time PCR of mouse IL-4 mRNA expression
	5′ -CTC GTT CAA AAT GCC GAT GA-3′	
Mouse TNF-α	5′-GGC AGG TCT ACT TTG GAG TCA TTG C-3′	Real-time PCR of mouse TNF-α mRNA expression
	5′-ACA TTC GAG GCT CCA GTG AA-3′	
Mouse CCL2	5′-GGC TCA GCC AGA TGC AGT TAA-3′	Real-time PCR of mouse CCL2 mRNA expression
	5′-CCT ACT CAT TGG GAT CAT CTT GCT-3′	
Mouse CCL8	5′-ACC TCA AAC AGT TTG CCC CA-3′	Real-time PCR of mouse CCL8 mRNA expression
	5′-TTC ACA TTT GCC GAG TCC G-3′	

Mouse CXCL9	5′-ACC TCA AAC AGT TTG CCC CA-3′	Real-time PCR of mouse CXCL9 mRNA expression
	5′-TTC ACA TTT GCC GAG TCC G-3′	
Mouse CXCL10	5′-TGC CGT CAT TTT CTG CCT CA-3′	Real-time PCR of mouse CXCL10 mRNA expression
	5′-TCA CTG GCC CGT CAT CGA TAT-3′	

Table 2. Number of offspring per litter and offspring survival rates (30 days postpartum) at high dose of infection (10^6 tachyzoites/dam).

Groups	Number of litters (no. of used mice), fertility rate	Mean number of offspring/litter (SD)	No. of surviving offspring /no. of offspring in each litter (Number of PCR positive offspring in each litter / number of analyzed samples in each litter)	Total no. of surviving offspring /total no. of offspring (%) (Total number of PCR positive offspring / total number of analyzed samples, %)
Negative control	, 83.3%5 (6)	6.4 (2.70)	0/2, 7/9, 0/8, 7/7, 6/6	20/32 (62.5%)
Nc1-infected	, 80% 4 (5)	6.5 (2.38)	2/8, 0/3, 0/7, 0/8) 7/7 ,7/7 3/3, 3/4,(	2/26 (7.7%) (20/21, 95.2%)
NcGRA7KO-infected	, 100%5 (5)	5.0 (3.67)	0/7, 0/8, 7/8, 0/1, 0/1) 1/1 ,1/1 ,4/8 ,3/6 ,5/5(	7/25 (28.0%) #, * (14/21, 66.7%) #

Mean number of offspring/litters in all experimental groups was analyzed with one-way ANOVA followed by the Tukey-Kramer post hoc test, but no significant differences were observed. Mice were injected with RPMI-1640 medium as negative control of uninfected dams. PCR was not performed to offspring from the negative control dams. Mortality rate of negative control was 37.5% (12/32). The pregnant mice were used for analysis of fertility rate. The total number of PCR positive offsprings/ litters among infected groups were analyzed with a χ^2 test, (# $P < 0.05$). Offspring survival rates at 30 days post-partum were analyzed with a χ^2 test, (* $P < 0.05$ against uninfected mice, # $P < 0.05$ against Nc1-infected mice). SD, standard deviation.

Table 3. Number of offspring per litter and offspring survival rates (30 days postpartum) at the low dose of infection (10^5 tachyzoites/dam).

Groups	**Number of litters (no. used mice), fertility rate**	**Mean number of offspring/litter (SD)**	**No. of surviving offspring/no. of offspring in each litter** **(Number of PCR positive offspring in each litter / number of analyzed samples in each litter)**	**Total no. of surviving offspring /total no. of offspring (Survival rate %)** **(Total Number of PCR positive offspring / total number of analyzed samples, %)**
Negative control	, 66.6%4 (6)	5.0 (2.8)	5/5, 0/1, 7/7, 6/7	18/20 (90.0%)
Nc1-infected	, 83.3%5 (6)	7.2 (0.4)	2/7, 0/7, 5/8, 0/7, 0/7 (7/7, 7/7, 8/8, 7/7, 7/7)	7/36 (19.4%) # (36/36, 100.0%)
NcGRA7KO-infected	, 71.4% (7) 5	4.4 (2.3)	0/6, 5/5, 0/2, 0/2, 6/7 (5/5, 3/5, 2/2, 0/1, 4/7)	11/22 (50.0%)$^{*,\#}$. (14/20, 70.0%)$^{\#}$

Mean number of offspring/litters in all experimental groups was analyzed with one-way ANOVA followed by the Tukey-Kramer post hoc test, but no significant differences were observed. Mice were injected with RPMI-1640 medium as negative control of uninfected dams. PCR was not performed to offspring from the negative control dam. Mortality rate of negative control was 10 % (2/20). The pregnant mice were used for analysis of fertility rate. The total number of PCR positive offsprings/ litters among infected groups were analyzed with a χ^2 test, (# $P < 0.05$). Offspring survival rates at 30 days post-partum were analyzed with a χ^2 test (* $P < 0.05$ against uninfected mice, # $P < 0.05$ against Nc1-infected mice). SD, standard deviation.

Table S1. The number of T lymphocytes and macrophage in placenta after 10 days post-infection with a high dose infection (10^6 tachyzoites/dam).

	Case	Tachyzoites	T lymphocyte			Macrophage		
			Decidua	Junctional zone	Labyrinth	Decidua	Junctional zone	Labyrinth
NcGRA7KO-infected	1	-	30	33	3	127	55	34
NcGRA7KO-infected	2	-	40	13	2	61	44	32
NcGRA7KO-infected	3	-	24	45	ND	55	40	ND
NcGRA7KO-infected	4	-	21	17	2	70	36	24
NcGRA7KO-infected	5	-	41	34	4	106	62	17
NcGRA7KO-infected	6	-	36	21	2	63	49	30
NcGRA7KO-infected	7	-	ND	21	1	ND	45	23
NcGRA7KO-infected	8	+	55	42	2	66	57	28
NcGRA7KO-infected	9	-	53	45	1	101	35	21
NcGRA7KO-infected	10	-	53	22	2	68	31	26
NcGRA7KO-infected	11	-	41	58	4	76	69	27
NcGRA7KO-infected	12	-	37	44	0	70	49	21
NcGRA7KO-infected	13	-	ND	ND	2	ND	ND	29
Mean			39.2	32.9	2.1	78.5	47.7	26.0
SD			10.9	13.5	1.1	21.6	11.0	4.8

Nc1-infected	1	-	ND	ND	3	ND	ND	16
Nc1-infected	2	-	26	18	2	68	31	25
Nc1-infected	3	-	14	23	2	58	30	23
Nc1-infected	4	-	23	22	1	33	44	31
Nc1-infected	5	-	13	3	2	71	49	22
Nc1-infected	6	-	14	17	2	60	47	25
Mean			18.0	16.6	2.0	58.0	40.2	23.7
SD			5.4	7.2	0.6	13.4	8.1	4.5
uninfected	1	-	2	2	0	30	21	23
uninfected	2	-	1	2	5	20	21	14
uninfected	3	-	ND	2	2	ND	12	17
uninfected	4	-	3	1	2	21	26	25
uninfected	5	-	0	1	2	36	21	18
uninfected	6	-	0	1	4	18	21	27
Mean			1.2	1.5	2.5	25.0	20.3	20.7
SD			1.2	0.5	1.6	6.9	4.1	4.6

The number of T lymphocytes and macrophage in placenta after 10 days post infection with high dose (10^6 tachyzoites/dam). The placenta was divided into three zones (decidua, junctional zone, labyrinth), CD3+ T lymphocytes and Iba1+ macrophages were counted in 3 high power fields. ND: not detected.

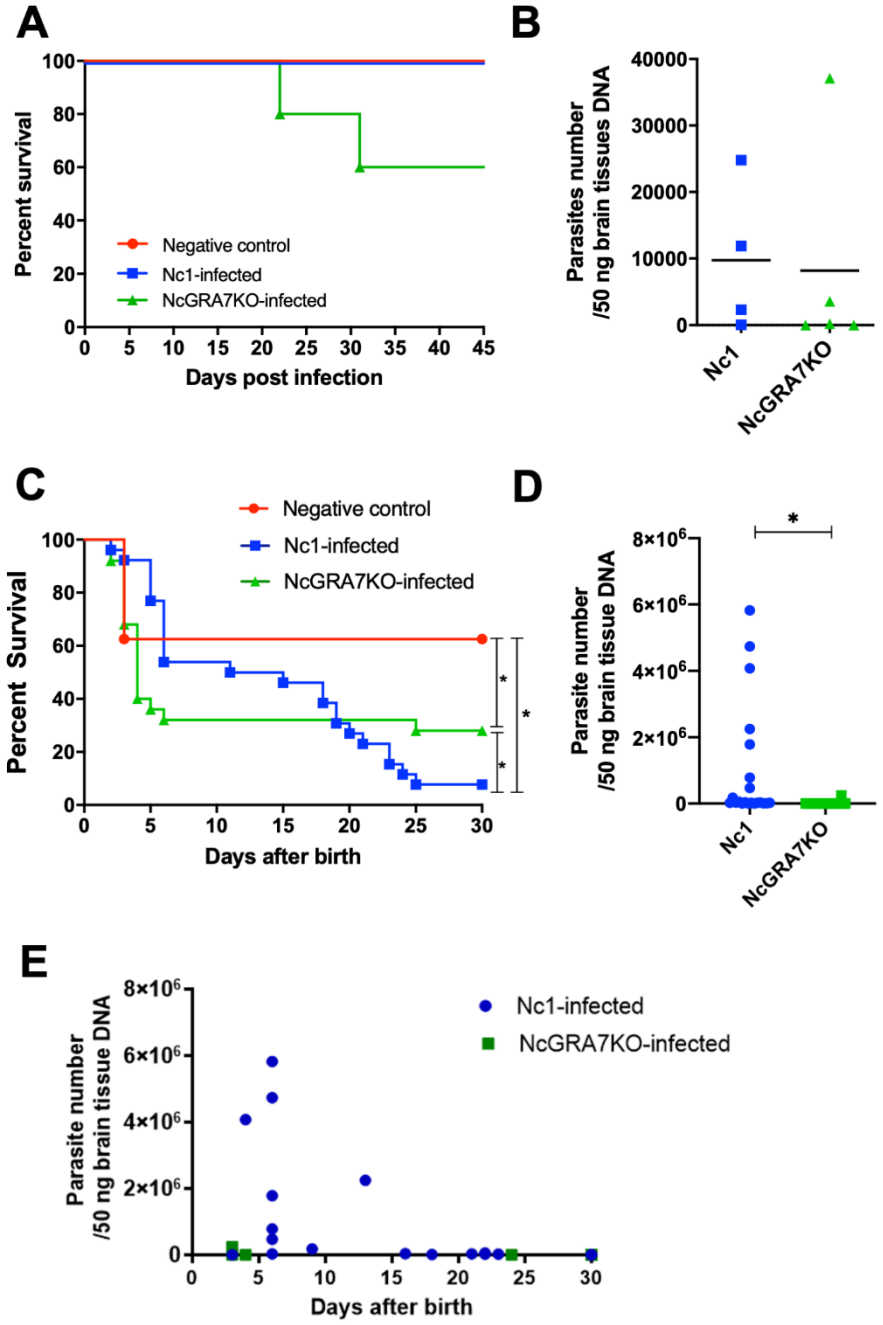


Fig. 1.Parasite virulence in mice under 1 × 10^6 parasite infection. Pregnant mice were infected with 1 × 10^6 *N. caninum* tachyzoites of the parental Nc1 strain, the NcGRA7-deficient parasite (NcGRA7KO), or they were injected with RPMI-1640 medium to represent the negative control on day 3.5 of pregnancy. **(A)** Survival rates in the dams were calculated until day 30 postpartum (45 days post infection) (negative control, 5/5, 100%; Nc1-infected, 4/4, 100%; NcGRA7KO-infected, 3/5, 60%). Statistically significant differences in the survival rates were analyzed with a χ^2 test but none were found. **(B)** Parasite burdens in brains of dams on day 30 postpartum. Values are the number of parasites in 50 ng of brain tissue DNA. Statistically significant differences between Nc1 and NcGRA7-infected

groups were analyzed by Student's t-test, but none were found. **(C)** Kaplan Meier survival curve in the offspring was generated until day 30 postpartum (Survival rates: Negative control, 20/32, 62.5%; Nc1, 2/26, 7.7%; NcGRA7KO, 7/25, 28.0%). Statistically significant differences were analyzed with a χ^2 test (* $P < 0.05$). **(D)** Parasite burdens in the brains of offspring. Parasite numbers were measured in the brains of the dead and surviving offspring on day 30 postpartum. Values are the number of parasites in 50 ng of brain tissue DNA. Undetectable values were expressed as "0". Statistically significant differences were analyzed with a Mann–Whitney U test (* $P < 0.05$). (**E**) Parasite number in the brain of offspring corresponding to the day of death after birth from day 0 until 30 days post-partum. Parasite numbers in the brains of the dead and surviving offspring were measured. Values are the number of parasites in 50 ng of brain tissue DNA. Undetectable values were expressed as "0".

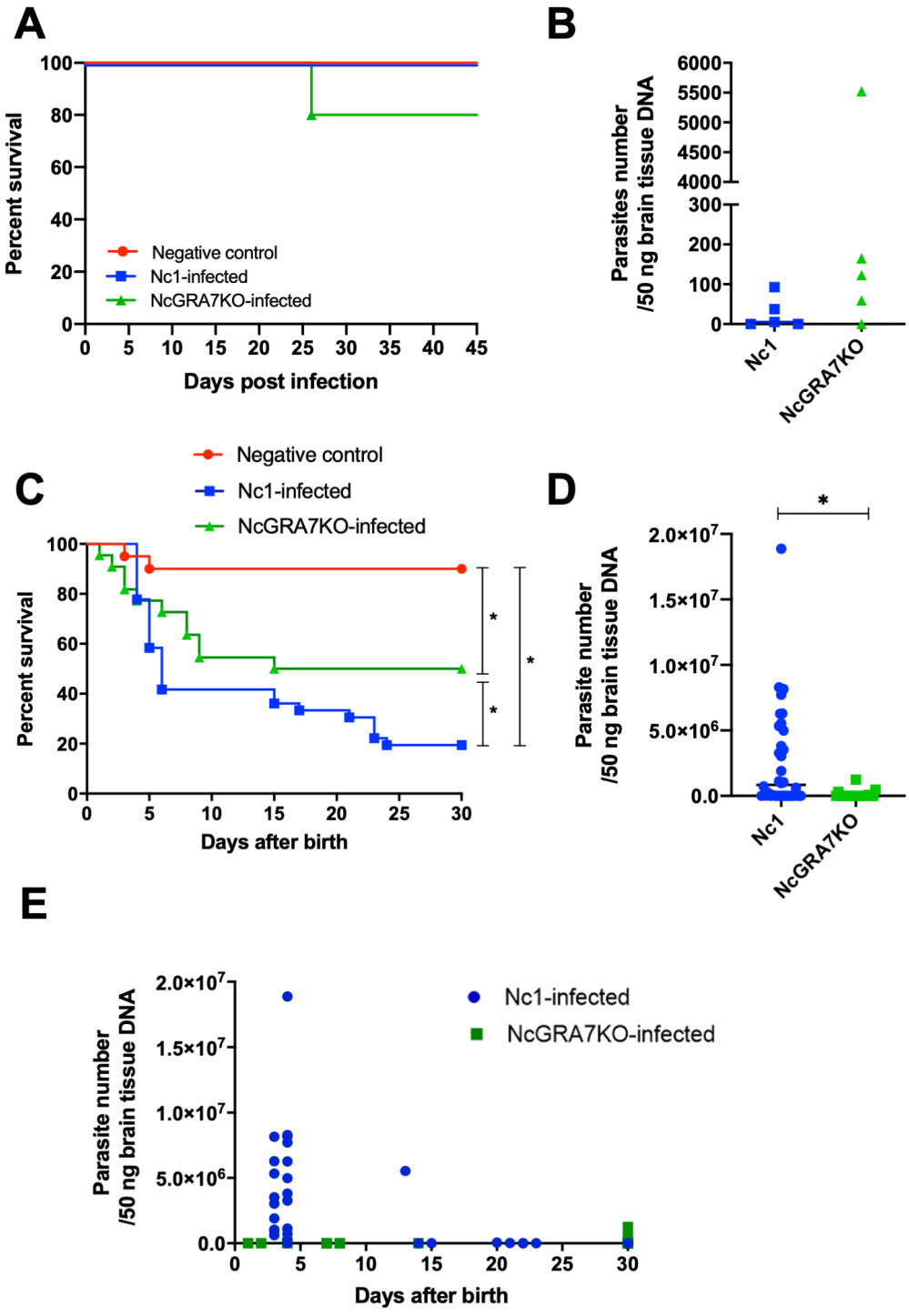


Fig. 2. Parasite virulence in mice under 1 × 10^5 parasite infection. Pregnant mice were infected with 1 × 10^5 *N. caninum* tachyzoites of the parental Nc1 strain or the NcGRA7-deficient parasite

(NcGRA7KO), or they were injected with RPMI-1640 medium to represent the negative control on day 3.5 of pregnancy. **(A)** Survival rates in the dams were calculated until day 30 postpartum (45 days post infection) (negative control, 4/4, 100%; Nc1-infected, 5/5, 100%; NcGRA7KO-infected, 4/5, 80%). Statistically significant differences in the survival rates were analyzed with a χ^2 test but none were found. **(B)** Parasite burdens in the brains of the dams on day 30 postpartum. Values are the number of parasites in 50 ng of brain tissue DNA. Statistically significant differences between Nc1 and NcGRA7-infected groups were analyzed by a Mann–Whitney U test, but none were found. **(C)** Kaplan Meier survival curve in the offspring were calculated until day 30 postpartum (Survival rates: Negative control, 18/20, 90.0%; Nc1, 7/36, 19.4%; NcGRA7KO, 11/22, 50.0%). Statistically significant differences were analyzed with a χ^2 test (* $P < 0.05$). **(D)** Parasite burdens in the brains of offspring. Parasite numbers were measured in the brains of the dead and surviving offspring on day 30 postpartum. Values are the number of parasites in 50 ng of brain tissue DNA. Undetectable values were expressed as "0". Statistically significant differences were analyzed with a Mann–Whitney U test (* $P < 0.05$). (**E**) Parasite number in the brain of offspring corresponding to the day of death after birth from day 0 until 30 days post-partum. Parasite numbers in the brains of the dead and surviving offspring were measured. Values are the number of parasites in 50 ng of brain tissue DNA. Undetectable values were expressed as "0".

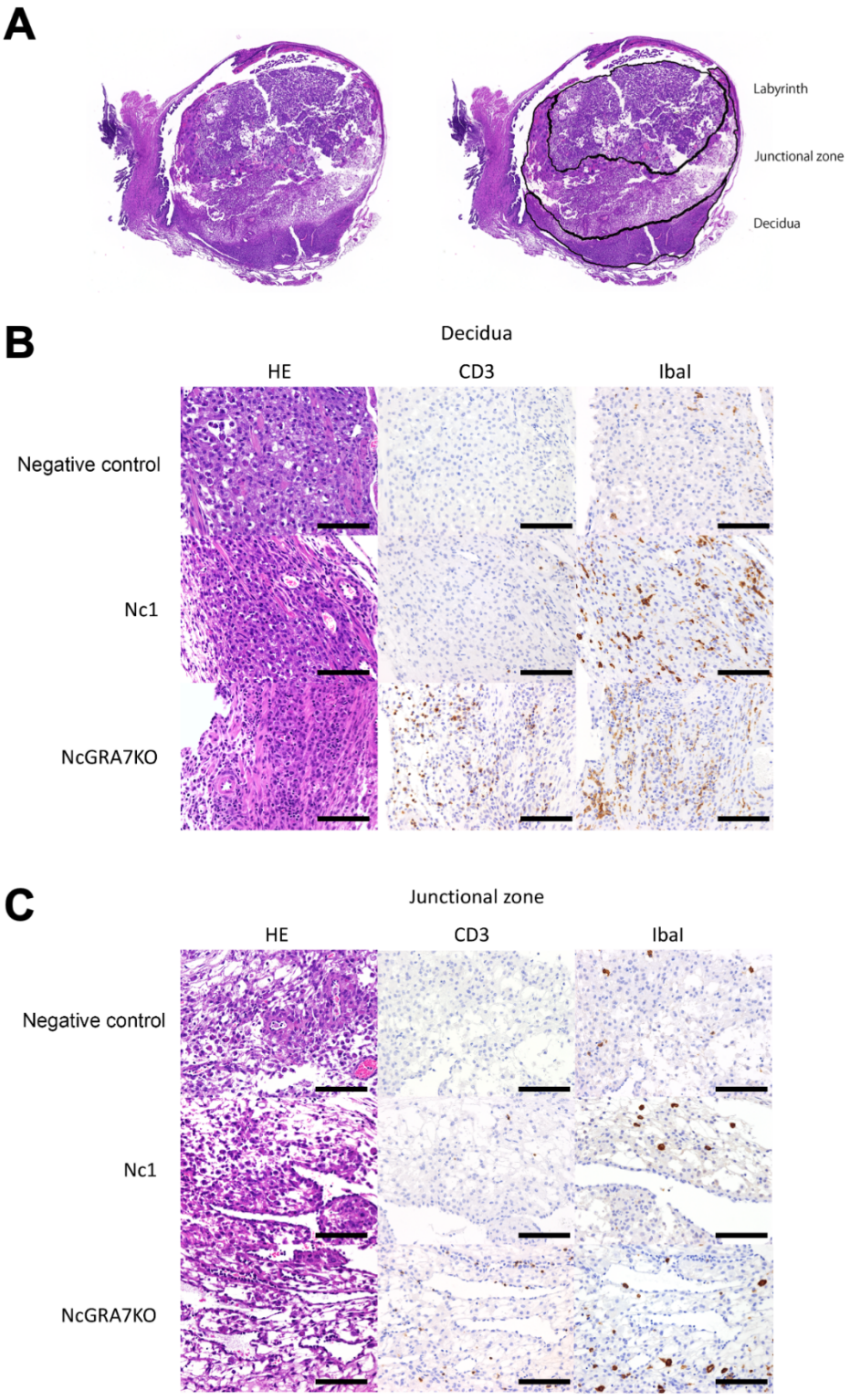


Fig. 3. Histopathological analysis of *N. caninum* in fetoplacental tissue on day 13.5 of pregnancy (10 days post-infection). **(A)** The placenta was divided into three layers (decidua, junctional zone, and labyrinth). A representative image of hematoxylin and eosin staining (HE). Bar = 1 mm. HE staining and immunohistochemistry targeting of CD3 and Iba1 in the decidua **(B)** and junctional zone **(C)** of RPMI-1640 medium-injected (negative control), parental strain Nc1-infected, and NcGRA7-deficient

(NcGRA7KO)-infected mice. **(B)** In decidua of Nc1-infected group, few T lymphocytes (arrow) and some macrophages (arrowhead) are infiltrated. In NcGRA7KO-infected group, T lymphocyte infiltration is more severe than Nc1-infected. Bar = 100 μm (C) In junctional zone, slight to mild inflammation were observed in both Nc1- and NcGRA7KO-infected groups. Bar = 100 μm, 3HPF: 3 high power fields.

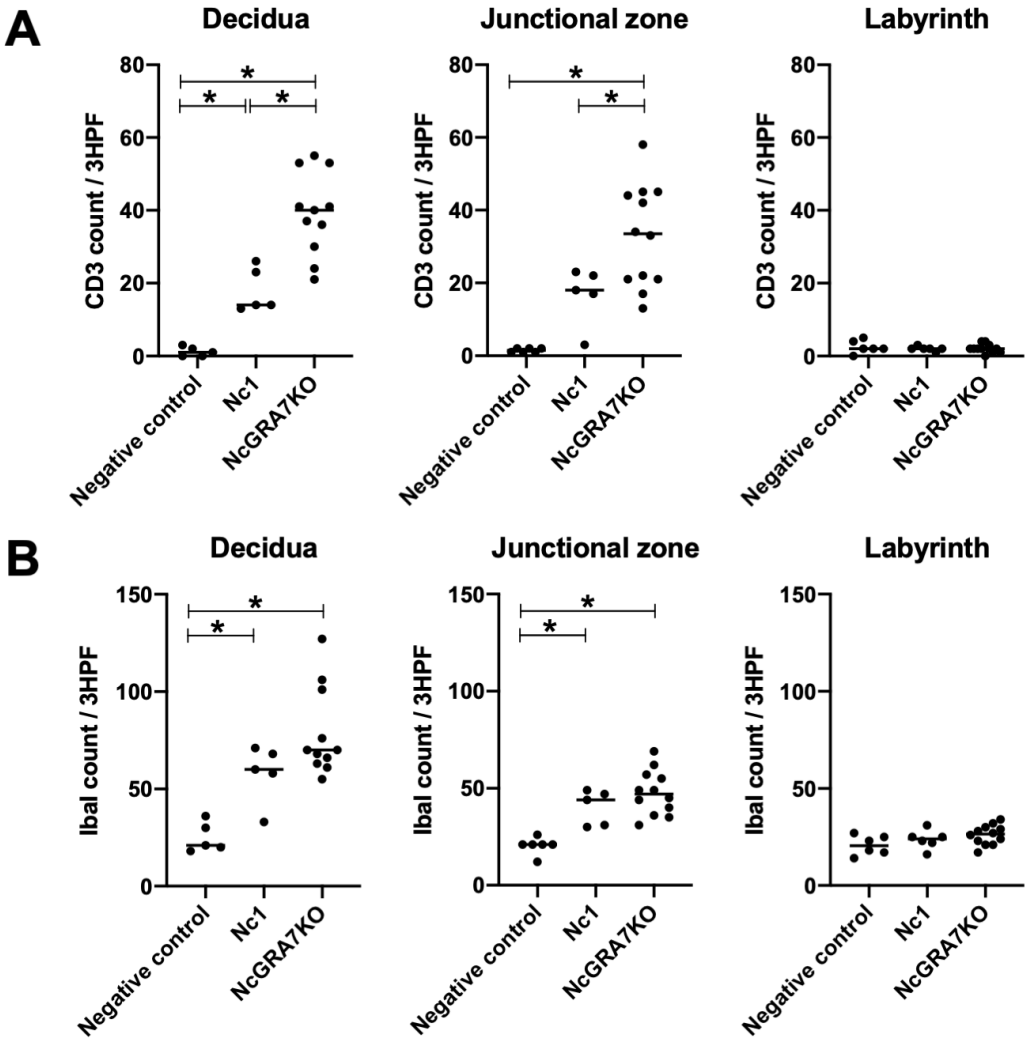

A
Decidua
Junctional zone
Labyrinth
CD3 count / 3HPF
0
20
40
60
80
*
*
*
*
*
Negative control
Nc1
NcGRA7KO
B
Decidua
Junctional zone
Labyrinth
Iba1 count / 3HPF
0
50
100
150
*
*
*
*
*
Negative control
Nc1
NcGRA7KO

Fig. 4. The number of T lymphocytes (A) and macrophages (B) in three high-power fields in three layers (decidua, junctional zone, and labyrinth) of placentas from RPMI-1640 medium-injected (negative control), parental strain Nc1-infected, and NcGRA7-deficient (NcGRA7KO)-infected mice. The immunohistochemistry analysis targeted CD3 (T lymphocytes) or Iba1 (macrophages) on day 13.5 of pregnancy (10 days post-infection). Negative control, n = 6; Nc1-infected, n = 6; NcGRA7KO-infected, n = 13. * Significant differences were analyzed by one-way ANOVA plus Tukey-Kramer hoc test ($P < 0.05$).

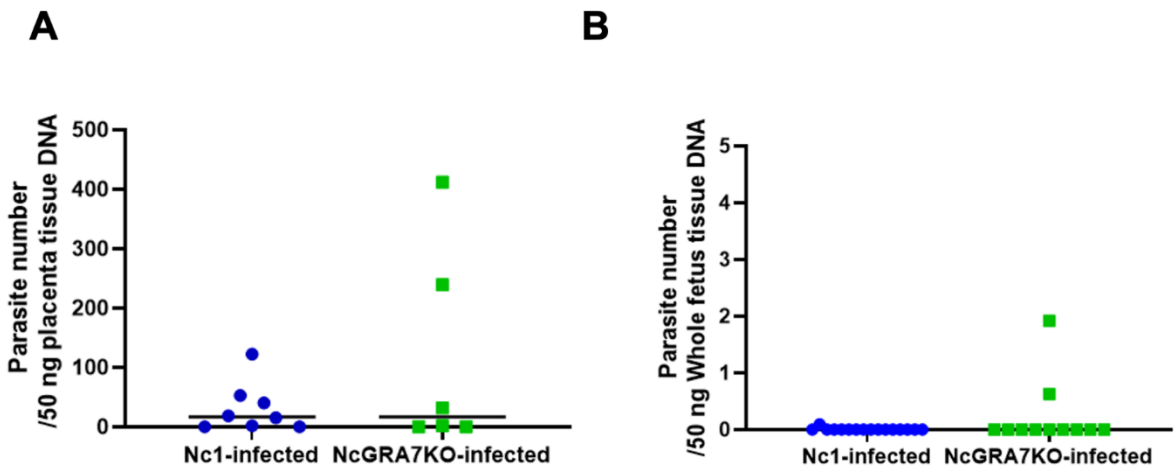


Fig. 5. Parasite burden in the placentas and fetuses of pregnant mice on day 13.5 of pregnancy and 10 days post-infection with 1 × 10^6 tachyzoites of Nc1 and NcGRA7-deficient (NcGRA7KO) parasites. **(A)** Values are the number of parasites in 50 ng of placental tissue DNA. (Nc1, n = 3; NcGRA7KO, n = 4). Statistically significant differences were analyzed by Student's t-test but none were found ($P < 0.05$). **(B)** Values are the number of parasites in 50 ng of tissue DNA from the body of each fetus (Nc1-infected, n = 16; NcGRA7KO-infected, n = 11). Statistically significant differences were analyzed by the Student's t-test, but none were found.

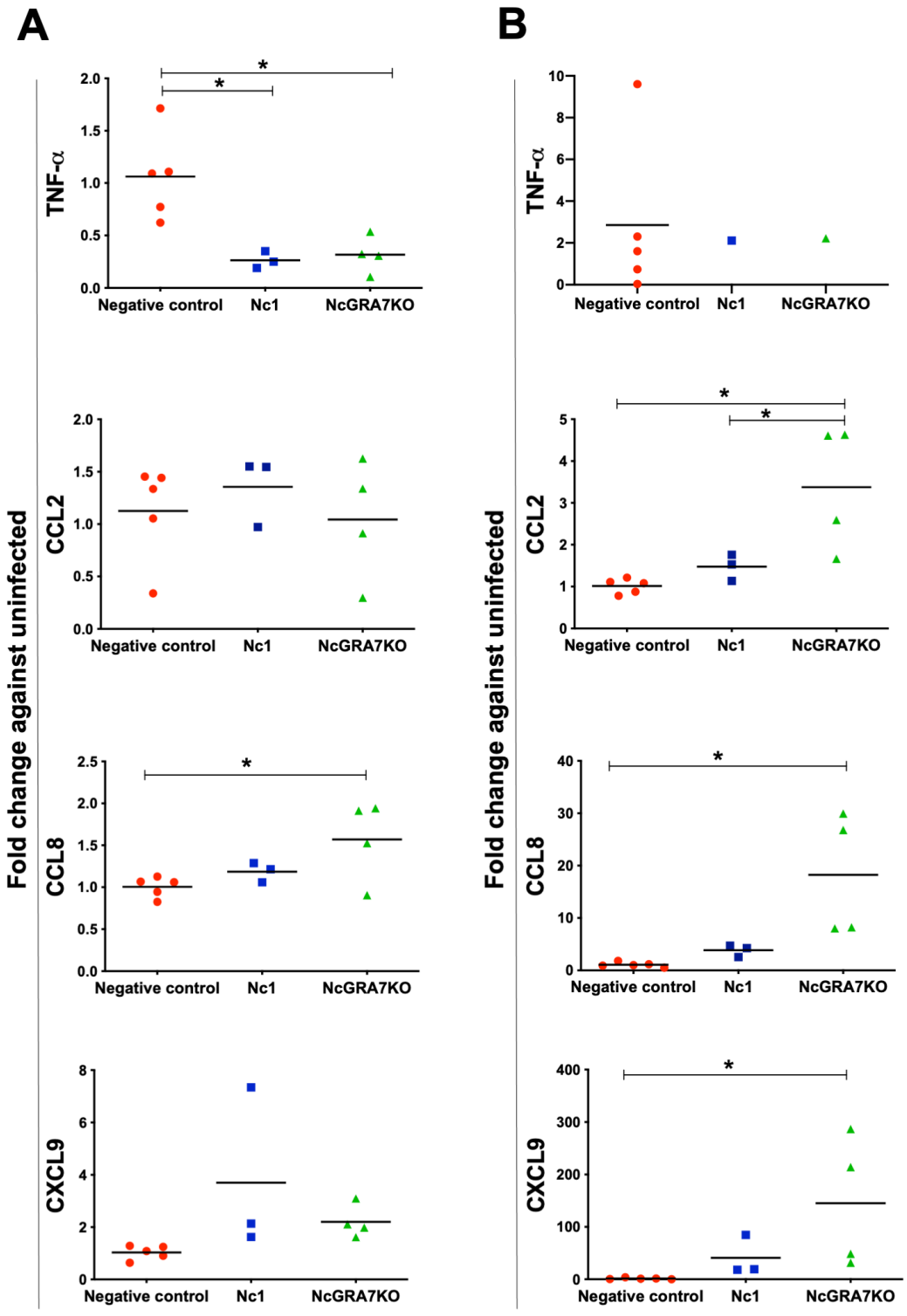


Fig. 6. Relative expression of TNF-α, CCL2, CCL8 and CXCL9 levels in the spleen (A) and placenta (B) of RPMI-1640 medium-injected pregnant mice (negative control) and pregnant mice on day 10 post-infection with 1×10^6 tachyzoites of Nc1 and NcGRA7-deficient (NcGRA7KO) parasites

(day 13.5 pregnancy). Pooled placenta samples were used for each dam. Negative control, n = 5; Nc1-infected, n = 3; NcGRA7KO-infected, n = 4. Undetectable values are not shown. Statistically significant differences were analyzed by one-way ANOVA plus Tukey-Kramer post hoc test (* $P < 0.05$).

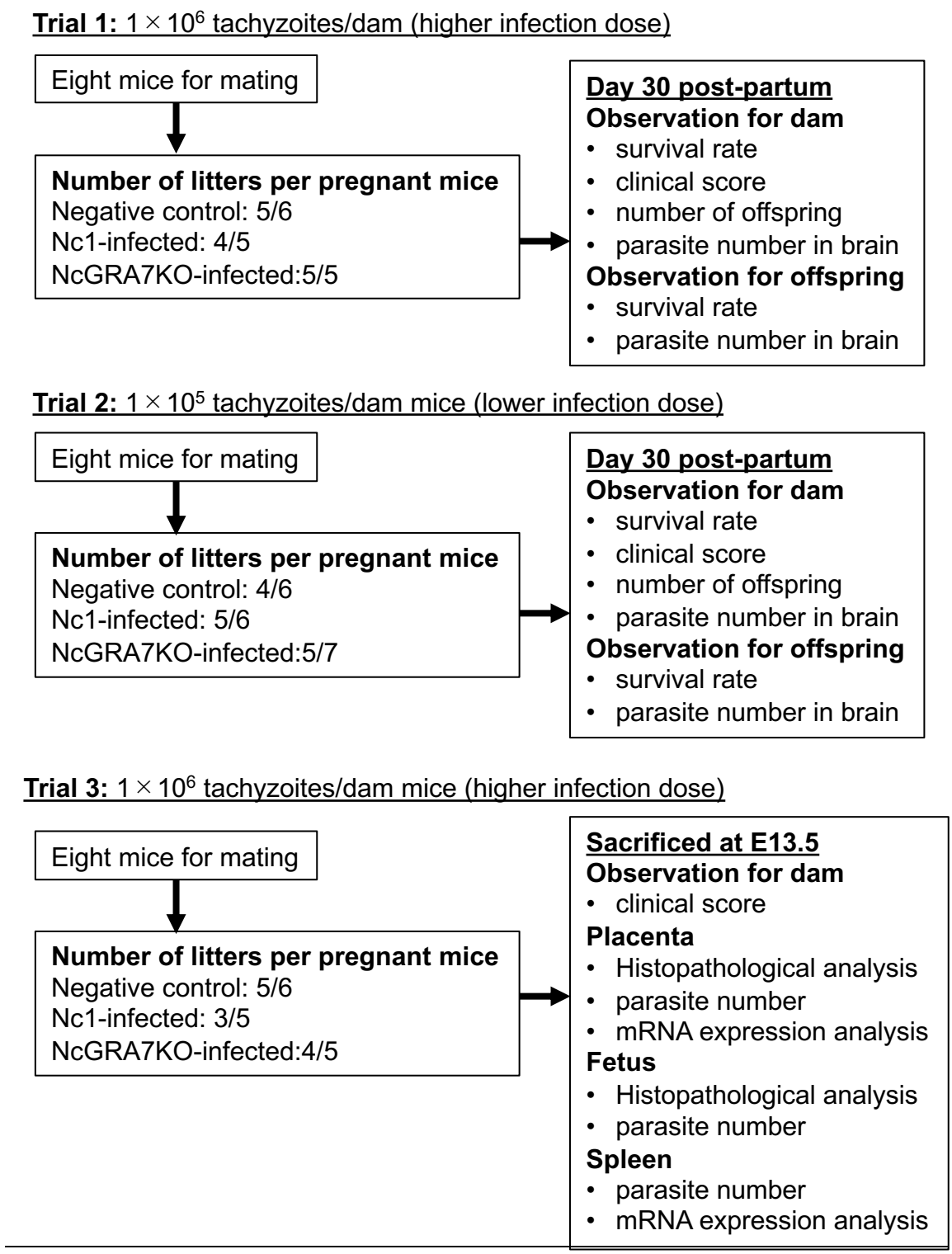


Fig S1. Flowchart explaining the numbers of mice, tissue sampling and observation. In all trials, eight mice were used for mating procedures and 5-7 mice were pregnant. Among them, 3-5 mice gave birth. In trials 1 and 2, the pregnant mice were challenged with Nc1 and NcGRA7KO (trial 1: 1×10^6 parasite/mouse, trial 2: 1×10^5 parasite/mouse), and injected with RPMI-1640 medium as negative control at day 3.5 of pregnancy (E3.5). Daily observation was performed until 30 days post-partum. Brain samples were collected from all dead offspring and surviving animals at 30 days after birth for quantitative PCR (qPCR) to determine *N. caninum* distribution. In trial 3, the pregnant mice were challenged with Nc1 and NcGRA7KO (1×10^6 parasite/mouse), and injected with RPMI-1640 medium

as negative control at E3.5. Daily observation was performed until E13.5 of pregnancy (10 days after the infection). Then, mice were sacrificed for histopathological and qPCR analyses of parasite numbers in placentas and fetuses and mRNA expression analysis of spleen and placenta.

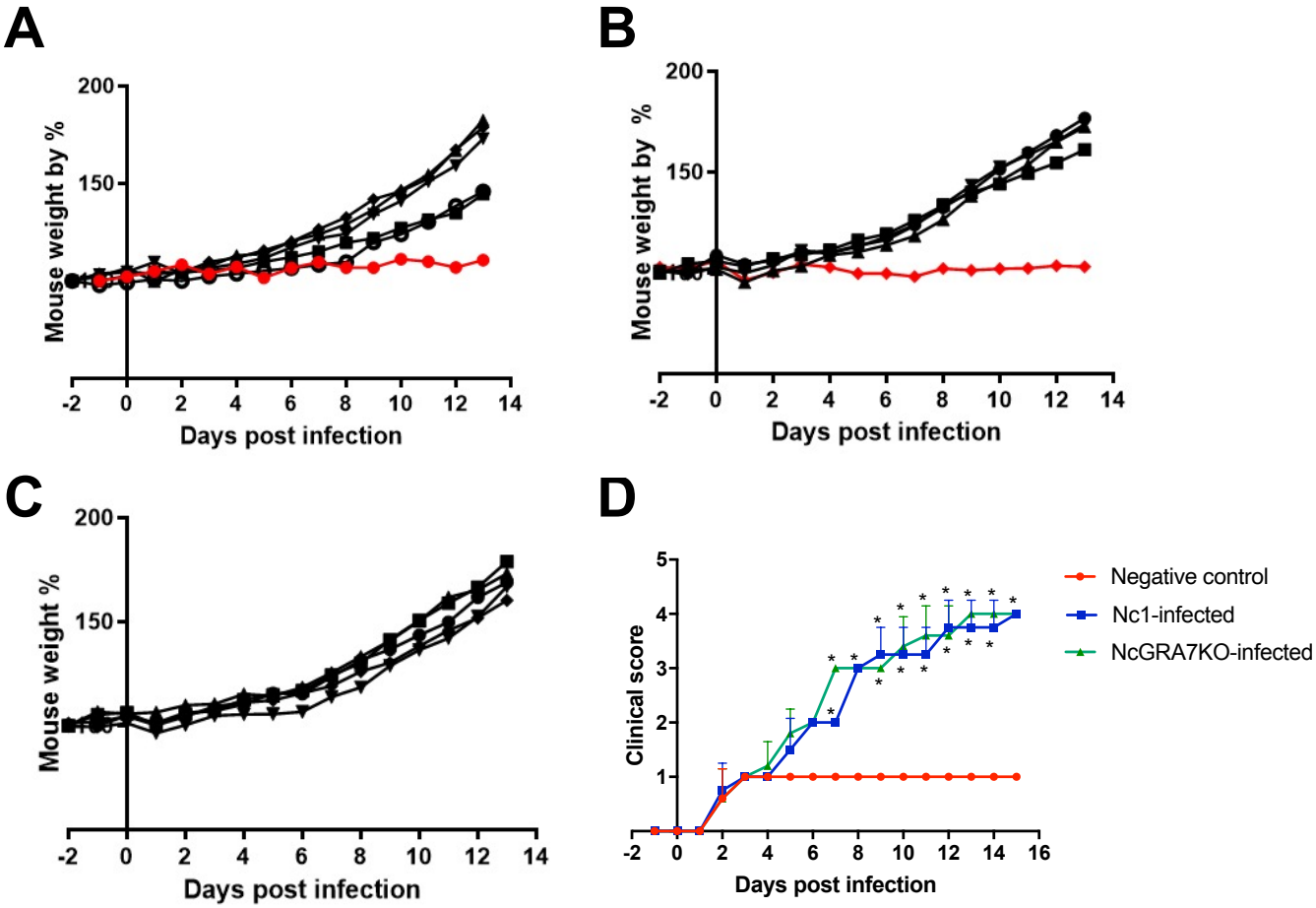


Fig. S2. Bodyweight and clinical scores of mice. Pregnant mice were infected with a high dose of *N. caninum* tachyzoites (1×10^6), parental strain Nc1, NcGRA7-deficient parasite (NcGRA7KO), and were injected with the RPMI-1640 medium as negative control at days 3.5 of pregnancy. The weight percentage (%) was calculated and expressed from the daily weight to the initial weight 2 days before infection until 13 days post-infection. **(A)** negative control mice, n = 6. **(B)** Nc1-infected mice, n = 5. **(C)** NcGRA7KO-infected mice, n = 5. The red lines represent the aborted or non-pregnant mice whereas the black or grey lines represent the confirmed pregnant mice. **(D)** Average and standard deviation (SD) of the clinical scores of the pregnant mice during *N. caninum* infection (negative control mice, n = 5; Nc1-infected mice, n = 4; NcGRA7KO-infected mice, n = 5). * Significant differences were analyzed by Two-way ANOVA plus Tukey-Kramer hoc test ($P < 0.05$). The clinical scores of both infected dams Nc1-infected and NcGRA7KO-infected were significantly higher than those of the uninfected animals from 5 to 15 days post-infection.

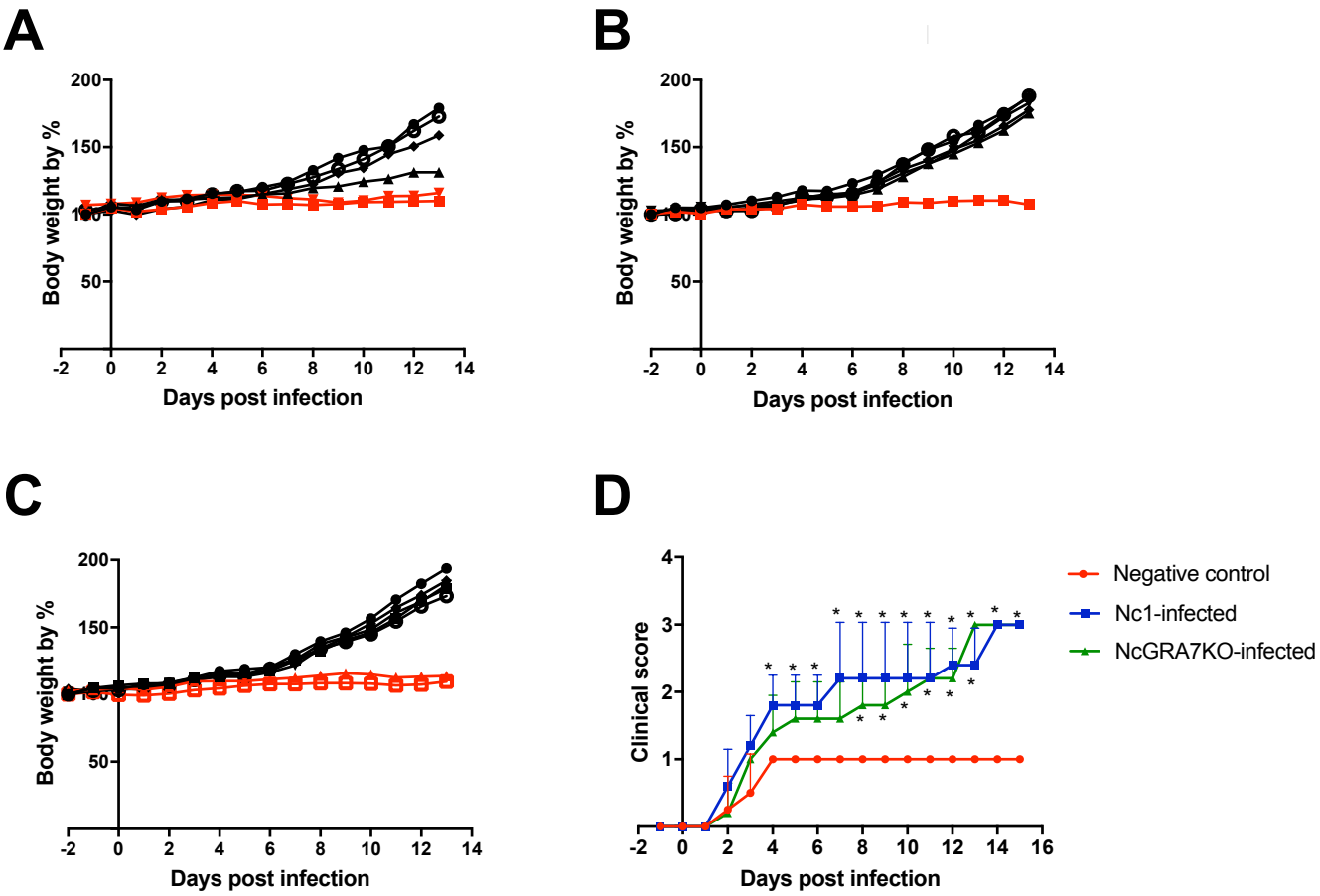


Fig. S3. Bodyweight and clinical scores of mice. Pregnant mice were infected with a high dose of *N. caninum* tachyzoites (1×10^5), parental strain Nc1, NcGRA7-deficient parasite (NcGRA7KO), and were injected with RPMI-1640 medium as negative control at day 3.5 of pregnancy. The weight % was calculated and was expressed from the daily weight to the initial weight 1 day before infection until 13 days post-infection. **(A)** Uninfected mice, n = 6. **(B)** Nc1-infected mice, n = 6. **(C)** NcGRA7KO-infected mice, n = 7. The red lines represent the aborted or non-pregnant dam mice whereas the black lines represent the confirmed pregnant dam mice. **(D)** Average and standard deviation (SD) of the clinical scores of the pregnant mice during *N. caninum* infection (negative control mice, n = 4; Nc1-infected mice, n = 5; NcGRA7KO-infected mice, n = 5). * Significant differences were analyzed by Two-way ANOVA plus Tukey-Kramer hoc test ($P < 0.05$). The clinical scores of the both infected dams were significantly higher than those of the uninfected animals from 5 to 15 days post-infection.

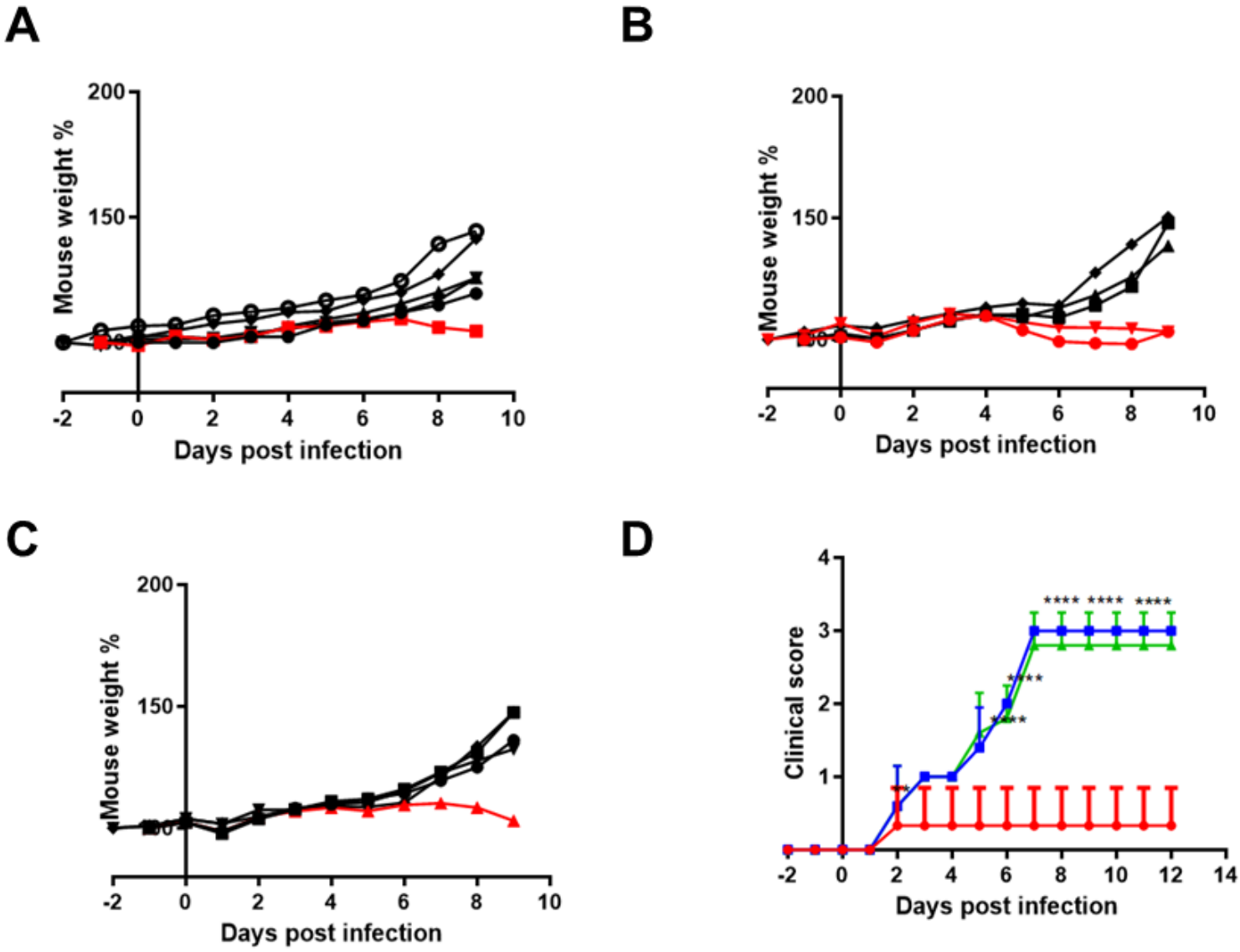


Fig. S4. Bodyweight and clinical scores of mice. Pregnant mice were infected with a high dose of *N. caninum* tachyzoites (1×10^6), parental strain Nc1, NcGRA7-deficient parasite (NcGRA7KO), and were injected with RPMI-1640 medium as negative control at day 3.5 of pregnancy. The weight % was calculated and was expressed from the daily weight to the initial weight 1 day before infection until 9 days post-infection. **(A)** Uninfected mice, n = 6. **(B)** Nc1-infected mice, n = 5. **(C)** NcGRA7KO-infected mice, n = 5. The red lines represent the aborted or non-pregnant dam mice whereas the black lines represent the confirmed pregnant dam mice. **(D)** Average and standard deviation (SD) of the clinical scores of the pregnant mice during *N. caninum* infection (negative control mice, n = 5; Nc1-infected mice, n = 3; NcGRA7KO-infected mice, n = 4). * Significant differences were analyzed by Two-way ANOVA plus Tukey-Kramer hoc test ($P < 0.05$). The clinical scores of the both infected dams were significantly higher than those of the uninfected animals from 6 to 12 days post-infection.

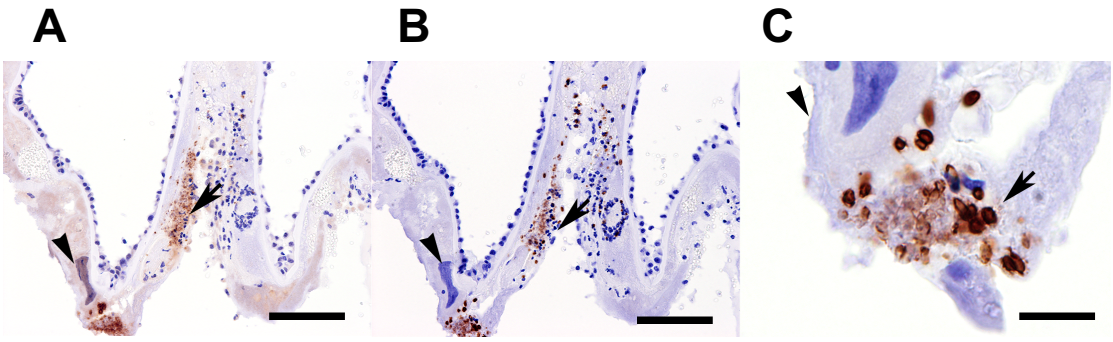


Fig. S5. Histopathological analysis showing the presence of *N. caninum* in mouse fetoplacental tissue on day 13 of pregnancy (10 days post-infection with the NcGRA7-deficient parasite). Immunohistochemistry targeting NcGRA6 (**A**) and NcSAG1 (**B, C**). *N. caninum* tachyzoites (arrow) are immunopositive for NcGRA6 and NcSAG1, and invade into trophoblasts. Arrowhead; trophoblast. Bar = 100 μm (A, B), 20 μm (C).

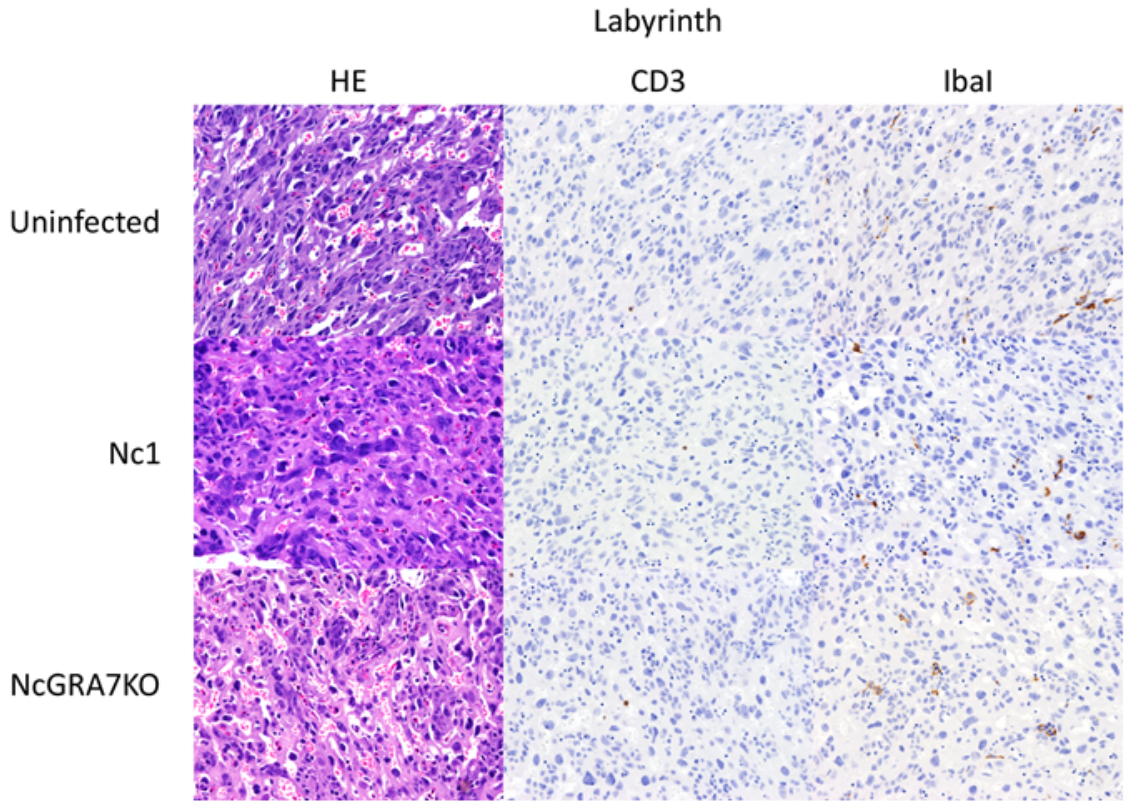


Fig. S6. Histopathological analysis showing fetoplacental tissue on day 13.5 of pregnancy (10 days post-infection). Hematoxylin and eosin (HE) stain and immunohistochemistry targeting CD3 and Iba1 in labyrinth tissue from uninfected mice, parental Nc1-infected mice, and NcGRA7-deficient parasite (NcGRA7KO)-infected mice. Small numbers of macrophages are scattered in labyrinth (arrowhead), though there is no different in uninfected, Nc1-, and NcGRA7KO-infected groups. Bar = 100 μm.

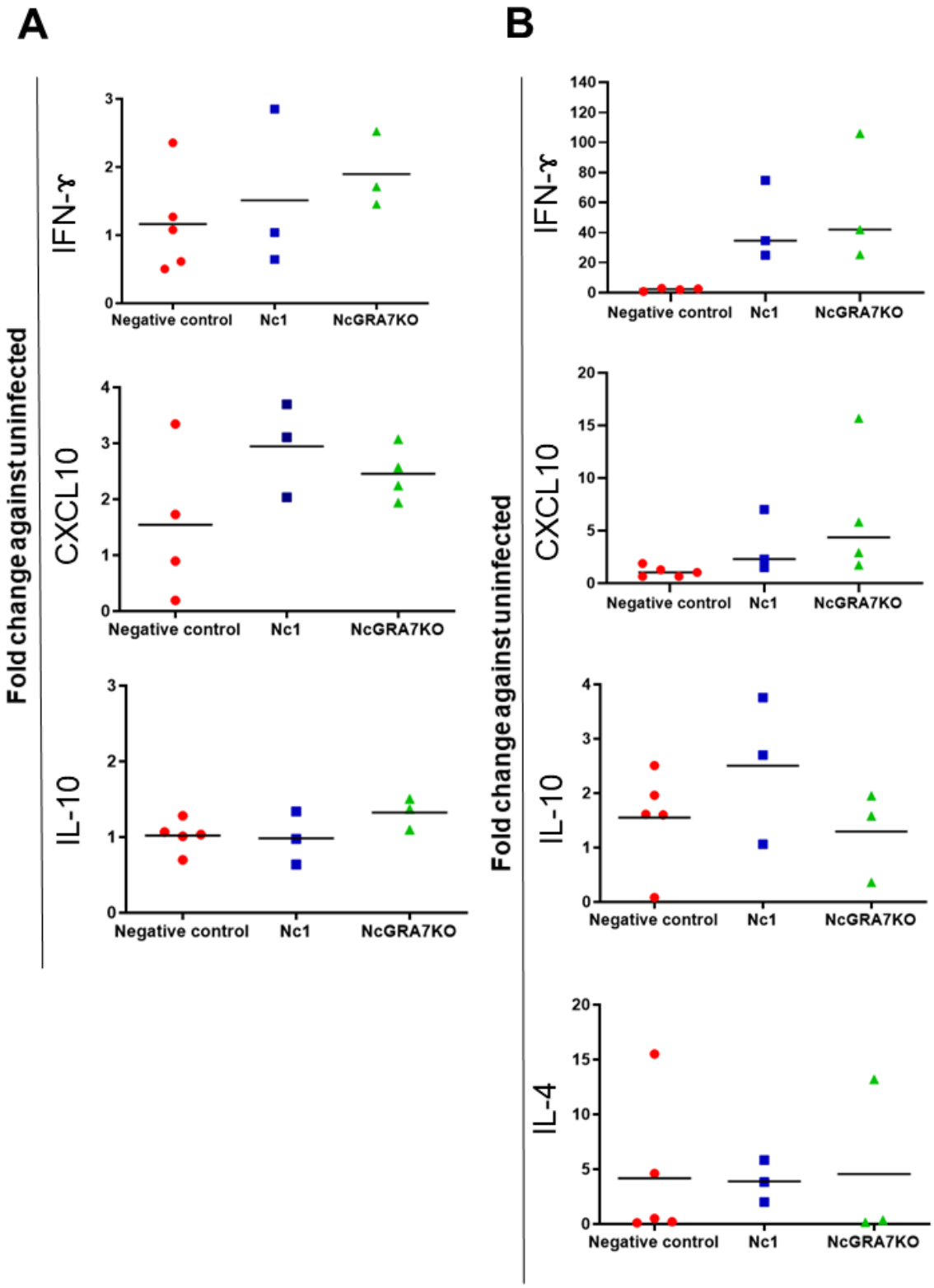


Fig. S7. Relative expression of IFN-γ, CXCL10, IL-10, and IL-4 levels in the spleen (A) and placenta (B) of RPMI-1640 medium-injected pregnant mice (negative control) and pregnant mice on day 10 post-infection with 1×10^6 tachyzoites of Nc1 and NcGRA7-deficient (NcGRA7KO) parasites (day 13.5 pregnancy). Pooled placenta samples were used for each dam. Negative control, n = 5; Nc1-

infected, n = 3; NcGRA7KO-infected, n = 4. Undetectable values are not shown. Statistically significant differences were analyzed by one-way ANOVA plus Tukey-Kramer post hoc test (* $P < 0.05$).

Chapter 2

Protective efficacy of an NcGRA7-deficient parasite as a live attenuated vaccine against *Neospora caninum* infection

2-1. Introduction

Neosporosis is a parasitic protozoan disease with a worldwide distribution (Dubey, 1999). Annually, neosporosis causes losses valuing approximately 1.3 billion US dollars in bovines through stillbirth, abortion, or the birth of weak calves (Reichel et al., 2013). Additionally, *Neospora caninum* infection can result in the delivery of healthy but persistently infected calves, which play a role in the persistence of the parasite through transplacental transmission to the next generation (Hemphill and Müller, 2015). Neosporosis vaccine development has been challenging. An inactivated vaccine (Neoguard®, Intervet International B.V., Boxmeer, the Netherlands) was developed and was commercially available until it was withdrawn from the market because only moderate protection against abortions was observed in field trials (Romero et al., 2004; Weston et al., 2012). Immunization with live tachyzoites showed protective efficacy against fetal death in cattle (Williams et al. 2007) and decreased the abortion rate and transplacental transmission after the infection of heifers before mating (Weber et al. 2013). Another study found that immunization with live tachyzoites before pregnancy partially protected against fetal loss (Rojo-Montejo et al. 2013). For commercially available options, pathogenicity of live tachyzoites is a main obstacle in the development of a commercial vaccine.

Notably, an efficient vaccine against *N. caninum* infection should fulfill the following requirements: prevention of tachyzoite proliferation and dissemination in pregnant cattle (or other animals); prevention or minimization of oocyst shedding by dogs; and prevention of tissue cyst formation in animals that have been infected with oocysts or tissue cysts (to avoid horizontal parasite transmission to final hosts). A vaccine that stimulates both protective cellular immunity and antibody responses should achieve these goals (Hemphill et al., 2006, Williams et al., 2007). A summary of candidate vaccines against *N. caninum* composed of live tachyzoites or different antigens from the

parasite secretory organelles, such as micronemes, rhoptries, and dense granules (GRAs), that were tested in mice was provided by Hemphill and Müller (2015). These vaccine studies exhibit a significant degree of variation in the mouse strain, *Neospora* parasite strain, time elapsed between vaccination and challenge infection, methods of challenge and vaccination, and vaccine adjuvants used by the studies. Additionally, vaccination with live or attenuated *N. caninum* tachyzoites is an efficient method of providing protection, in both mice and cattle, against acute *N. caninum* infection and preventing transplacental parasite transmission.

One strategy for increasing the efficacy and safety of live *N. caninum* vaccines is the disruption of parasite virulence factors. In a previous study, NcGRA7-deficient *N. caninum* (NcGRA7KO) showed lower virulence in mice (Nishikawa et al., 2018). Therefore, the aim of the present study was to evaluate the use of NcGRA7KO as a live attenuated vaccine against *N. caninum* infection in both non-pregnant and pregnant model using BALB/c mice.

2-2. Materials and methods

Ethics statement

This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The study protocol was approved by the Committee on the Ethics of Animal Experiments at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan (permit numbers 21-30, 21-36). To decrease animal suffering, all surgical operations, blood collection, and cervical dislocation were performed under isoflurane anesthesia.

Mice

Female and male BALB/c mice, aged 10–12 weeks old, were bred and housed under specific-pathogen-free conditions in the animal facility of the National Research Center for Protozoan Diseases at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. The mice were initially obtained from Clea Japan (Tokyo, Japan). The animals were kept under standard laboratory conditions

and fed commercial food and water ad libitum on a 12/12-h light/dark cycle at 21 °C under 40% relative humidity.

Parasites and cell cultures

NcGRA7KO, generated by the clustered regularly interspaced short palindromic repeats (CRISPR)-associated gene 9 (CRISPR/CAS9) system (Nishikawa et al., 2018), and a green fluorescent protein (GFP)-expressing Nc1 strain of *N. caninum* (Nc1-GFP) were maintained in African green monkey kidney epithelial (Vero) cells as previously described (Nishikawa et al., 2003; 2008; Leesombun et al., 2016). The parasites were cultured in Eagle's minimum essential medium (Sigma, St. Louis, MO, USA) supplemented with 8% heat-inactivated fetal bovine serum (Nichirei Biosciences, Tokyo, Japan), 100 U/ml penicillin, and 10 mg/ml streptomycin. Parasites and host cell debris were washed with cold phosphate-buffered saline (PBS), and the final pellet resuspended in cold PBS was passed through a 27-gauge needle and a 5.0-µm-pore size filter (Millipore, Bedford, MA, USA). After 72 h, the parasites were washed with PBS by centrifugation at 1,500 ×*g* for 10 min before being filtered again, and the number of parasites was then hemocytometrically counted for each experiment.

Experimental design

To estimate the safety of live vaccines using the two parasite lines (Experiment 1, Table 4), Nc1 and NcGRA7KO, six mice per group were used. Female mice were injected subcutaneously with 0.1 ml of RPMI-1640 medium containing 1×10^5 Nc1 or NcGRA7KO parasites or with RPMI-1640 medium alone as an unvaccinated control. At 45 days post-vaccination (dpv), all mice were challenged with 2×10^6 Nc1-GFP tachyzoites in 0.4 ml/mice, and postmortem examination was performed at 30 days post-challenge infection (dpi; corresponding to 76 dpv). The choice of parasite challenge dose was made on the basis of reports from a previous study (Marugán-Hernández et al., 2011).

For experiments in non-pregnant animals (Experiment 2, Table 1), female mice were injected subcutaneously with a high dose (1×10^5) of NcGRA7KO tachyzoites in a 0.1-ml volume. After 3 weeks, blood was taken from the tail and used for an evaluation of antibody production by indirect enzyme-linked immunosorbent assay (iELISA). Mice lacking seropositivity for parasite-specific antibodies

were excluded from the challenge experiment. The daily bodyweight measurement and clinical score of each dam were recorded from −2 to 20 dpv (mating time starts). Clinical scores were assigned according to the extent of hunching, piloerection, warm-seeking behavior, ptosis, sunken eyes, ataxia, latency of movement, flaccidity, latent touch reflexes, skin and eye reflexes, and belly lying observed in the mice and were represented by a score ranging from 0 (no symptoms) to 10 (complete symptoms), in accordance with previous studies (Abe et al., 2005; Hermes et al., 2008; Fereig et al., 2019). At 45 dpv, all mice were challenged intraperitoneally with 2×10^6 Nc1-GFP tachyzoites, and a postmortem examination was performed at 30 dpi (76 dpv). Brain tissue for measuring parasite burden and blood for use in a serum analysis were collected from all mice. The same protocols were performed on male animals (Experiment 3, Table 4). Animals were sacrificed in accordance with the ARRIVE guidelines 2.0 and the reported study (Talbot et al., 2020).

For experiments in pregnant animals, we used two groups of mice injected subcutaneously with 0.1 ml of RPMI-1640 medium alone (unvaccinated control group) or containing NcGRA7KO parasites (vaccinated group; Experiment 4: 1×10^5 tachyzoites/mouse, Experiment 5: 1×10^4 tachyzoites/mouse) (Table 4). At 3 weeks post-vaccination, blood was collected from the mice for use in an iELISA to estimate the level of antibody production. The mice found to be seronegative were excluded from the experiment and mating procedures. At 1-month post-vaccination, mating procedures were begun. Estrus was synchronized in female animals as previously described (Whitten 1956;1958; Marsden and Bronson, 1964). Male and female mice were co-housed (one female with one male per cage) after 18:00 for 14 h to mate. This mating procedure was repeated for four successive days, and females were checked for vaginal mucoid plugs or swelling at 8:00–9:00 the morning after each mating day, as described previously (Lopez-Perez et al. 2008). The mice with visible vaginal plugs were considered to be in day 0.5 of pregnancy, whereas the mice without a vaginal plug were excluded from the experiment and kept under observation to confirm that they were not pregnant. In Experiment 4, 10 mice were used in mating per group; in Experiment 5, 8–9 mice were used. In both experiments, 6–8 mice from each group were estimated, by observation of the vaginal mucoid plug, to be pregnant. Only 3–6 mice out of the 8 vaginal-plug-positive mice were confirmed to be pregnant by a rise in bodyweight until the start of the challenge experiment at 10 days post-mating, indicating that some presumed pregnant mice may

have aborted or been non-pregnant. On day 10.5 of pregnancy, all confirmed pregnant animals were challenged intraperitoneally with 1×10^5 Nc1-GFP tachyzoites (0.4 ml), and a postmortem examination was performed at 30 days postpartum (dpp) (Fereig et al., 2021).

To evaluate the vaccine efficacy, the survival and clinical score of dams and the survival and number of their offspring were observed daily until 30 dpp. Parasite burdens were then quantified in the brain tissues from all dams and offspring at their time of death or at 30 dpp. The bodyweight of each dam was recorded daily from −2 to 20 dpv with NcGRA7KO and compared with the initial bodyweight of the same mouse at 0 dpv. The average clinical scores of dams were determined by monitoring the clinical signs manifested in each mouse from −2 to 20 dpv (until mating).

iELISA using recombinant NcSAG1 and NcGRA6 antigens to detect N. caninum-specific antibodies

To detect antibodies specific to *N. caninum*, iELISAs were carried out as previously described (Fereig et al., 2020). Briefly, glutathione *S*-transferase (GST)-fused recombinant antigens, NcSAG1-GST (18.2 μM) and NcGRA6-GST (11.2 μM), and GST (19.2 μM) were diluted to a final concentration of 0.1 μM in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6). The wells of the ELISA plate (Nunc, Roskilde, Denmark) were coated with 50 μl of the antigens and incubated overnight at 4 °C. The plates were then blocked with PBS containing 3% skim milk for 1 h at 37 °C. After being washed with PBS containing 0.05% Tween 20, the plates were incubated with serum samples diluted 1:250. The bound antibody was detected by incubating the samples with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Bethyl, Montgomery, TX, USA) diluted 1:8,000 and ABTS [2,2′-azinobis (3-ethylbenzthiazolinesulfonic acid)] (Sigma, St. Louis, MO, USA). The color was allowed to develop at room temperature. The optical density (OD) value was measured by an MTP-500 microplate reader (Corona Electric, Hitachinaka, Japan) at 415 nm. The ELISA results were determined for each sample by subtracting the OD value of GST protein from the OD value of recombinant NcSAG1 or recombinant NcGRA6.

DNA isolation and quantitative (q)PCR to determine the* N. caninum *distribution

DNA was extracted from the brains of dams and their offspring from all experiments. Each organ was thawed in 10 volumes of extraction buffer (0.1 M Tris-HCl [pH 9.0], 1% SDS, 0.1 M NaCl, 1 mM EDTA) and 20 μg/ml proteinase K at 50 °C. The resulting DNA was purified by phenol-chloroform extraction and ethanol precipitation. Parasite DNA was then amplified with *N. caninum* Nc5 gene-specific primers (forward 5′ -ACT GGA GGC ACG CTG AAC AC-3′ and reverse 5′ -AAC AAT GCT TCG CAA GAG GAA-3′ (Nishimura et al., 2015) for the parasites used for vaccination and with Nc1-GFP gene-specific primers (5′ -TGGCCCTGTCCTTTTACCAGA-3′ and reverse 5′ -TCCATGCCATGTGTAATCCCA-3′) for the parasites used for challenge infection. Amplification, data acquisition, and data analysis were performed with the ABI Prism 7900HT sequence detection system (Applied Biosystems, Waltham, Massachusetts, USA), and the cycle threshold (Ct) values were calculated as described previously (Nishimura et al., 2015). Standard curves were constructed using 10-fold serial dilutions of *N. caninum* DNA extracted from 10^5 parasites; thus, each curve ranged from 0.01 to 10,000 parasites. Parasite numbers were calculated from the standard curve.

Statistical analysis

GraphPad Prism 8.3.4 software (GraphPad Software Inc., La Jolla, CA, USA) was used for data analysis. Data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using a Mann–Whitney U test and one-way or two-way analysis of variance (ANOVA) followed by the Tukey-Kramer hoc test for group comparisons. Survival rates and statistical comparisons were assessed using a logrank Mantel–Cox test. Statistically significant differences, defined by p-values of < 0.05, are marked in the figures with asterisks and defined in each corresponding figure legend together with the name of the statistical test that was used.

2-3. Results

Parasite virulence and comparative protective efficacy of high-dose Nc1 and NcGRA7KO vaccination in non-pregnant mice

To confirm the parasite virulence and investigate the protective efficacy of both the parenteral strain Nc1 and NcGRA7KO, I performed Experiment 1 on five experimental groups, as described in Table 4. Animals were vaccinated (infected) with 1×10^5 of either Nc1 or NcGRA7KO tachyzoites or mock-vaccinated with RPMI media, and some groups were later challenged with 2×10^6 Nc1-GFP at 45 dpv. The parasite virulence was compared among the two unchallenged vaccinated groups that were sacrificed at 30 dpv (Groups D and E) and the unvaccinated group (Group A). The % bodyweights of the unchallenged NcGRA7KO-vaccinated mice and the unvaccinated mice were each significantly higher than that of the Nc1-vaccinated mice (Fig. S8A), whereas the clinical scores of the unchallenged NcGRA7KO-vaccinated animals were significantly lower than those of the unchallenged Nc1-vaccinated mice but higher than those of the unvaccinated mice (Fig. S8B). At 30 dpv, there were no deaths observed among any of the unchallenged experimental groups (Fig. S8C). Although the number of parasites in the brains of unchallenged mice vaccinated with NcGRA7KO trended lower than that of unchallenged mice vaccinated with Nc1, this difference is not statistically significant (Fig. S8D). All vaccinated mice were seropositive for *N. caninum* infection at 30 dpv (Fig. S9).

In the challenge experiment conducted on vaccinated mice, the bodyweights of the NcGRA7KO-vaccinated mice were significantly higher than those of the Nc1-vaccinated mice, while the clinical scores of the NcGRA7KO-vaccinated mice were significantly lower than those of the Nc1-vaccinated mice, both before and after challenge (Fig. 7). Additionally, although the survival rate of the challenged NcGRA7KO-vaccinated animals trended higher than that of the challenged mice vaccinated with Nc1, this difference was not statistically significant (Fig. 7C). The number of challenged Nc1-GFP parasites in the brains of NcGRA7KO-vaccinated mice trended lower than that in the brains of the unvaccinated animals, but this difference was not statistically significant either. Notably, the Nc1-GFP parasite number in the mice that had been vaccinated with Nc1 was significantly lower compared with that in the unvaccinated animals (Fig. 7D), suggesting early death of the mice might result in lower parasite number.

Protective efficacy of high-dose and low-dose vaccination with NcGRA7KO in non-pregnant female mice

To expand upon the above results, I performed Experiment 2, as described in Table 1, in which the protective efficacy of both low and high doses (1×10^4 and 1×10^5, respectively) of NcGRA7KO parasite were assessed in a non-pregnant mouse model. The bodyweight percentage was not different between the vaccinated and unvaccinated animals until 63–68 dpv (19–25 dpi), when the bodyweight of the low-dose-vaccinated group was significantly higher than that of unvaccinated animals (Fig. 8A). The clinical scores of unvaccinated animals were significantly higher than those of vaccinated mice from 50–72 dpv (Fig. 8B). The survival rate of high-dose-vaccinated group (3/6, 50.0%) trended lower than those of the low-dose-vaccinated (6/6, 100.0%) and unvaccinated (5/6, 83.3%) groups, but these differences were not statistically significant (Fig. 8C). The amounts of parasite DNA in the vaccinated mice trended lower than that in the unvaccinated mice for both vaccination parasites and Nc1-GFP challenge parasites, but these differences were not statistically significant either (Figs. 8D, E). All mice used in Experiment 2 were seropositive for *N. caninum* infection at 25 dpv (Fig. S10A).

Protective efficacy of high-dose and low-dose vaccination with NcGRA7KO in male mice

Experiment 2 described above was repeated in male mice (Experiment 3 in Table 4). Before the challenge infection, there were no significant differences in the bodyweight change between the low-dose-vaccinated and unvaccinated animals, whereas the high-dose-vaccinated animals showed a significant decrease in the bodyweight % as compared with the unvaccinated animals (Fig. 9A). After the challenge infection, the bodyweight of the unvaccinated animals was significantly decreased compared with the low-dose-vaccinated animals at 66–76 dpv (20–30 dpi) (Fig. 9A). Mice that received the low vaccination dose were the healthiest among all the experimental groups; the clinical scores of the vaccinated animals were significantly lower than those of the unvaccinated animals at 48–73 dpv (3–27 dpi) (Fig. 9B).

No significant difference was observed in the survival rate between the unvaccinated animals (6/6, 100%) and either the high-dose-vaccinated (5/6, 83.3%) or low-dose-vaccinated (6/6, 100.0%) animals (Fig. 9C). The amount of DNA from the vaccination parasites in the vaccinated animals trended lower than that in the unvaccinated ones, but this difference did not reach statistical significance (Fig. 9D). In contrast, both vaccination groups had a significantly lower amount of DNA from the Nc1-GFP

(challenge) parasites compared with the unvaccinated animals (Fig.9E). All mice used in Experiment 3 were seropositive for *N. caninum* infection at 25 dpv (Fig. S10B).

Protective efficacy of high-dose vaccination with NcGRA7KO parasites in pregnant mice

To evaluate the effect of the NcGRA7KO vaccine in pregnant animals, pregnant mice were vaccinated subcutaneously with 1×10^5 NcGRA7KO tachyzoites and then challenged with Nc1-GFP at day 10.5 of pregnancy (Experiment 4 in Table 4). The bodyweight change was not different between the unvaccinated and vaccinated dams, except at 23 dpv (Fig. S11A). Additionally, there was no significant difference in the clinical scores between unvaccinated and vaccinated groups before the challenge infection, except for at 12 and 13 dpv (Fig. S11B). Before mating, I confirmed the antibody production in the experimental mice by iELISA on blood samples collected at 7 and 20 dpv; the one mouse with a seronegative result was excluded from the mating experiment (Fig. S11C, D, and E). Pregnancy was estimated by observation of a vaginal mucoid plug and was confirmed by a daily rise in bodyweight until 14 days post-mating [Unvaccinated (5/8), NcGRA7KO-vaccinated (3/8)] (Fig. S12, Tables 5 and 6).

The survival rate of the NcGRA7KO-infected dams (3/3, 100%) was the same as that of the unvaccinated dams (5/5, 100%) (Fig. 10A). There was no significant difference in the amount of DNA from the vaccination or challenge parasites in the brains of the vaccinated and unvaccinated animals (Figs. 10B and C); the mean number of offspring per litter was not different between the experimental groups either (Table 5). The fertility rates were as follows: unvaccinated, 5/8 (62.5%); NcGRA7KO-vaccinated, 3/8 (37.5%).

I also investigated the early mortality of offspring from the experimental mice (Figure 4D). The offspring survival rate at 30 days post-birth of the NcGRA7KO-vaccinated group (3/11, 27.8%) was not different from that of the unvaccinated group (9/23, 39.1%) (Fig. 10D, Table 5). However, the unvaccinated group had a lower birth rate and a higher rate of abnormal pregnancy outcomes (stillbirth, delayed birth, or no birth) compared with the vaccinated group, which had no abnormal pregnancy outcomes (Table 5). The amount of DNA from Nc1-GFP parasites in the brains of offspring was significantly lower in the vaccinated group than in the unvaccinated group (Fig. 10E). The vertical

transmission rate was monitored, and parasite DNA was detected in the brains of 17/23 (73.9%) offspring from unvaccinated dams, but parasite DNA was detected in the brains of only (2/7, 28.5%) offspring from NcGRA7KO-vaccinated dams (Table 5).

These results show that, despite the lack of significant difference in the survival rates of offspring between the experimental groups, there was significantly less vertical transmission of the challenge parasites to the offspring of vaccinated dams than to the offspring of unvaccinated ones. Moreover, some abnormal pregnancy outcomes, such as no birth, stillbirth, and delayed birth, were observed in the unvaccinated dams (Table 5). Thus, a high-dose (1×10^5 tachyzoites) with live NcGRA7KO tachyzoites may affect mouse conception and offspring survival, but it fails to provide adequate protection to mice against challenge infection with *N. caninum*.

Protective efficacy of low-dose vaccination with NcGRA7KO parasites in pregnant mice

To assess the vaccine efficacy of a low-dose vaccination with 1×10^4 tachyzoites from NcGRA7KO, Experiment 5 was conducted as shown in Table 4. The bodyweight change was not different between the unvaccinated and vaccinated dams (Fig. S13A). Additionally, there was no significant difference in the clinical scores between unvaccinated and vaccinated animals through 25 dpv, except for at 23 dpv (Fig. S13B). Before mating, we confirmed the antibody production by iELISA using blood samples collected at 22 dpv; all vaccinated mice were seropositive for *N. caninum* after vaccination (Fig. S13C). Pregnancy was estimated by observation of a vaginal mucoid plug and was confirmed by a daily rise in the bodyweight until 17 days post-mating (Fig. S14).

The survival rate of the NcGRA7KO-infected dams (4/4, 100%) was the same as that of the unvaccinated dams (100%, 5/5) (Fig. 11A). The amount of vaccination parasite DNA in the brain was not significantly different between the unvaccinated and vaccinated dams (Fig. 11B), whereas there was significantly less challenge parasite (Nc1-GFP) DNA in the brains of the vaccinated animals (Fig. 11C). There was no significant difference in the mean number of offspring per litter between the experimental groups (Table 6). The fertility rates were as follows: unvaccinated, 5/6 (83.3%); NcGRA7KO-vaccinated, 4/8 (50.0%).

I also investigated the early mortality of offspring from the experimental mice (Fig. 11D). The offspring survival rate at 30 days post-birth for the NcGRA7KO-vaccinated group (18/24, 75.0%) was significantly higher than that for the unvaccinated group (2/29, 6.9%) (Fig. 11D, Table 6). The amount of DNA from Nc1-GFP (challenge) parasites in brains of offspring was significantly lower in the NcGRA7KO-vaccinated group than in the unvaccinated group (Fig. 11E). The vertical transmission rate was monitored, and parasite DNA was detected in the brains of 19/23 (82.6%) offspring from unvaccinated dams, but parasite DNA was detected in the brains of a significantly lower percentage (4/22, 18.2%) of offspring from NcGRA7KO-vaccinated dams (Table 6). These results indicate that low-dose vaccination with live tachyzoites from NcGRA7KO parasites has protective efficacy against *N. caninum* infection, which is confirmed by the lower vertical transmission rate and higher survival rate in the offspring of vaccinated dams than in the offspring of unvaccinated ones.

2-4. Discussion

An effective vaccine against neosporosis should aim not only to prevent abortion or vertical transmission in infected animals but also to prevent the subsequent infection of uninfected animals (Innes et al., 2002). Multiple types of live vaccines against neosporosis have been developed, in either cattle or mouse models, including less virulent strains, attenuated strains, or genetically modified strains (Monney et al., 2011).

Toxoplasma gondii, an intracellular protozoan parasite that is closely related to *N. caninum*, can induce protective immunity against *N. caninum* infection. For example, the *Toxoplasma* microneme protein-deficient line, TgMic1-3KO, induced protection against *N. caninum* infection (Penarete-Vargas et al., 2010). However, the first commercial vaccine for toxoplasmosis, Toxovax™, which is composed of live attenuated *Toxoplasma* tachyzoites and is used mainly for sheep in most European countries and New Zealand, is a non-persistent strain of *T. gondii*, S48 (Buxton, 1993). This vaccine does not induce any cross-over protection against *N. caninum* infection (Innes et al., 2001a; 2001b).

In experimental animal models, protective efficacy has been induced by immunization with live *N. caninum* tachyzoites. The immunization of mice with naturally attenuated Nc-Spain 1 H tachyzoites induced protective immunity against neosporosis (Rojo-Montejo et al., 2012). In cattle, immunization

with Nc-Spain 1H live tachyzoites also induced a protective immune response, which partially reduced the rates of *N. caninum*-associated abortions and transplacental transmission of the parasite (Rojo-Montejo et al., 2013). Another study reported the inoculation of pregnant heifers with 6.25×10^7 live tachyzoites from the Nc-6 Argentina isolate of *N. caninum*, which provided partial protection against transplacental transmission following challenge in early gestation (Hecker et al., 2013). The vaccination of heifers with live Nc-Nowra tachyzoites before breeding induces protection in 55.6% to 85.2% of vaccinated animals, depending on the immunization route (subcutaneous or intravenous) and vaccine strain growth conditions; this vaccination also reduced the rates of abortion and presence of parasite DNA in calves as determined by PCR (Weber et al., 2013). Another study reported that the immunization of cattle with live tachyzoites from the Nc-Nowra strain before pregnancy conferred 100% protection from fetal death, whereas vaccination with parasite lysate failed to provide any protection (Williams et al., 2007). Thus, the use of a live attenuated vaccine based on gene-editing technology has great potential for practical application against neosporosis.

In my study, the comparative efficacy of vaccination with Nc1 or NcGRA7KO was evaluated in non-pregnant mice and that of vaccination with NcGRA7KO was evaluated in non-pregnant and pregnant mouse models. Vaccination with NcGRA7KO was safer than vaccination with Nc1, as demonstrated by the higher survival rate and lower number of brain parasites in vaccinated mice (Figure 1C). The vertical transmission rate in mice that received a low-dose vaccination with live tachyzoites from NcGRA7KO was 18.2%, which is significantly lower than this rate in unvaccinated mice (82.6%) (Table 6). These data are similar to the results of vaccination with the transgenic strain Nc-1 SAG4c2.1 (vertical transmission rate: 10.8%) (Marugan-Hernandez et al., 2011). Our findings support the further assessment of NcGRA7KO parasites as a candidate live vaccine against infection with *N. caninum*.

2-5. Summary

Live vaccination is the most protective method against bovine neosporosis, the major cause of bovine abortion globally. Here, parenteral stain Nc1 and a NcGRA7-deficient *Neospora caninum* (NcGRA7KO), which is less virulent in mice, I evaluated as potential live vaccines. Pregnant and non-pregnant BALB/c mice were subcutaneously inoculated with high (1×10^5) or low (1×10^4) doses of

tachyzoites. At both doses, NcGRA7KO-vaccinated animals showed higher bodyweight gain and fewer clinical signs than unvaccinated animals against the challenge with Nc1-GFP. Moreover, the parasite burden of Nc1-GFP in the brain of vaccinated animals was lower than that of unvaccinated animals. For high-dose vaccination in pregnant mouse model against the challenge with Nc1-GFP, there was no significant difference in the survival rates of offspring between vaccinated (27.8%) and unvaccinated dams (39.1%), whereas for low-dose vaccination, the offspring from vaccinated dams had a significantly higher survival rate (75.0%) than those from unvaccinated dams (6.9%). The amount of challenge (Nc1-GFP) parasite DNA in the brain of offspring from vaccinated dams was significantly lower than that of offspring from unvaccinated dams. These results suggest that NcGRA7KO parasites have potential for use as a live vaccine against *N. caninum* infection.

Table 4. Experimental design for vaccination and challenge with *N. caninum* in different mouse models

	Mouse group	Mouse model	Parasite vaccination dose	Number of animals used / group	Challenge by Nc1-GFP tachyzoites/mouse (dose)	Fertility rate (%)
Experiment 1	Group A (unvaccinated)	Non - pregnant	Not vaccinated	6	Yes, (2×10^6)	
	Group B (Nc1-vaccinated)		1×10^5	6		
	Group C (NcGRA7KO-vaccinated)		1×10^5	6		
	Group D (Nc1-vaccinated)		1×10^5	6	No challenge	
	Group E (NcGRA7KO-vaccinated)		1×10^5	6		
Experiment 2	Group A (unvaccinated)	Non - pregnant	Not vaccinated	6	Yes, (2×10^6)	
	Group B (NcGRA7KO-vaccinated)		1×10^4	6		
	Group C (NcGRA7KO-vaccinated)		1×10^5	6		
Experiment 3	Group A (unvaccinated)	Male model	Not vaccinated	6	Yes, (2×10^6)	
	Group B (NcGRA7KO-vaccinated)		1×10^4	6		
	Group C (NcGRA7KO-vaccinated)		1×10^5	6		
Experiment 4	Group A (unvaccinated)	Pregnant	Not vaccinated	10	Yes, (1×10^5)	5/8 (62.5%)
	Group B (NcGRA7KO-vaccinated)		1×10^5	10		3/8, (37.5%)
Experiment 5	Group A (unvaccinated)	Pregnant	Not vaccinated	8	Yes, (1×10^5)	5/6 (83.3%)
	Group B (NcGRA7KO-vaccinated)		1×10^4	9		4/8, (50.0%)

The numbers of mice, vaccination and challenge doses, and timing of mice sacrifice for each performed experiment.
In Experiment 1, 6 animals per group were vaccinated with Nc1 or NcGRA7KO parasites or were injected with RPMI-1640 medium as an unvaccinated control. Groups A–C were challenged, and groups D and E were not challenged. Daily observations were performed until 76 days post-vaccination (dpv; 30 days post-

challenge infection [dpi]). Brain samples were collected from all animals at the time of death or at 76 dpv for use in quantitative (q)PCR to determine the *N. caninum* distribution.

In Experiments 2 and 3, 6 animals were used for each group, and all animals were vaccinated with a high or a low dose of NcGRA7KO parasites. Animals were sacrificed in accordance with the animal ARRIVE guidelines; these mice are indicated by black-colored symbols in Figures 3 and 4. Daily observations were performed until 76 dpv (30 dpi). Brain samples were collected from all mice at their time of death or scarification at 76 dpv for use in qPCR to determine the *N. caninum* distribution.

In all pregnant model experiments (Experiments 4 and 5), animals were vaccinated with NcGRA7KO parasites; 8–10 mice were used for mating procedures, and 6–8 mice were estimated as pregnant by observation of a vaginal mucoid plug. Among them, 3–6 mice gave birth. The presumed pregnant mice were challenged with Nc1-GFP (1×10^5 parasites/mouse) or were injected with RPMI-1640 medium as an unvaccinated control at day 10.5 of pregnancy (E10.5). Daily observations were performed until 30 days postpartum. Brain samples were collected from all dams and offspring at the time of death or at 30 days postpartum for use in qPCR to determine the *N. caninum* distribution.

Table 5. Survival rates of offspring (30 days postpartum) following high-dose live tachyzoite vaccination (10^5 tachyzoites/dam) (Experiment 4).

Groups	**Number of litters (no. used mice), fertility rate %**	**Birth rate (%)**	**Abnormal pregnancy**			**Mean number of offspring/litter (SD)**	**No. of surviving offspring/no. of offspring in each litter** **(Number of PCR positive offspring in each litter/number of analyzed samples in each litter)**	**Total no. of surviving offspring /total no. of offspring (Survival rate %)** **(Total Number of PCR positive offspring / total number of analyzed samples, %)**
			No birth	**Still birth**	**Delayed birth**			
Unvaccinated	, 62.5%) 8 (5	4/5 (80%)	(1/5, 20%)	(1/5, 20%)	(1/5, 20%)	4.6 (4.2)	0/0, 0/2, 0/11, 4/4, 5/6 (0/0), (2/2), (6/11), (1/4), (2/6)	9/23 (39.1%) (17/23, 73.9%)
NcGRA7KO-vaccinated	, 37.5%) 8 (3	3/3 (100%)	(0/3, 0%)	(0/3, 0%)	(0/3, 0%)	3.6 (1.1)	0/5, 0/3 , 3/3 (0/3), (1/1), (0/3)	3/11 (27.8 %) (2/7, 28.5%)

The mean number of offspring/litters in all experimental groups was analyzed with a Student's *t*-test, but no significant differences among groups were observed. The fertility rate was analyzed with a Fisher's test, but no significant differences among groups were observed. One mouse in the unvaccinated group did not give birth; its litter is expressed as "0/0". Some offspring could not be obtained owing to the cannibalistic behavior of dams, specifically 4 out of 11 pups from the NcGRA7KO-vaccinated group. The survival rates of offspring were analyzed with a logrank Mantel–Cox test, but no significant differences among groups were observed. The vertical transmission rate (PCR-positive offspring/total number of analyzed samples/group) of the challenged parasites (Nc1-GFP) was monitored in each group; the difference between the NcGRA7KO-vaccinated group and the unvaccinated group was assessed with a Fisher's exact test, but it was found to lack statistical significance ($p = 0.0680$). The offspring survival rates at 30 days postpartum were analyzed with a logrank Mantel–Cox test against unvaccinated mice; SD, standard deviation.

Table 6. Survival rates of offspring (30 days postpartum) following low-dose live tachyzoite vaccination (10^4 tachyzoites/dam) (Experiment 5)

Groups	**Number of litters (no. of used mice) , fertility rate)**	**Mean number of offspring/litter (SD)**	**No. of surviving offspring /no. of offspring in each litter (Number of PCR positive offspring in each litter / number of analyzed samples in each litter)**	**Total no. of surviving offspring /total no. of offspring (%) (Total number of PCR positive offspring / total number of analyzed samples, %)**
Unvaccinated	, 83.3%) 6 (5	5.8 (3.0)	0/7, 0/1, 0/9, 2/7, 0/5 (4/5 ,0/1, 5/7, 6/6, 4/4)	2/29 (6.9%) (19/23, 82.6%)
NcGRA7KO-vaccinated	, 50.0%)84 (	6.0 (1.8)	4/4, 5/7, 8/8, 1/5 (0/4 ,2/3, 2/7, 0/8)	18/24 (75.0%) * (4/22, 18.2%) #

The mean number of offspring/litters in all experimental groups was analyzed with a Student's *t*-test, but no significant differences between the unvaccinated and vaccinated groups were observed. The fertility rate was analyzed with a Fisher's test, but no significant differences among groups were observed. Some offspring could not be obtained owing to the cannibalistic behavior of dams, specifically 6 out of 29 pups from the unvaccinated dam group and 2 out of 24 pups from the NcGRA7KO-vaccinated dams group. The survival rates of offspring were analyzed with a logrank Mantel–Cox test, and a significant difference was observed between the offspring of NcGRA7KO-vaccinated dams and those of unvaccinated dams ($p < 0.0001$). The vertical transmission rate (PCR-positive offspring/total number of analyzed samples/group) of the challenged parasites (Nc1-GFP) w s monitored in each group; a statistically significance difference was observed between the NcGRA7KO-vaccinated group and the unvaccinated group, as analyzed with a Fisher's exact test ($p < 0.0001$); SD, standard deviation.

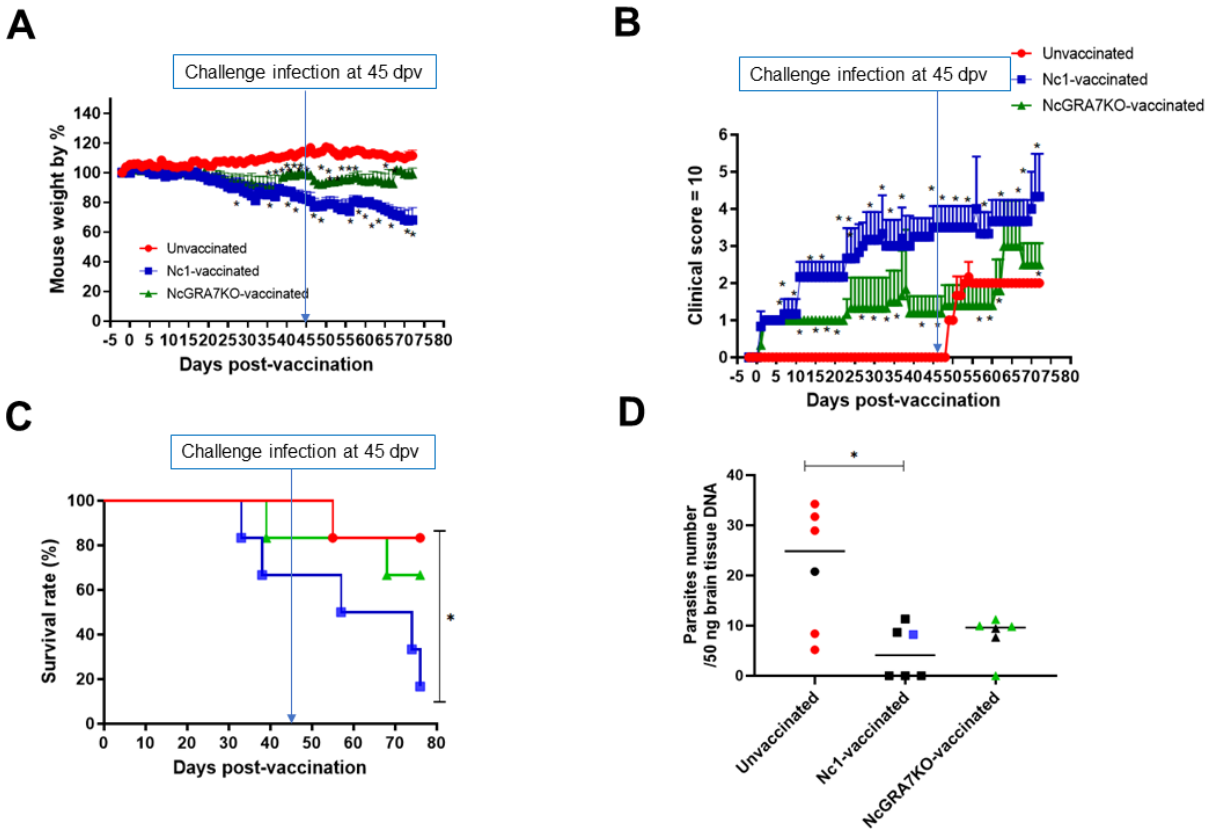


Fig. 7. **Protective efficacy of live parasite vaccination against challenge infection with *N. caninum* in non-pregnant female mice**. Mice were vaccinated with *N. caninum* tachyzoites (1×10^5) of parental strain Nc1 or NcGRA7-deficient parasite (NcGRA7KO) or were injected with RPMI-1640 medium as an unvaccinated control. These mice were then challenged with 2×10^6 Nc1-GFP at 45 days post-vaccination (dpv) and were kept under observation for 1 month (30 days post-challenge infection [dpi]). **(A)** The bodyweight percentage (%) was calculated from the initial bodyweight at day 0 (vaccination day) and the daily bodyweight up until 76 dpv (30 dpi). Values are presented as the means ± SD. Unvaccinated mice, n = 6; Nc1-vaccinated mice, n = 6; NcGRA7KO-vaccinated mice, n = 6. Differences from the unvaccinated animals were analyzed for statistical significance by a two-way ANOVA plus Tukey-Kramer post hoc test; $*p < 0.05$. **(B)** Clinical scores of the mice during *N. caninum* vaccination and challenge infection. Values are presented as the means ± SD. Differences from the unvaccinated group were analyzed by a two-way ANOVA plus Tukey-Kramer post hoc test; $*p < 0.05$. **(C)** Kaplan Meier survival curves through 76 dpv were calculated for all groups (Survival rates: Unvaccinated, 5/6, 83.3%; Nc1, 1/6, 16.67%; NcGRA7KO, 4/6, 66.67%). The statistical significance of differences was analyzed with a logrank Mantel–Cox test; $*p < 0.05$. **(D)** Parasite burdens in the brains of female mice at 30 dpi (76 dpv). Values are the number of parasites in 50 ng of brain tissue DNA. Black symbols represent animals that died were euthanized owing to infection severity in accordance with the ARRIVE guidelines . The statistical significance of differences was analyzed with a one-way ANOVA followed by Tukey-Kramer post hoc test; $*p < 0.05$.

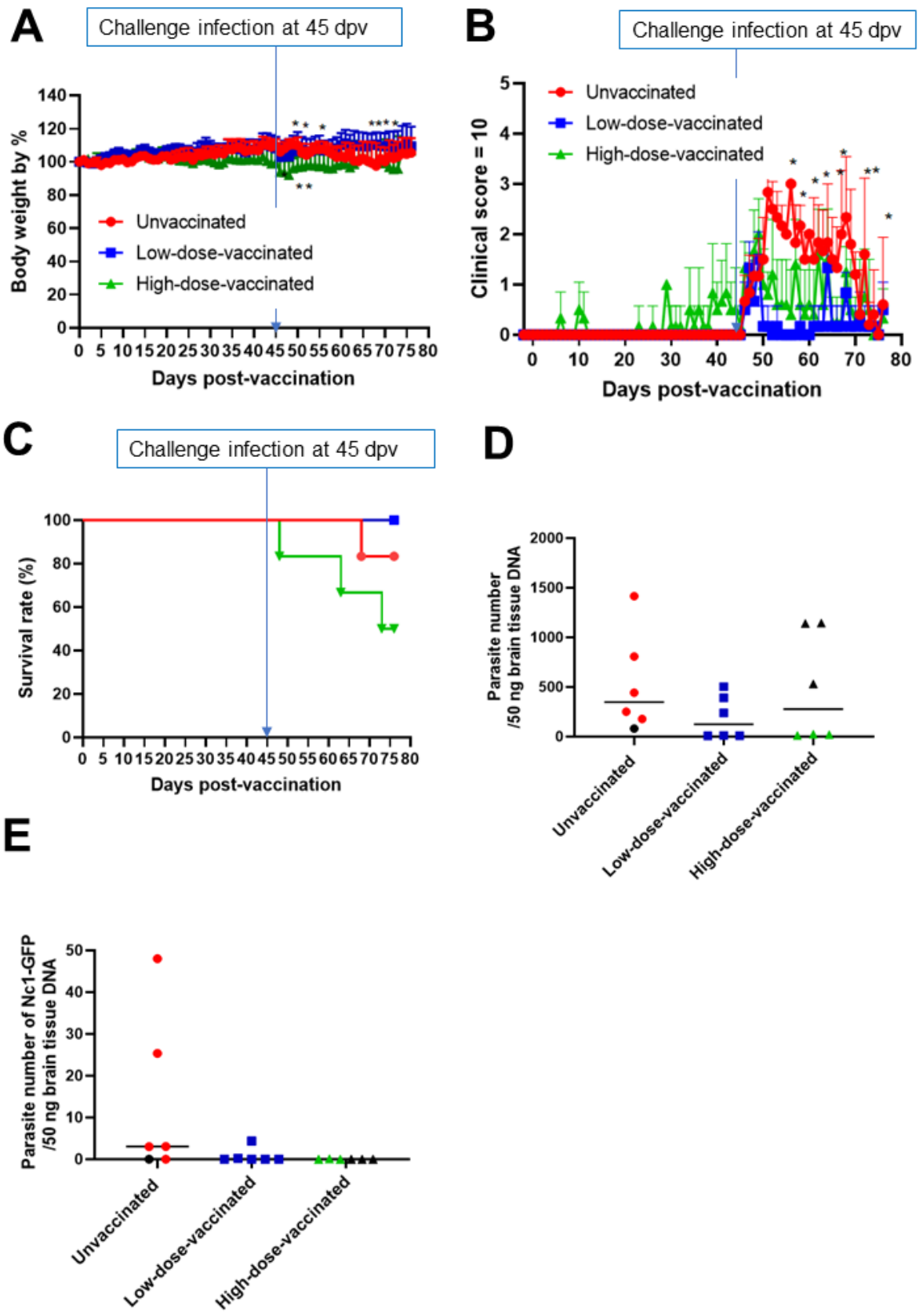


Fig. 8. Protective efficacy of live parasite vaccination with low and high doses against challenge infection with *N. caninum* in non-pregnant female mice. Mice were vaccinated subcutaneously with low or high doses (1×10^4 or 1×10^5, respectively) of live *N. caninum* tachyzoites from NcGRA7-deficient

parasites (NcGRA7KO) or were injected with RPMI-1640 medium as an unvaccinated control. All mice were then challenged intraperitoneally with 2×10^6 Nc1-GFP at 45 days post-vaccination (dpv) and were observed for 30 days post-challenge infection (dpi). **(A)** The bodyweight percentage (%) was calculated from the initial bodyweight at day 0 (vaccination day) and the daily bodyweight up until 76 dpv (30 dpi); values are presented as the means ± SD. Unvaccinated mice, n = 6; Low-dose-vaccinated mice, n = 6. High-dose-vaccinated mice, n = 6. The statistical significance of differences from the unvaccinated animals was analyzed by a two-way ANOVA plus Tukey's Kramer post hoc test; $*p < 0.05$. **(B)** Clinical scores of the mice during *N. caninum* vaccination and challenge infection. Values are presented as the means ± SD. The statistical significance of differences from the unvaccinated group was analyzed by a two-way ANOVA plus Tukey-Kramer post hoc test; $*p < 0.05$. **(C)** Kaplan Meier survival curves through 76 dpv (30 dpi) was calculated for all groups (Survival rates: Unvaccinated, 5/6, 83.3%; Low-dose-vaccinated group, 6/6, 100%; High-dose-vaccinated group, 3/6, 50.0%). The statistical significance of differences in survival rates was analyzed with a logrank Mantel–Cox test, but no differences were identified as significant. **(D)** Parasite burdens in the brains of female mice. Parasite burdens (vaccination parasites) were measured in the brains of mice at their time of sacrifice owing to disease severity or on 76 dpv (30 dpi). Values are the number of parasites in 50 ng of brain tissue DNA. Black symbols represent animals that were sacrificed owing to disease severity in accordance with the ARRIVE guidelines. Unvaccinated mice, n = 6; Low-dose-vaccinated mice, n = 6; High-dose-vaccinated mice, n = 6. The statistical significance of differences was analyzed with a one-way ANOVA followed by Tukey-Kramer post hoc test, but no differences were identified as significant. **(E)** Parasite burdens (challenge parasite; Nc1-GFP) were measured in the brains of female mice at their time of sacrifice owing to disease severity or on 76 dpv (30 dpi). Values are the number of parasites in 50 ng of brain tissue DNA. Unvaccinated mice, n = 6; Low-dose-vaccinated mice, n = 6; High-dose-vaccinated mice, n = 6. Black symbols represent animals that were sacrificed owing to disease severity in accordance with the ARRIVE guidelines. The statistical significance of differences was analyzed with a one-way ANOVA followed by Tukey-Kramer post hoc test, but no differences were identified as significant.

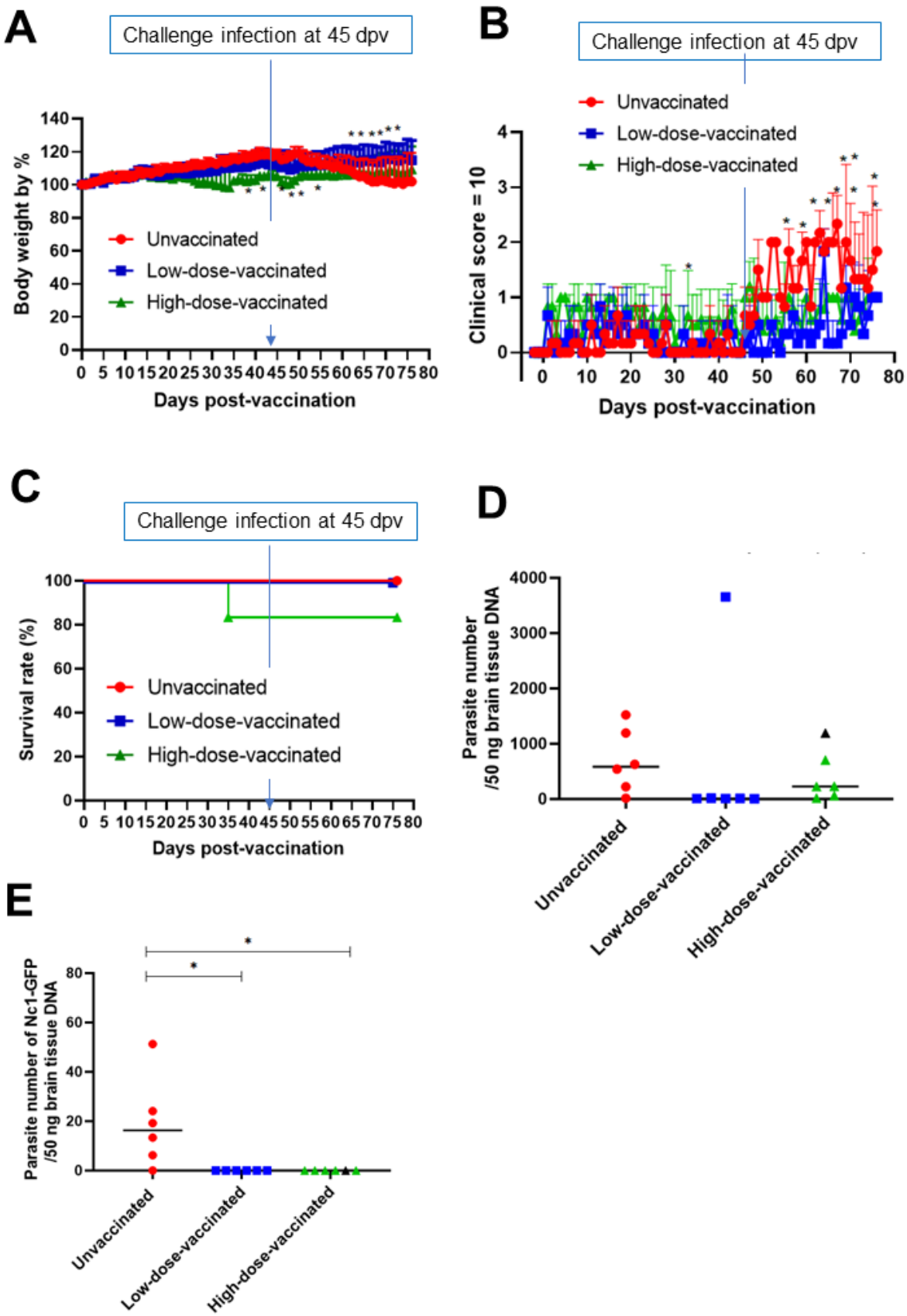


Fig. 9. **Protective efficacy of live parasite vaccination against challenge infection with *N. caninum* in male mice**. Mice were vaccinated subcutaneously with low or high doses (1×10^4 or 1×10^5,

respectively) of live *N. caninum* tachyzoites from NcGRA7-deficient parasites (NcGRA7KO) or were injected subcutaneously with RPMI-1640 medium as an unvaccinated control. All mice were then challenged intraperitoneally with 2×10^6 Nc1-GFP at 45 days post-vaccination (dpv). **(A)** The bodyweight percentage (%) was calculated from the initial bodyweight at day 0 (vaccination day) and the daily bodyweight up until 76 dpv (30 dpi); values are presented as the means ± SD. Unvaccinated mice, n = 6; Low-dose-vaccinated mice, n = 6; High-dose-vaccinated mice, n = 6. The statistical significance of differences from the unvaccinated animals was analyzed by a two-way ANOVA plus Tukey's Kramer post hoc test; $*p < 0.05$. **(B)** Clinical scores of the mice during *N. caninum* vaccination and challenge infection. Values are presented as the means ± SD of the clinical scores of the mice during *N. caninum* challenge infection. The statistical significance of differences from the unvaccinated group was analyzed by a two-way ANOVA plus Tukey-Kramer post hoc test; $*p < 0.05$. **(C)** Kaplan Meier survival curves through 76 dpv were calculated for all groups (Survival rates: Unvaccinated, 6/6, 100.0%; Low-dose-vaccinated group, 6/6, 100%; High-dose-vaccinated group, 5/6, 83.3%). The statistical significance of differences in survival rates was analyzed with a logrank Mantel–Cox test, but no differences were identified as significant. **(D)** Parasite burdens (vaccination parasites) were measured in the brains of male mice at their time of sacrifice owing to disease severity or on 76 dpv (30 dpi). Values are the number of parasites in 50 ng of brain tissue DNA. Unvaccinated mice, n = 6; Low-dose-vaccinated mice, n = 6; High-dose-vaccinated mice, n = 6. Black symbols represent animals that were sacrificed owing to disease severity in accordance with the ARRIVE guidelines. The statistical significance of differences was analyzed with a one-way ANOVA followed by Tukey-Kramer post hoc test, but no differences were identified as significant. **(E)** Parasite burdens (challenge parasite; Nc1-GFP) were measured in the brains of male mice at their time of sacrifice owing to disease severity or on 76 dpv (30 dpi). Values are the number of parasites in 50 ng of brain tissue DNA. Unvaccinated mice, n = 6; Low-dose-vaccinated mice, n = 6; High-dose-vaccinated mice, n = 6. Black symbols represent animals that were sacrificed owing to disease severity in accordance with the ARRIVE guidelines. The statistical significance of differences was analyzed with a one-way ANOVA followed by Tukey-Kramer post hoc test; $*p < 0.05$.

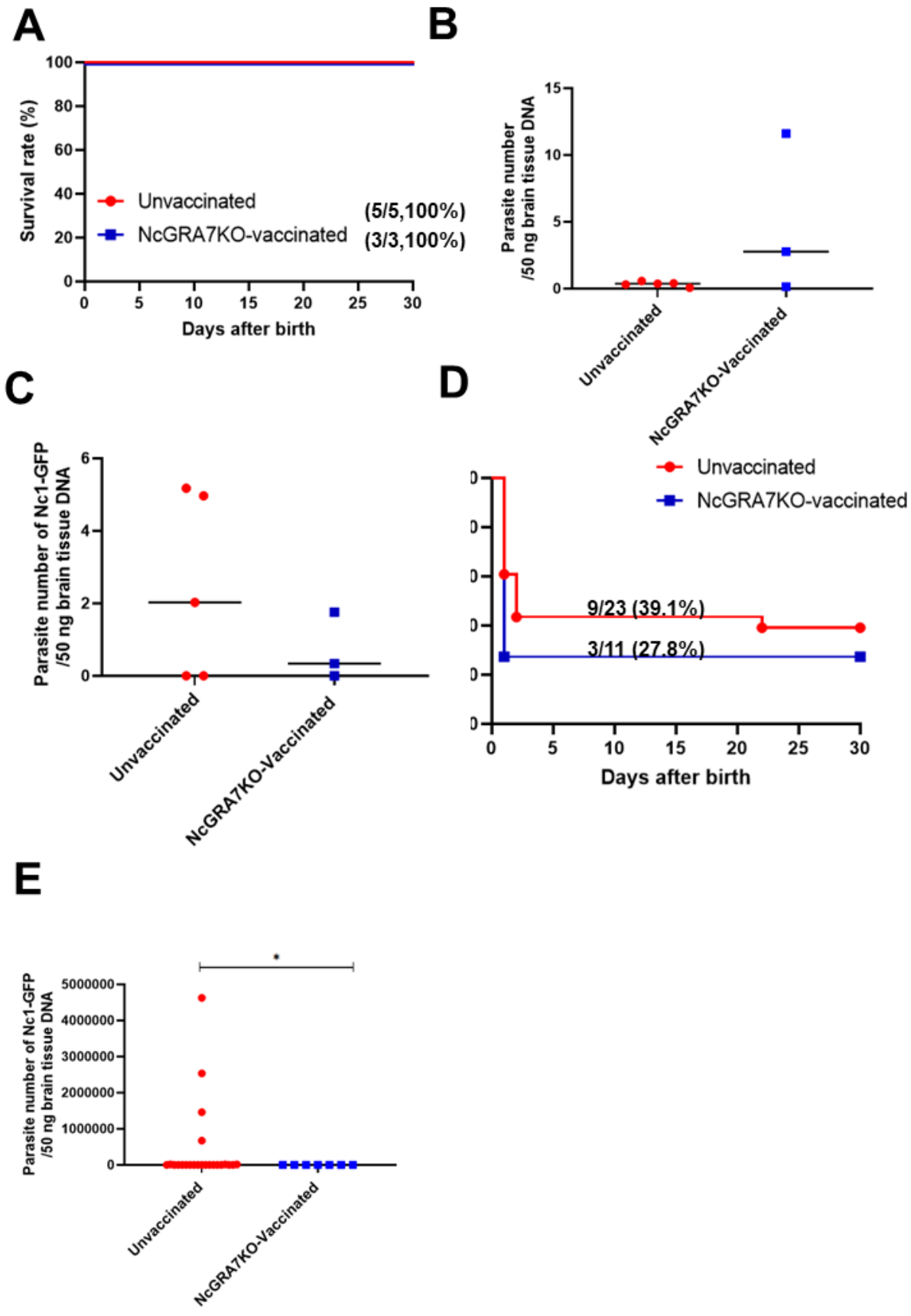

A
Survival rate (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
Days after birth
Unvaccinated
NcGRA7KO-vaccinated
(5/5,100%)
(3/3,100%)
B
Parasite number /50 ng brain tissue DNA
15
10
5
0
Unvaccinated
NcGRA7KO-Vaccinated
C
Parasite number of Nc1-GFP /50 ng brain tissue DNA
6
4
2
0
Unvaccinated
NcGRA7KO-Vaccinated
D
Unvaccinated
NcGRA7KO-vaccinated
9/23 (39.1%)
3/11 (27.8%)
0
5
10
15
20
25
30
Days after birth
E
Parasite number of Nc1-GFP /50 ng brain tissue DNA
*
5000000
4000000
3000000
2000000
1000000
0
Unvaccinated
NcGRA7KO-Vaccinated

Fig. 10. **Efficacy of high-dose live vaccination in pregnant mice**. Mice were vaccinated with 1×10^5 *N. caninum* tachyzoites from NcGRA7-deficient parasites or were injected with RPMI medium as an unvaccinated control. All the mice were then challenged intraperitoneally with 1×10^5 Nc1-GFP at day 10.5 of pregnancy. **(A)** Kaplan Meier survival curves through day 30 postpartum (dpp) were calculated for the unvaccinated and NcGRA7KO-vaccinated dam groups (Survival rates: Unvaccinated, 5/5, 100.0%; NcGRA7KO, 3/3, 100.0%). The statistical significance of differences in survival rates was analyzed with a logrank Mantel–Cox test, but no differences were identified as significant. **(B)** Parasite burdens (vaccination parasites) were measured in the brains of female mice at 30 dpp. Values are the number of parasites in 50 ng of brain tissue DNA. (Unvaccinated, n = 5; NcGRA7KO-vaccinated, n = 3). The statistical significance of differences was analyzed with a one-way ANOVA followed by Tukey-Kramer post hoc test, but no differences were identified as significant. (**C**) Parasite burdens (challenge parasites; Nc1-GFP) were measured in the brains of dams at 30 dpp. Values are the number of parasites in 50 ng of brain tissue DNA. (Unvaccinated, n = 5; NcGRA7KO-vaccinated, n = 3). The statistical significance of differences was analyzed with a one-way ANOVA followed by Tukey-Kramer post hoc test, but no differences were identified as significant. **(D)** Survival curves through 30 dpp of offspring from unvaccinated or NcGRA7KO-vaccinated dams (Survival rates: offspring from unvaccinated dams, 9/23, 39.1%; offspring from NcGRA7KO-vaccinated dams, 3/11, 27.8%). The statistical significance of differences in survival rates was analyzed with a logrank Mantel–Cox test, but no differences were identified as significant. (**E**) Parasite burdens (challenge parasites; Nc1-GFP) were measured in the brains of offspring at their time of their death or at 30 dpp. Values are the number of parasites in 50 ng of brain tissue DNA. (offspring from unvaccinated dams, n = 23; offspring from NcGRA7KO-vaccinated, n = 7). The statistical significance of differences was analyzed with a Mann Whitney U test; $*p < 0.05$.

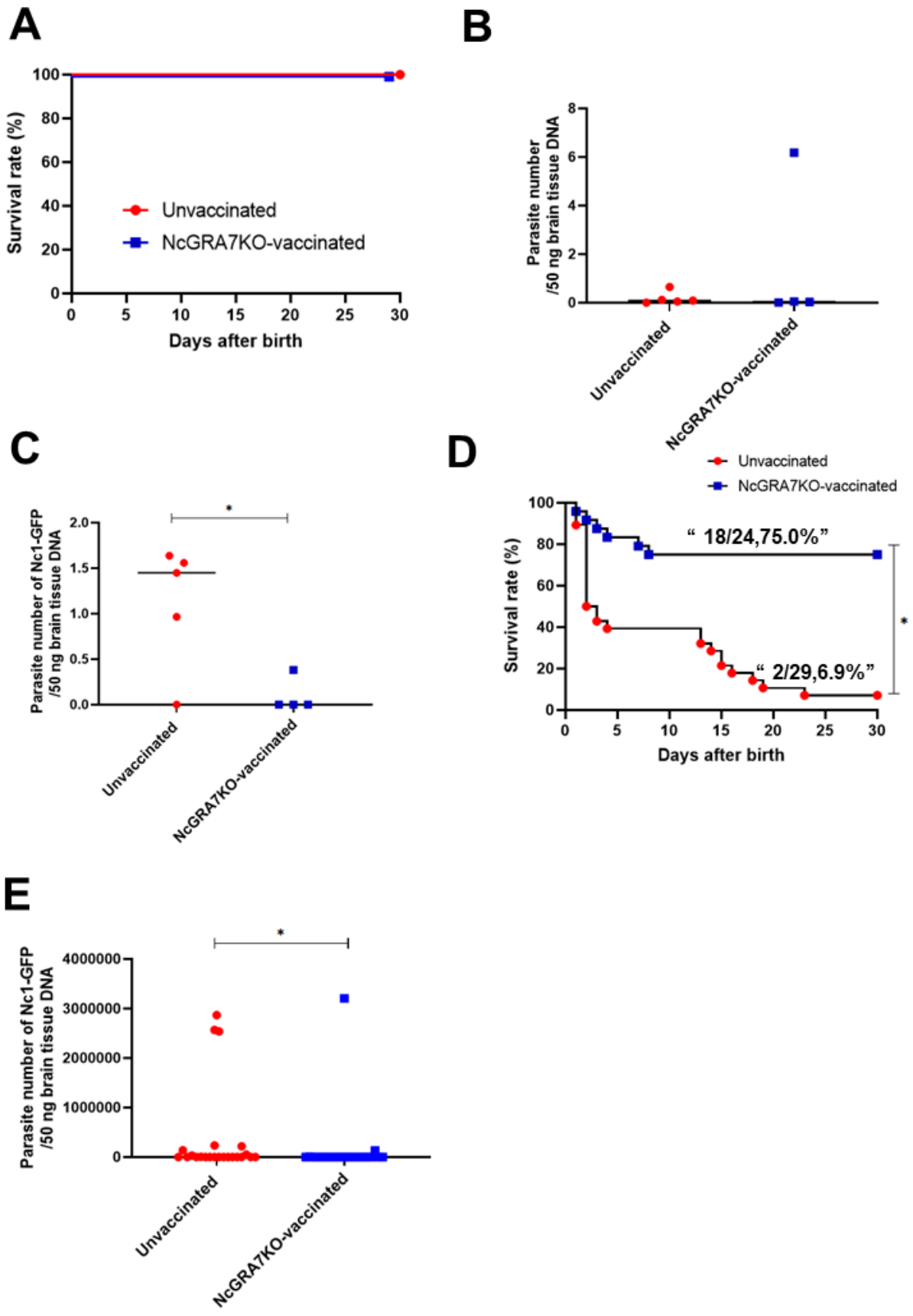
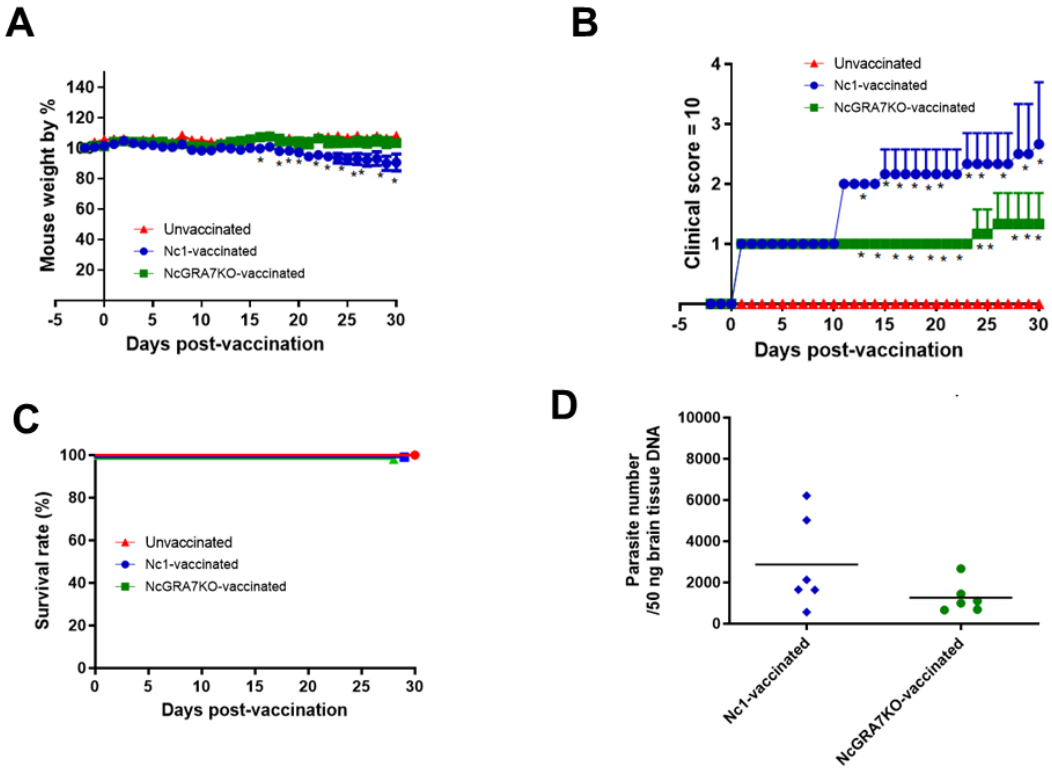
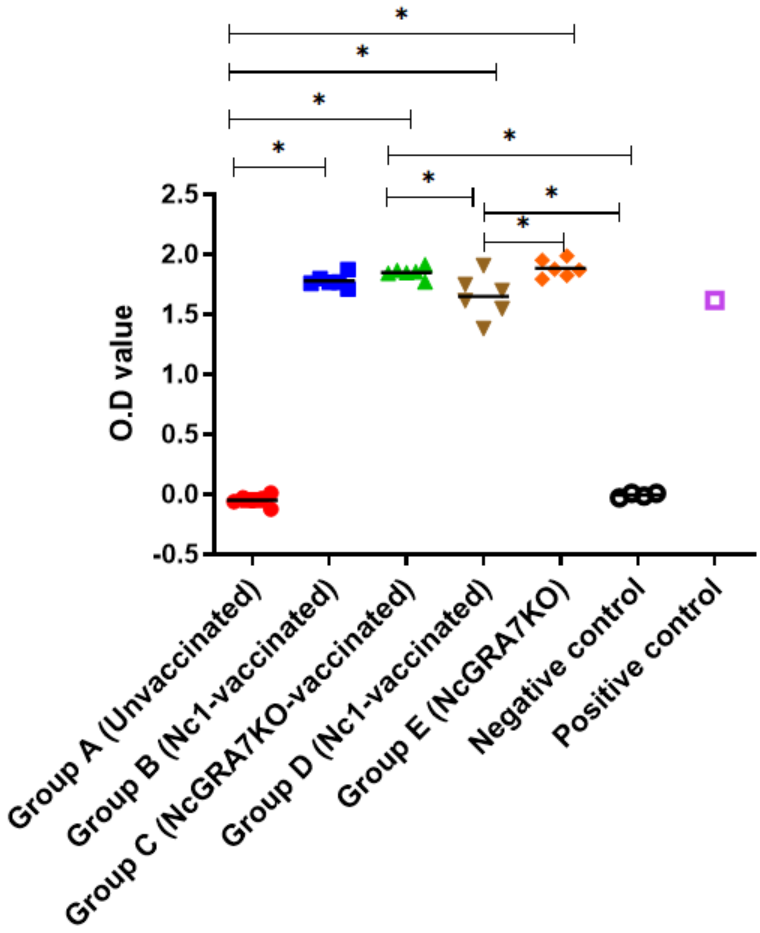

A
Survival rate (%)
Unvaccinated
NcGRA7KO-vaccinated
Days after birth
B
Parasite number /50 ng brain tissue DNA
Unvaccinated
NcGRA7KO-vaccinated
C
Parasite number of Nc1-GFP /50 ng brain tissue DNA
*
Unvaccinated
NcGRA7KO-vaccinated
D
Unvaccinated
NcGRA7KO-vaccinated
Survival rate (%)
" 18/24,75.0%"
" 2/29,6.9%"
*
Days after birth
E
Parasite number of Nc1-GFP /50 ng brain tissue DNA
*
Unvaccinated
NcGRA7KO-vaccinated

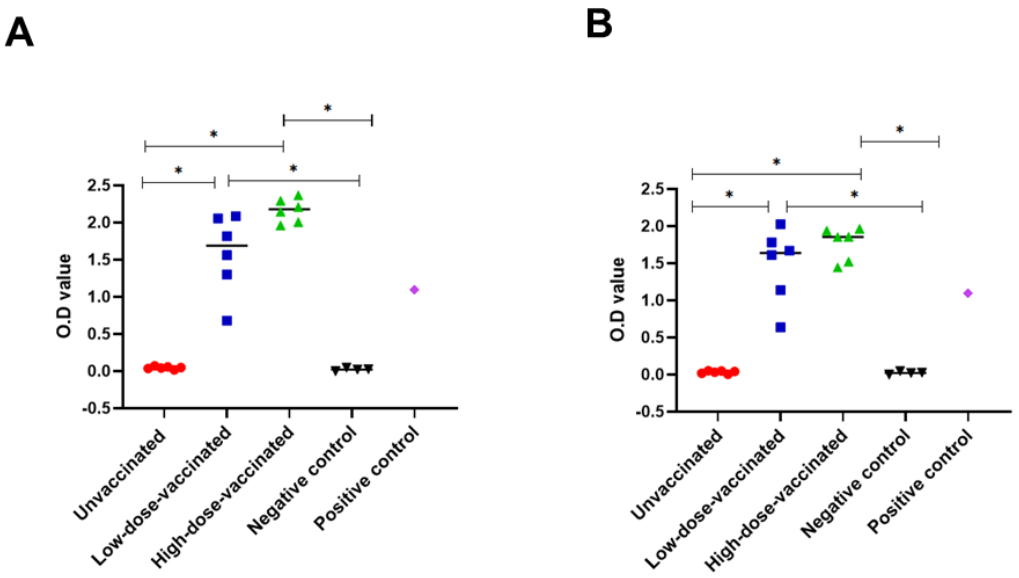
Fig. 11. **Efficacy of low-dose live vaccination in pregnant mice**. Mice were vaccinated with 1×10^4 *N. caninum* tachyzoites from NcGRA7-deficient parasites or were injected with RPMI medium as an unvaccinated control. All the mice were then challenged intraperitoneally with 1×10^5 Nc1-GFP at day 10.5 of pregnancy. **(A)** Kaplan Meier survival curves through 30 days postpartum (dpp) of the unvaccinated and NcGRA7KO-vaccinated groups (Survival rates: Unvaccinated, 5/5, 100.0%; NcGRA7KO, 4/4, 100.0%). The statistical significance of differences in survival rates was analyzed with a logrank Mantel–Cox test, but no differences were identified as significant. **(B)** Parasite burdens (vaccination parasites) were measured in the brains of dams at 30 dpp. Values are the number of parasites in 50 ng of brain tissue DNA. (Unvaccinated, n = 5; NcGRA7KO-vaccinated, n = 4). The statistical significance of differences was analyzed with a one-way ANOVA followed by Tukey-Kramer post hoc test, but no differences were identified as significant. (**C**) Parasite burdens (challenge parasites; Nc1-GFP) were measured in the brains of dams at 30 dpp. Values are the number of parasites in 50 ng of brain tissue DNA. (Unvaccinated, n = 5; NcGRA7KO-vaccinated, n = 4). The statistical significance of differences was analyzed with a one-way ANOVA followed by Tukey-Kramer post hoc test; $*p < 0.05$. **(D)** Kaplan Meier survival curves through 30 dpp of offspring from unvaccinated or NcGRA7KO-vaccinated dams (Survival rates: offspring from unvaccinated dams, 2/29, 6.9%; offspring from NcGRA7KO-vaccinated dams, 18/24, 75.0%). The statistical significance of differences in survival rates was analyzed with a logrank Mantel–Cox test; $*p < 0.05$. (**E**) Parasite burdens (challenge parasites; Nc1-GFP) were measured in the brains of offspring at their time of death or at 30 dpp. Values are the number of parasites in 50 ng of brain tissue DNA. (Unvaccinated, n = 23; NcGRA7KO-vaccinated, n = 22). The statistical significance of differences was analyzed with a Mann Whitney U test; $*p < 0.05$.



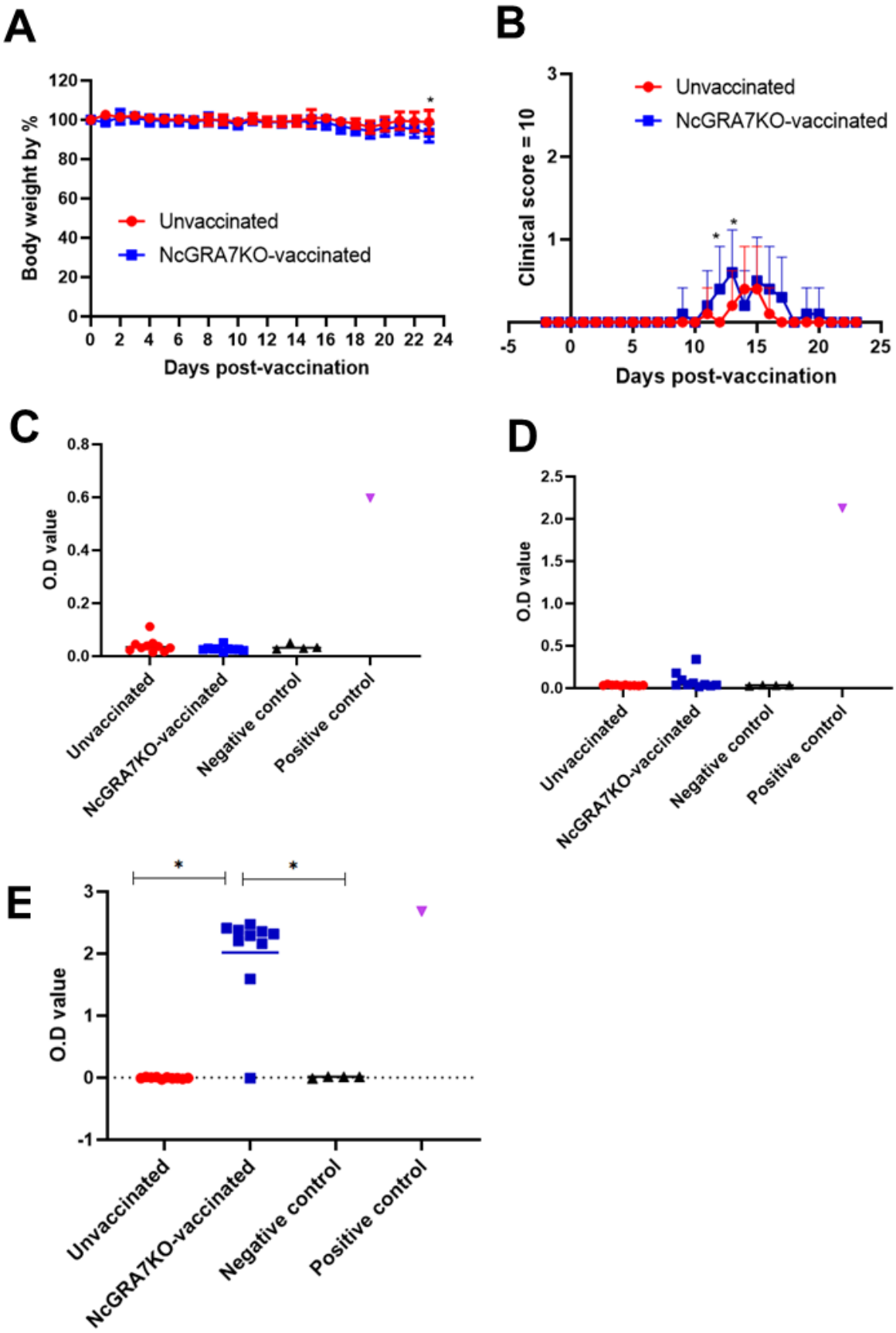
SFig 8. Virulence assessment of parental strain Nc1 or NcGRA7-deficient (NcGRA7KO) *N. caninum* parasites in non-pregnant female mice. Mice were vaccinated with *N. caninum* tachyzoites (1×10^5), from parental strain Nc1 or NcGRA7-deficient (NcGRA7KO) parasites, or were injected with RPMI-1640 medium as an unvaccinated control (Experiment 1; n = 6 per group). **(A)** The % of initial (day 0; vaccination day) bodyweight, up until 30 days post-vaccination (dpv). Values are presented as the means ± SD. Differences from the unvaccinated animals were assessed for statistical significance by a two-way ANOVA plus Tukey-Kramer post hoc test; * $p < 0.05$. **(B)** Clinical scores of pregnant mice during *N. caninum* infection. Values are presented as the means ± SD. Differences from the unvaccinated animals were analyzed for statistical significance by a two-way ANOVA plus Tukey-Kramer post hoc test; *$p < 0.05$. **(C)** Kaplan Meier survival curves spanning through 30 dpv for all groups were calculated (Survival rates: uninfected, 6/6, 100.0%; Nc1, 6/6, 100%; NcGRA7KO, 6/6, 100%). The differences among survival rates were analyzed with a logrank Mantel–Cox test, but none were found to be statistically significant. **(D)** Parasite burdens in the brains of mice. Parasite numbers in the brains of the mice at 30 dpv were measured. Values are the number of parasites in 50 ng of brain tissue DNA. Differences among groups were analyzed with a Mann-Whitney U test, but none were found to be statistically significant.



SFig. 9. Indirect enzyme-linked immunosorbent assay (iELISA) using recombinant antigen NcGRA6 conducted on sera from *N. caninum*-vaccinated mice (Experiment 1) at 30 days post-vaccination (dpv). An iELISA based on NcGRA6 was performed on sera collected from dams at 30 dpv with *N. caninum* (Unvaccinated, n = 6; Nc1-vaccinated, n = 6; NcGRA7KO-vaccinated, n = 6; negative control, n = 4; positive control, n = 1). Unvaccinated mice **(Group A)** and mice vaccinated with 1×10^5 tachyzoites of either the parental strain Nc1 challenged **(Groups B)** unchallenged **(Group D)** or the NcGRA7-deficient line (NcGRA7KO) challenged **(Groups C)** and unchallenged **(Group E)** of *N. caninum*. The negative control samples were sera from uninfected mice. The positive control sample was a reference serum sample from our laboratory. Differences between the tested groups and the unvaccinated mice were analyzed by a one-way ANOVA plus Tukey's multiple comparisons; *$p <$ 0.05.



SFig. 10. Indirect enzyme-linked immunosorbent assay (iELISA) using recombinant antigen NcGRA6 conducted on sera from *N. caninum*-vaccinated mice. Mice were vaccinated with high or low doses of *N. caninum* tachyzoites (1×10^4 or 1×10^5, respectively) of the NcGRA7-deficient line (NcGRA7KO). The negative control samples were sera from uninfected mice. The positive control sample was a reference serum from our laboratory. (**A–B**) iELISAs based on NcGRA6 were performed with sera from female mice (Experiment 2) **(A)** or male mice (Experiment 3) **(B)** collected at 25 days post-vaccination with NcGRA7KO. (Unvaccinated, n = 6; NcGRA7KO high dose-vaccinated, n = 6; NcGRA7KO low dose-vaccinated, n = 6, negative control, n = 4, positive control, n = 1). Differences among the tested groups were analyzed by a one-way ANOVA plus Tukey–Kramer post hoc analysis; *$p < 0.05$.


A
Body weight by %
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Days post-vaccination
Unvaccinated
NcGRA7KO-vaccinated
*
B
Clinical score = 10
3
2
1
-5 0 5 10 15 20 25
Days post-vaccination
Unvaccinated
NcGRA7KO-vaccinated
* *
C
O.D value
0.8
0.6
0.4
0.2
0.0
Unvaccinated
NcGRA7KO-vaccinated
Negative control
Positive control
D
O.D value
2.5
2.0
1.5
1.0
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Unvaccinated
NcGRA7KO-vaccinated
Negative control
Positive control
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O.D value
3
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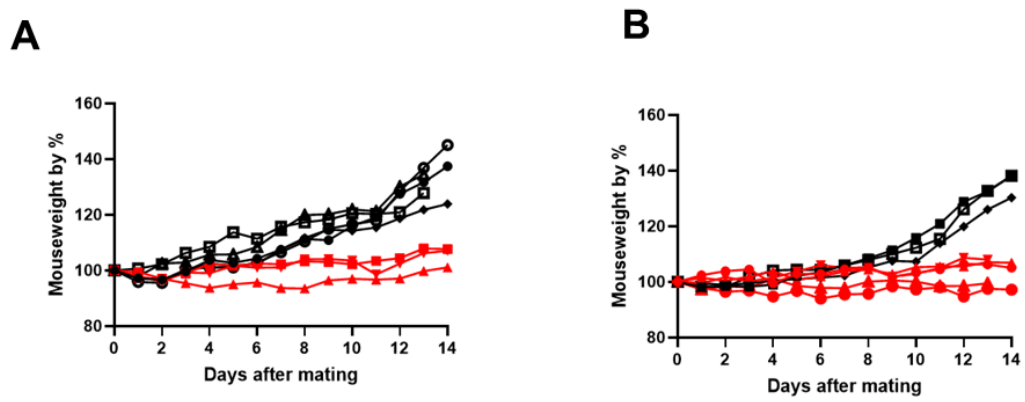
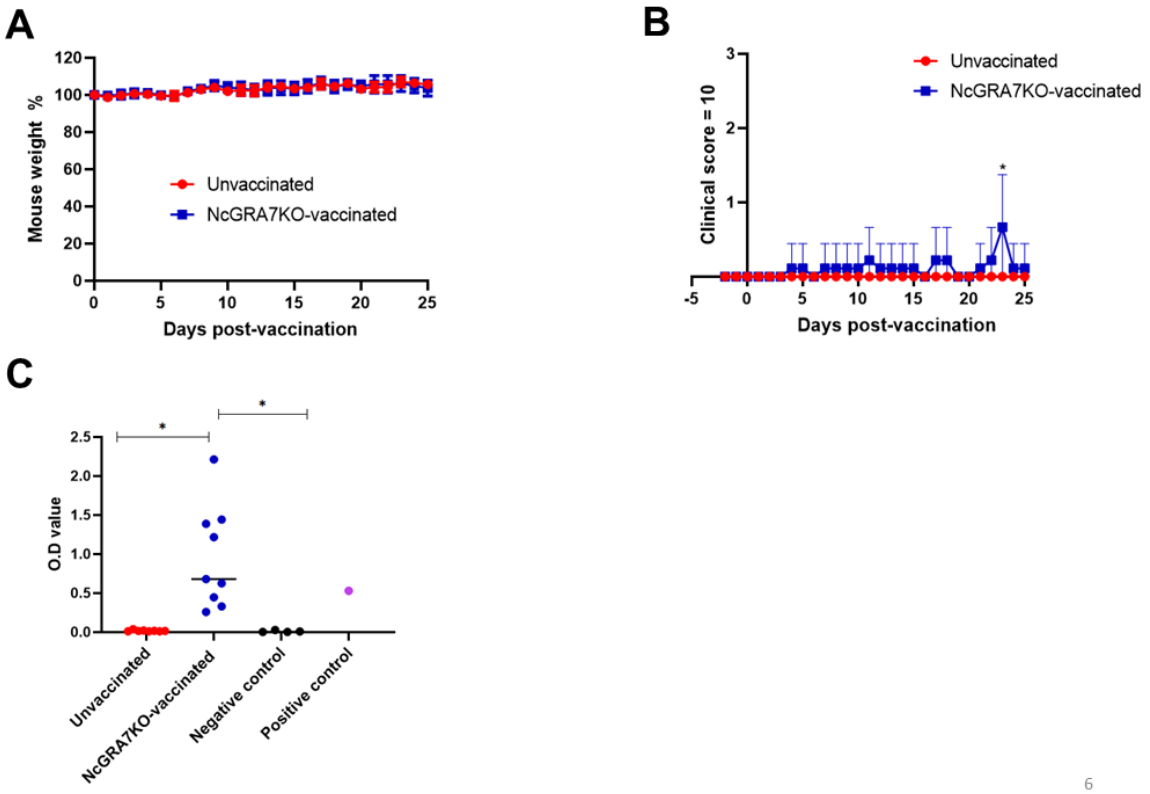
Unvaccinated
NcGRA7KO-vaccinated
Negative control
Positive control

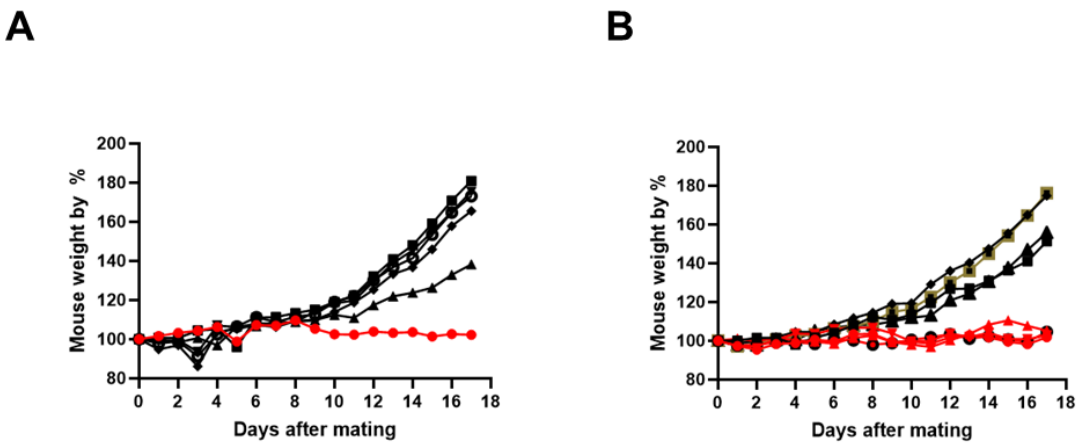
SFig. 11. Efficacy of high-dose live vaccination in pregnant mice (Experiment 4). Mice were vaccinated with 1×10^4 *N. caninum* NcGRA7-deficient (NcGRA7KO) tachyzoites or were injected with RPMI-1640 medium as an unvaccinated control. These mice were then challenged intraperitoneally with 1×10^5 Nc1-GFP at day 10.5 of pregnancy (Experiment 4). **(A)** The bodyweight % was calculated from the daily weight with reference to the initial weight at day 0 (vaccination day) up until 23 days post-vaccination. Unvaccinated mice, n = 10; NcGRA7KO-vaccinated mice, n = 10. Values are presented as the means ± SD. The statistical significance of differences from the unvaccinated animals was calculated by a two-way ANOVA plus Bonferroni comparisons' test; *$p < 0.05$. **(B)** Clinical scores of the pregnant mice during *N. caninum* infection. Values are presented as the means ± SD. The statistical significance of differences from the unvaccinated animals were analyzed by a two-way ANOVA plus Bonferroni comparisons test; *$p < 0.05$. **(C–E)** Results of indirect enzyme-linked immunosorbent assays (iELISAs) using recombinant antigen NcGRA6 or NcSAG1 conducted on sera from *N. caninum*-vaccinated mice. The negative control samples were sera from uninfected mice, while the positive control sample was a reference serum sample from our laboratory. iELISAs based on NcGRA6 were performed on sera collected at 7 dpv **(C)**, and iELISAs based on NcSAG1 were performed on sera collected at 7 dpv **(D)** or 20 dpv **(E)** (Unvaccinated animals, n = 10; NcGRA7KO-vaccinated animals, n = 10; negative control, n = 4; positive control, n = 1). Differences among the tested groups were analyzed by a one-way ANOVA plus Tukey–Kramer post hoc analysis; *$p < 0.05$.



SFig. 12. Bodyweight changes in high-dose live vaccinated pregnant mice (Experiment 4). (**A–B**) Mice were vaccinated with a high dose of *N. caninum* tachyzoites (1×10^5) from the NcGRA7-deficient parasite (NcGRA7KO) (**A**) or were injected with RPMI-1640 medium as an unvaccinated control (**B**), and all mice were then intraperitoneally challenged with Nc1-GFP (1×10^5) tachyzoites at day 10.5 of pregnancy. The bodyweight percentage (%) was calculated daily up until 14 days post-mating. n = 8/group. The red lines represent the aborted or non-pregnant mice, and the black lines represent the confirmed pregnant mice.



SFig. 13. Efficacy of low-dose live vaccination in pregnant mice (Experiment 5). The mice were vaccinated with 1×10^4 *N. caninum* tachyzoites of the NcGRA7-deficient parasite (NcGRA7KO) or were injected with RPMI-1640 medium as an unvaccinated control. These mice were then challenged intraperitoneally with Nc1-GFP at day 10.5 of pregnancy. **(A)** The bodyweight % was calculated from day 0 (vaccination day) until 23 days post-vaccination (dpv). Unvaccinated mice, n = 8, NcGRA7KO-infected mice, n = 9. Values are presented as the means ± SD. The statistical significance of differences from the unvaccinated animals was calculated by a two-way ANOVA plus Bonferroni comparisons' test, but no significant differences were found. **(B)** Clinical scores of the pregnant mice during *N. caninum* vaccination. Values are presented as the means ± SD. The statistical significance of differences from the unvaccinated animals was calculated by a two-way ANOVA plus Bonferroni comparisons test; *$p < 0.05$. **(C)** Indirect enzyme-linked immunosorbent assay (iELISA) using recombinant antigen NcGRA6 was conducted on sera collected from mice at 22 dpv with a low dose (1×10^4) of *N. caninum* tachyzoites of the NcGRA7-deficient line (NcGRA7KO) or medium alone as an unvaccinated control. The negative control samples were sera from uninfected mice. The positive control sample was a reference serum from our laboratory. NcGRA7KO-vaccinated, n = 9; unvaccinated, n = 8; negative control, n = 4, positive control, n = 1. Differences among the tested groups from the unvaccinated mice were analyzed by a one-way ANOVA plus Tukey–Kramer post hoc analysis; *$p < 0.05$.



SFig. 14. Bodyweight changes in low-dose live vaccinated pregnant mice (Experiment 5). (**A–B**) Mice were vaccinated with a low dose (1×10^4) of *N. caninum* tachyzoites from the NcGRA7-deficient parasite (NcGRA7KO) (**A**) or were injected with RPMI-1640 medium as an unvaccinated control (**B**), and all mice were then intraperitoneally challenged with Nc1-GFP (1×10^5) tachyzoites at day 10.5 of pregnancy. The bodyweight percentage (%) was calculated daily up until 17 days post-mating. Unvaccinated mice, n = 6; NcGRA7KO-vaccinated mice, n = 8. The red lines represent the aborted or non-pregnant mice, and the black lines represent the confirmed pregnant mice.

Chapter 3

Extracts of wild Egyptian plants from the desert inhibit the growth of *Toxoplasma gondii* and *Neospora caninum* in vitro

3-1. Introduction

Toxoplasmosis, caused by the obligate intracellular protozoan parasite *Toxoplasma gondii*, is a disease of zoonotic potential and global veterinary and medical concern (Liu et al., 2015) *T. gondii* is considered a single species in the genus *Toxoplasma*, phylum Apicomplexa (Howe and Sibley, 1995). Approximately 30% of the global human population has contracted the parasite as evidenced by the presence of anti-*T. gondii* antibodies. While *T. gondii* infections are usually asymptomatic, they can lead to adverse effects especially in patients who are immunocompromised or who obtain the disease congenitally (Safarpour et al., 2020). In humans, *T. gondii* infection occurs through three main pathways: ingestion of undercooked meat contaminated with cysts, consumption of food or water contaminated with the infective stage (oocysts), or congenitally via vertical transmission from infected mothers to their offspring (Moncada and Montoya , 2012; Sepúlveda-Arias et al., 2014) Transplacental transmission occurs when women are infected during pregnancy, (Hill and Dubey, 2002) and the timing of infection during pregnancy affects the progress of the disease, with infection in the first trimester more severe than infection during the second or third trimesters (Remington et al., 1995).

Neospora caninum, the causative agent of neosporosis, is an obligate intracellular tissue cyst-forming coccidian parasite of the phylum Apicomplexa (Dubey and Schares, 2011). *N. caninum* is transmitted by two main pathways: transplacentally (vertically) and horizontally. Vertical transmission occurs when the parasite passes through the placental barrier to the fetus, while horizontal transmission involves either direct ingestion of the oocysts shed by the final host or ingestion of contaminated food or drinking water (Dubey et al., 2007; Donahoe et al., 2015)

T. gondii and *N. caninum* are closely related parasites, sharing many morphological and biological features in addition to clearly conserved genomes (Mugridge et al., 1999; Speer et al., 1999; Reid et al., 2012). While they differ in the ranges of their final hosts, they both have zoonotic potential and both utilize

vertical and horizontal transmission to maintain natural infection in infected livestock (Dubey and Schares, 2011; Robert-Gangneux and Darde, 2012). *N. caninum* has a wide global distribution and in dogs causes severe neuromuscular signs while in cattle it causes abortions and neonatal deaths, leading to large economic losses in the beef and dairy industries (Dubey and Schares, 2011; Dubey et al., 2007; Reichel et al., 2013). Unlike *T. gondii*, the zoonotic potential of *N. caninum* has not yet been confirmed (Chu and Quan, 2021) though antibodies against *N. caninum* have been detected in people (Dubey et al., 2007; Tranas et al., 1999). In addition, when 201 umbilical cord samples were analyzed, two umbilical cord blood samples (1%) tested positive for *N. caninum* despite *N. caninum* not being found in the associated placental tissues (Duarte et al., 2020).

Sulphadiazine has been found to have important inhibitory effects on *T. gondii* (IC_{50} = 2.5 µg/ml) and to be associated with a reduction of the growth of these intracellular parasites and an alteration to their normal morphology (Derouin and Chastang, 1989). In contrast, sulfonamides demonstrate little activity against *N. caninum* tachyzoites at 100 µg/ml (Lindsay et al., 1994). Pyrimethamine and trimethoprim show potent activity against *T. gondii* and cause striking parasite morphological changes with IC_{50}s of 0.04 and 2.3 µg/ml, respectively. Additionally, trimethoprim is effective against *N. caninum* tachyzoites at 10 µg/ml (Lindsay et al., 1994). While sulphadiazine and pyrimethamine can be used in combination for the treatment of toxoplasmosis in people, reported side effects include agranulocytosis, Stevens-Johnson syndrome, toxic epidermal necrolysis, and hepatic necrosis (Alday and Doggett, 2017). Chemoprophylactic and chemotherapeutic chemical agents that have been used in the treatment of toxoplasmosis and neosporosis in pregnant and non-pregnant small and large domestic ruminants have been summarized (Sánchez-Sánchez et al., 2018). Except for Toxovax® (MSD Animal Health, Upper Hutt, New Zealand), no effective vaccine for toxoplasmosis has been developed, despite multiple trials being performed (Chu and Quan, 2021). There is also no effective commercially available vaccine for neosporosis. A commercial inactivated vaccine (Neoguard®, Intervet International B.V., Boxmeer, the Netherlands) was developed but withdrawn from the market since only moderate protection against abortions was observed in field trials (Romero et al., 2004; Weston et al., 2012). Therefore, developing novel chemotherapeutics from natural resources that are of low or no toxicity is beneficial for treating both toxoplasmosis and neosporosis.

A large number of medicinal plants that produce natural products with potent anti-parasitic activity have been identified and researched (Newman and Cragg, 2007). Plant extracts or secondary metabolites that were considered as an alternative to commercial drugs were evaluated during the search for antiparasitic candidates. From 1981 to 2006, 1,184 new drugs were registered of which 28% were either natural products or their derivatives. In addition, another 24% of these novel drugs had pharmacophores derived from natural products (Newman and Cragg, 2007; Wink, 2012). The desert plants in this study have been reported to have wide medicinal uses (see Table S2). Therefore, the current study aimed to evaluate the in vitro efficacy of plant extracts collected from Egypt against the growth of tachyzoites of both *T. gondii* and *N. caninum*.

3-2. Materials and methods

Parasites

A green fluorescent protein-expressing RH strain of type I *T. gondii* (RH-GFP) and GFP-expressing Nc1 strain of *N. caninum* (Nc1-GFP) were maintained in African green monkey kidney epithelial (Vero) cells according to previously reported methods (Nishikawa et al., 2003; Nishikawa et al., 2008; Leesombun et al., 2016).

Collection of plant materials and extraction

The plants used in this study were obtained from the field in two regions of the southern region of Egypt in the Qena Governorate (Latitude: 26° 09' 51.05" N, Longitude: 32° 43' 36.16" E). The collection was done from two sites along the Qena-Sohag and Qena-Safaga desert roads, Eastern desert, Egypt, and collection was done under the approval of the Faculty of Veterinary Medicine, South Valley University, Qena. The collection was done following the guidelines and rules of South Valley University, Qena, Egypt. A map that identifies the sites of collection is shown (Fig. S15). Twelve plant samples were collected in May 2019 during the plant flowering season. The collected plant samples were identified by the South Valley University herbarium, Faculty of Science, Qena, Egypt, and an

official letter of identification was issued. Identification was performed according to the reported literature (Boulos, 1999a; 1999b; 2002; 2009). Plant taxonomy and species were further updated according to Plants of the World Online (POWO, 2019).

Plants samples were left to dry in the shade for 3 to 10 days. A fine powder was obtained from dried leaves, flowers, fruit, or seed parts using a kitchen blender. 100 grams of each powdered plant material was dissolved in either 80% methanol, 70% ethanol, or distilled water for a minimum of 1 to 3 days with a ratio of 1:10 (100 g of plant powder per 1 liter of the solvent used). The plant supernatant was further collected and filtrated by a glass filtration apparatus and was then collected in a wide conical flask. It was then dissolved in a wide petri dish at room temperature for 1 to 3 days. The final crude extract was collected in centrifuge tubes and stored at -30°C until use. To test the antiprotozoal potential of the various plant extracts, they were solubilized individually in the solvent dimethyl sulfoxide (DMSO) to prepare stock solutions (100 mg/ml). The previously reported medicinal uses and the Latin binomial names of the wild plants used in this study are shown in Table S2.

Determination of cytotoxicity of plant extracts

To determine the cytotoxic potential of the plant extracts, cytotoxicity was evaluated against human cells using human foreskin fibroblast (HFF) cells. Cell suspensions (1×10^5 cells/ml) in Dulbecco's Modified Eagle medium (DMEM, Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 10% fetal bovine serum (FBS) (Nichirei Bioscience, Tokyo, Japan) were plated at 100 µl/well in 96-well plates and incubated at 37°C in a 5% CO_2 atmosphere. The plant extracts were added to the cells for 72 h at final concentrations of two-fold serial dilution starting from 1,000 µg/ml. To evaluate cell viability, cell proliferation inhibition (%) was calculated as described previously (Leesombun et al.,2016; Pagmadulam et al., 2020).

Determination of anti-*Toxoplasma gondii* and anti-*Neospora caninum* activities of plant extracts in vitro

HFF cells (cell suspensions 1×10^5 cells/ml in DMEM supplemented with 10% FBS) were plated at 100 µL/well in 96-well plates, then *T. gondii* RH-GFP and *N. caninum* Nc1-GFP (5×10^4 tachyzoites/well) were added at five multiples of infection (MOI) for 4 hours. The extracellular parasites were then washed by changing the medium and the new medium with 10% FBS was added to each well. Only the plant extracts prepared in DMEM were added to the infected cells (final concentrations: 0.25–100 µg/ml), and a medium with 0.5% dimethyl sulfoxide was used as the negative control. After being incubated for 72 hours, the fluorescence intensities of RH-GFP and Nc1-GFP were measured using a Glomax multi-detection system microplate reader (Promega Corporation, Madison, WI, USA). The percentage inhibition of parasite growth was calculated according to the previous methods (Leesombun et al.,2016; Pagmadulam et al., 2020). The mean IC_{50} values were calculated for RH-GFP and Nc1-GFP. Data represent the mean values ± SD for three independent experiments.

Effects of plant extracts against the growth of extracellular parasites in vitro

RH-GFP and Nc1-GFP tachyzoites (2×10^5) were pre-treated with either one of the four different extracts (*A. judaica*, *C. droserifolia, T. africanum, and V. tortilis*) (100 µg/mL), Sulfadiazine (1 mg/ml) or MEM alone for 1 hour at 37°C. Then, the pre-treated parasites were added to Vero cells at 1 ml/well in a 12-well plate (M. O. I = 2). At 2 to 3 hours post-infection, the extracellular parasites were washed away and MEM supplemented with 8% FBS was added. After 24 h, the infected cells were analyzed by indirect fluorescent antibody test to measure the infection rates. The infection rate for RH-GFP and Nc1-GFP was measured by counting the number of *T. gondii*-GRA7-positive and *N. caninum*-GRA7-positive Vero cells per 100-140 Vero cells in ten randomly different fields for each plant candidate against the untreated parasites, respectively. Estimation of the infection rate values was performed blindly for all slides. The infection rate values were calculated by IFAT as follows: [(number of parasite-positive Vero cells) / (100 randomly selected Vero cells)] × 100 (Leesombun et al.,2016).

Statistical analysis

Graph Pad Prism 6.0 and its updated version 8.3.4 software (Graph Pad Software Inc. La Jolla, CA, USA) were used for the analysis of the IC_{50} values for the percentage inhibition of the parasites and host cells. The final mean IC_{50}s of anti-RH-GFP and anti-Nc1-GFP, and against HFF cells were calculated based on three independent experiments and the data represent the mean ± SD. For mean infection rate calculation (mean infection rate %), statistical analyses were performed using a One-way analysis of variance (ANOVA) and a Tukey-Kramer post hoc analysis ($P < 0.05$).

3-3. Results

In vitro anti-*Toxoplasma gondii* activities of plant extracts

The activities of different types of Egyptian plant extracts were evaluated against the growth of *T. gondii and N. caninum* in vitro. Among the twelve plant extracts tested, four extracts, from *A. judaica*, *C. droserifolia*, *T. africanum,* and *V. tortilis,* showed potent activity against RH-GFP growth in vitro with mean IC_{50}s of 2.1, 12.5, 21.8, and 24.5 µg/ml, respectively. Moreover, their mean SI values were 150.8, 29.6, 18.9, and 22.6, respectively (Table 7).

Plant extracts showing partial efficacy against *T. gondii* tachyzoites were an aqueous extract from *Calotropis procera,* an ethanolic extract from *Pulicaria undulata,* an ethanolic extract from *Ocimum basilicum,* a methanolic extract from *P. undulata,* and a methanolic extract from *Forsskaolea tenacissima,* their mean IC_{50}s were 7.2 µg/ml, 15.4 µg/ml, 46.6 µg/ml, 46.9 µg/ml, and 64.5 µg/ml, respectively. Moreover, their mean SI values were 5.7, 3.6, 5.4, 4.2, and 8.0, respectively (Table 7). The methanolic extract from *C. procera* exhibited weak efficacy against *T. gondii* in vitro with a mean IC_{50} of 1.9 µg/ml and a mean SI of 1.5, as it exhibited high cytotoxicity against HFF cells with a mean IC_{50} of 2.9 µg/ml. We considered the extracts from *Aerva javanica, Anabasis setifera,* and *Ochradenus baccatus,* along with both methanolic and ethanolic extracts of *Citrullus colocynthis,* to have no efficacy against the in vitro growth of *T. gondii* as the IC_{50}s were > 100 µg/ml (Table 7).

Extracts with high SI values (> 10) were further analyzed for their anti-*Toxoplasma* effects by examining the intensity of fluorescence of RH-GFP after 72 hours of treatment. *A. judaica*, *C. droserifolia*, *T. africanum*, and *V. tortilis* inhibited RH-GFP signal growth at a concentration of 100

μg/mL (Fig. 12A) when compared with the untreated parasites, and more than sulfadiazine did when tested at a concentration of 1 mg/ml.

To examine the effect of each plant extract on extracellular *T. gondii*, purified parasites were pre-treated extracellularly with 100 μg/mL of each extract (Fig. 12B). The mean parasite infection rate values after 24 hours of pre-treatment with *A. judaica*, *C. droserifolia*, *T. africanum*, and *V. tortilis* were 6.7%, 6.8%, 9.6%, and 10.6%, respectively, which were significantly lower than the mean parasite infection rate of the untreated parasites (29.12%) (Fig. 12B).

In vitro anti-*Neospora caninum* activities of plant extracts

Plant extracts with potent and partial activities against RH-GFP growth were evaluated against the growth of *N. caninum* (Nc1-GFP) tachyzoites in vitro since this parasite is closely related to *T. gondii*. The results showed that *C. droserifolia, T. africanum*, *A. judaica*, and *V. tortilis* exhibited potent efficacy against the growth of Nc1-GFP with mean IC_{50}s of 1.0 μg/ml, 3.1 μg/ml, 8.6 μg/ml, and 17.2 μg/ml, respectively. In addition, their mean SI values were 370.9, 133.2, 36.8, and 32.2, respectively (Table 8). The growth inhibition of extracts from *A. judaica*, *C. droserifolia*, *T. africanum*, and *V. tortilis* plants against the growth of Nc1-GFP at a concentration of 100 μg/ml after 72 hours or treatment was shown (Fig. 13A). Moreover, methanolic and aqueous extracts of *C. procera* and an ethanolic extract of *P. undulata* exhibited partial efficacy against Nc1-GFP with mean IC_{50}s of 0.9 μg/ml, 1.8 μg/ml, and 3.0 μg/ml, respectively. In addition, their mean SI values were 3.0, 23.0, and 18.5, respectively (Table 8).

The extracts with potent activity were also tested against extracellular *N. caninum*. The mean parasite infection rate values after 24 hours of pre-treatment with *A. judaica*, *C. droserifolia, T. africanum, and V. tortilis* extracts were 5.9%, 11.8%, 12.9%, and 15.1%, respectively, which were significantly lower than the values from the untreated parasites (39.77%) (Fig 13B).

Cytotoxicity of plant extracts against HFF cells

The cytotoxic potential of all plant extracts was evaluated by two-fold serial dilutions from 1000 μg/ml and the mean IC_{50} values were calculated (Table 7). The methanolic and aqueous extracts

from *C. procera*, the ethanolic extract from *P. undulata*, both the methanolic and ethanolic extracts from *C. colocynthis*, *the* methanolic extract from *P. undulata*, and the ethanolic extract from *O. basilicum* showed the highest cytotoxicity against HFF cells with mean IC_{50}s of 2.9 µg/ml, 41.5 µg/ml, 55.5 µg/ml, 65.6 µg/ml, 88.0 µg/ml, 197.5 µg/ml, and 252.6 µg/ml, respectively. Extracts from plants *A. judaica, C. droserifolia, A. javanica, T. africanum, F. tenacissima*, and *V. tortilis* exhibited moderate to weak toxicity against HFF cells with mean IC_{50}s of 316.8 µg/ml, 370.9 µg/ml, 378.1 µg/ml, 413.0 µg/ml, 519.0 µg/ml, and 554.5 µg/ml, respectively. Extracts from *A. setifera and O. baccatus* were nontoxic or safe for HFF cells as cytotoxicity was not observed and their mean IC_{50}s were >1,000 µg/ml of 1263.6 µg/ml, and 1179.0 µg/ml, respectively (Table 7).

3-4. Discussion

Among all the tested plants, *A. judaica* showed the highest efficacy against the growth of *T. gondii* with a mean IC_{50} of 2.1 µg/ml (Table 7) and potent efficacy against Nc1-GFP with a mean IC_{50} of 8.6 µg/ml (Table 8). The *Artemisia* genus belongs to the plant family Asteraceae. The genus includes about 500 species, many of which are known for their medicinal properties, (Hussain et al., 2017) and some plants in this genus have been reported to have medicinal value against protozoan parasites. *A. judaica* has been traditionally used to treat different diseases, Recently, its ethanolic extract inhibits the protozoan parasite *Blastocystis* at a concentration range from 250 µg/ml to 4,000 µg/ml (Mokhtar et al., 2019). These doses of *A. judaica* were higher than those used in our present study (100 µg/ml) against the Apicomplexan parasites. *A. argyi* has been reported to have an anti-amoebic effect (Kolören et al., 2019). Artemisinin and its derivatives from *A. annua* have been reported to treat many different species of protozoan parasites such as *Leishmania* sp., *Trypanosoma* sp., *T. gondii, N. caninum, Eimeria tenella, Acanthamoeba castellanii, Naegleria fowleri, Cryptosporidium parvum, Giardia lamblia,* and *Babesia* sp. (Loo et al., 2017). In my study, *A. judaica* demonstrated potent effects against both *T. gondii* and *N. caninum* in vitro with mean IC_{50}s of 2.1 µg/ml and 8.6 µg/ml, respectively (Tables 7 and 8), despite a lack of information about the presence of artemisinin in *A. judaica* plants.

Essential oils from *A. judaica* have several biological activities, including anthelmintic, anti-inflammatory, analgesic, and antipyretic effects (Batanouny et al., 1999). The essential oils isolated

from the aerial portions of *A. judaica* growing on the northern coast of Egypt exhibit anti-oxidative activity, as determined by the thiocyanate and scavenging effects on 2,2-diphenyl-1-picrylhydrazyl (El-Massry et al., 2002). The potent effect of *A. judaica* against both *T. gondii* and *N. caninum* may be attributed to one of its chemical biological constituents and/or other reported medicinal attributes.

C. droserifolia is another species with medicinal value widely used traditionally in Egypt and Jordan. It has been reported that an ethanol extract can treat hyperglycemia in diabetic male albino rats (Shtaiwi et al., 2013). Phytochemical screening studies on *Cleome* species have shown that it contains diverse, beneficial secondary products such as terpenoids, flavonoids, phenolics, and alkaloids, and suggest the use of plants from this genus in pharmacological investigations (Aboushoer et al., 2010).

Extracted oil from *C. droserifolia* diluted in methanol was reported to have antimicrobial efficacy against Gram-positive bacteria including *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Bacillus cereus,* and *Micrococcus luteus* with minimum inhibitory concentrations (MICs) of 23.7 µg/ml, 28.7 µg/ml, 36.0 µg/ml, 14.7 µg/ml, and 35.3 µg/ml, respectively. It also exhibited efficacy against Gram-negative bacteria such as *Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, and Serratia marcescens* with MICs of 19.3 µg/ml, 17.0 µg/ml, 11.3 µg/ml, and 14.3 µg/ml, respectively. These results demonstrate that *C. droserifolia* may have components that can kill pathogenic microorganisms and suggest the need for future therapeutic investigations of *Cleome* species (Muhaidat et al., 2015). In my study, I demonstrated the same results when crude methanolic extract was prepared from *Cleome* leaves. This exhibited potent efficacy against both *T. gondii* and *N. caninum* using lower concentrations than in the previously mentioned study (Abdallah Emad et al., 2013), with mean IC_{50}s of 12.5 µg/ml and 1.0 µg/ml, respectively (Tables 7 and 8).

The qualitative analysis of *T. africanum* plant extract showed that this extract contained many phytochemicals such as tannins, saponins, flavonoids, terpenoids, and phenolic compounds (Abdallah Emad et al., 2013). A 100 mg/ml concentration of the methanolic extract of *T. africanum* was previously reported to have an antibacterial effect against Gram-negative bacteria such as *Proteus vulgaris*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* with mean inhibition zones of 9.5 mm, 10.5 mm, 12.5 mm, 10.5 mm, and 14.0 mm, respectively. These

effects were also exhibited against gram-positive bacteria and fungi such as *Bacillus cereus*, *Salmonella typhi*, and *Candida albicans* with mean inhibition zones of 13.5 mm, 11.5 mm, and 17.5 mm, respectively (Abdallah Emad et al., 2013).

In my study, *T. africanum* was tested against two different protozoal parasites at lower concentrations starting from 100 µg/ml, and its mean IC_{50} against *T. gondii and N. caninum* was 21.8 µg/ml and 3.1 µg/ml, respectively (Tables 7 and 8). Furthermore, the microwave-ultrasonic oil extraction method used for *T. africanum* elicited an antioxidant activity (Jaradat et al., 2016), which might explain its low cytotoxicity.

The plant *C. procera* (Aiton) Dryand belongs to the Apocynaceae family, and the chemical constituents of *C. procera* include triterpenoids, flavonoids, cardiac glycosides, cardenolides, anthocyanins, α-amyrin, β-amyrin, lupeol, β-sitosterol, flavanols, mudarine, resins, a powerful bacteriolytic enzyme called calactin, and a nontoxic proteolytic enzyme called calotropin (Mali et al., 2019). The reported pharmacological effects include antimicrobial, anthelmintic, anti-inflammatory, analgesic, antipyretic, anticancer, anti-angiogenic, antidiabetic, hypolipidemic, gastroprotective, hepatoprotective, renoprotective, antidiarrheal, antioxidant, anticonvulsant, enhanced wound healing, antifertility, and smooth muscle relaxation (Mali et al., 2019). *C. procera* was reported to have many pharmacological effects such as antischistosomal activity (Yousif et al., 2007). The ethanol extract form of *C. procera* showed high efficacy against the schizont stage of other Apicomplexa parasites; *Plasmodium falciparum* with IC_{50} values ranging from 0.11 mg/ml to 0.47 mg/ml against MRC20 chloroquine-sensitive strains, and 0.52 mg/ml to 1.22 mg/ml against MRC76- chloroquine-resistant strains of *P. falciparum* (Sharma and Sharma, 2000). Furthermore, the methanolic extract of *C. procera* was previously reported to treat *Leishmania tropica* with an IC_{50} of 95.2 µg/ml (Ilaghi et al., 2021). *C. procera* was found to have a high percentage of inhibition (92.5%) against blood parasites such as *Theileria annulata* infection in cattle compared with 75% for buparvaquone after 3 weeks of treatment (Durrani et al., 2009). Despite the previously reported wide medicinal uses of *C. procera* plant extracts, both methanolic and aqueous extracts from *C. procera* flowers collected from desert roads in my study showed high cytotoxicity and their mean SI against *T. gondii* were 1.5 and 5.7, respectively (Table 7), suggesting its weak effect against *T. gondii*. Only the aqueous extract showed efficacy against *N.*

caninum tachyzoite growth in vitro with a SI of 23.0 (Table 8). A recent study evaluated the essential oil from *O. basilicum* (basil), supplied by the National Research Center of Medicinal and Aromatic Plants (Qalyubia governorate, northern Egypt) against the growth of *T. gondii* in vitro, and the inhibition to RH-GFP with 50 µg/ml and 10 µg/ml were 50.03% ± 4.46 and 22.53% ± 1.53, respectively (Elazab et al., 2020). While in my study the ethanolic extract from *O. basilicum* crude extract collected from southern Egypt (around Qena governorate) showed partial effect against RH-GFP with a mean IC_{50} of 46.6 µg/ml and a mean SI value of 5.4 (Table 7).

The antiprotozoal species specificity of plant extracts was observed among the potent candidates for the treatment of both *T. gondii* and *N. caninum*. We observed that *A. judaica* plant extract showed the greatest efficacy against *T. gondii* growth in vitro with a mean IC_{50} of 2.1 µg/ml and a mean SI of 150.8 (Table 7), while its effect against *N. caninum* was 4-fold lower with a mean IC_{50} of 8.6 and a mean SI of 36.8 (Tables 7 and 8). However, both *C. droserifolia* and *T. africanum* extracts demonstrated more potent efficacy against *N. caninum* than *T. gondii*; their mean IC_{50}s against Nc1-GFP were 1.0 µg/ml and 3.1 µg/ml, respectively, and their SI values were 370.9 and 131.9, respectively (Table 8). Their mean IC_{50}s against RH-GFP were 12.4 µg/ml and 21.8 µg/ml, respectively, and their mean SIs were 29.9 and 18.9, respectively (Table 7). These findings may demonstrate that *A. judaica* is species-specific for *T. gondii*, while *C. droserifolia* and *T. africanum* extracts were species-specific for *N. caninum*.

C. colocynthis, obtained from a herbal drug store in Mansoura city, Egypt, was previously reported to have a high inhibitory effect against RH-GFP in vitro of 97.07% ± 8.7 and 65.73% ± 4.45 when tested at 50 µg/ml and 10 µg/ml, respectively (Elazab et al., 2020). However, I found that *C. colocynthis* demonstrated high cytotoxicity with no efficacy against both RH-GFP and Nc1-GFP (Tables 7 and 8). These contrasting findings suggest that the actions of an extract from the same plant origin may differ depending on the site and time of collection, environmental climate, method of extraction used, and the portion of the plant extracted. My data reveal that different wild plant extracts collected from Egypt with low or no cytotoxicity demonstrate potent activity against the growth of both *T. gondii* and *N. caninum* tachyzoites in vitro. These plants may be useful in the development of medications used to treat toxoplasmosis and neosporosis in the future. I plan to try to characterize and

identify the main active components of the potent crude extracts and evaluate their efficacies in vivo against both parasites in subsequent studies.

3-5. Summary

Wild medicinal plants from the desert have been traditionally used as antimicrobial agents in Egypt and this use of medicinal plants can help overcome issues associated with commercial drugs such as toxicity and resistance. However, little is known about the antiprotozoal efficacy of these plants. Here, I evaluated the in vitro activity of extracts from certain wild Egyptian desert plants against *Toxoplasma gondii* and *Neospora caninum*. Of the 12 plant extracts tested, the methanolic extracts from *Artemisia judaica, Cleome droserifolia, Trichodesma africanum,* and *Vachellia tortilis* demonstrated potent activity against the growth of *T. gondii*, with half-maximal inhibitory concentrations (IC_{50}s) of 2.1 µg/ml, 12.5 µg/ml, 21.8 µg/ml, and 24.5 µg/ml, respectively. Their mean selectivity index (SI) values were 150.8, 29.6, 18.9, and 22.6, respectively. These extracts, along with an ethanolic extract of *P. undulata,* were then evaluated against *N. caninum. C. droserifolia*, an ethanolic extract of *P. undulata*, *T. africanum*, *A. judaica*, and *V. tortilis* demonstrated potent efficacy against the growth of *N. caninum,* with mean IC_{50}s of 1.0 µg/ml, 3.0 µg/ml, 3.1 µg/ml, 8.6 µg/ml, and 17.2 µg/ml, respectively. Their mean SI values were 370.9, 18.5, 133.2, 36.8, and 32.2, respectively. These findings indicate that *A. judaica* is most potent against *T. gondii*, while both *C. droserifolia* and *T. africanum* extracts were more effective to *N. caninum*. My data suggest these extracts could provide an alternative treatment for *T. gondii* and *N. caninum* infections.

Table 7. Mean IC_{50}s of Egyptian plant extracts against *Toxoplasma gondii* (RH-GFP) and HFF cells in vitro

Plant Extract	Plant family	Plant part	Mean IC_{50} (µg/ml)		Mean Selectivity index (SI)
			RH-GFP [a] (±SD)	HFF cells [b] (±SD)	
Aerva javanica (Burm.f.) Juss. ex Schult.	Amaranthaceae	leaves	>100	378.1 (134.0)	>3.7
Anabasis setifera Moq.	Amaranthaceae	leaves	>100	1,263.6 (194.9)	>12.6
Artemisia judaica L.	Asteraceae	leaves	2.1 (0.6)	316.8 (88.8)	150.8
Calotropis procera (Aiton) Dryand.	aq.	Apocynaceae	7.2 (3.3)	41.5 (22.6)	5.7
	M80%	flowers	1.9 (0.1)	2.9 (1.5)	1.5
Citrullus colocynthis (L.) Schrad.	E70%	Cucurbitaceae	seeds	>100	88.0 (14.3)	>0.8
	M80%		seeds	>100	65.6 (7.2)	>0.6
Cleome droserifolia (Forssk.) Delile	Cleomaceae	leaves	12.5 (9.4)	370.9 (95.3)	29.6
Forsskaolea tenacissima L.	Urticaceae	leaves	64.5 (12.8)	519.0 (141.9)	8.0
Ochradenus baccatus Delile	Resedaceae	fruit	>100	1,179.0 (245.4)	>11.7
Ocimum basilicum L. (E70%)	Lamiaceae	leaves	46.6 (28.3)	252.6 (13.9)	5.4
Pulicaria undulata (L.) C.A.Mey.	(E70%)	Asteraceae	flowers	15.4 (14.5)	55.5 (10.7)	3.6
	(M80%)		flowers	46.9 (7.9)	197.5 (61.3)	4.2
Trichodesma africanum (L.) Sm.	Boraginaceae	leaves	21.8 (14.0)	413.0 (96.9)	18.9
Vachellia tortilis subsp. raddiana (Savi) Kyal. & Boatwr.	Fabaceae	seeds	24.5 (0.5)	554.5 (110.5)	22.6
Sulfadiazine			99.4 µg/ml*	>1,000*	N.D

[a] The mean IC_{50} and standard deviation values against *T. gondii* (RH-GFP) were calculated from the average of three independent experiments after a 72-hour culture of the parasites with a plant extract.

[b] The mean IC50 against HFF cells was calculated from the average of three independent experiments after a 72-hour treatment. Experiments with extracts that had no effect with IC50 >100 µg/ml were not replicated. Except for those indicated to have an ethanolic or aqueous extract, all the plant extracts were methanolic. Average IC_{50}s from all plant extracts were calculated from three independent experiments with near values. IC_{50}: half maximal inhibitory concentration 50; SD: standard deviation; SI: selectivity index; RH-GFP, a green fluorescent protein expressing-RH strain of *T. gondii*; HFF: human foreskin fibroblast; M80%; 80% methanol; E70%; 70% ethanol; aq.; aqueous, *data from (Leesombun et al., 2019).

Table 8. Mean IC_{50}s of Egyptian plant extracts against *Neospora caninum*-GFP and HFF cells in vitro

Plant Extract	Plant family	Plant part	Mean IC_{50} (µg/ml)		Mean Selectivity index (SI)
			Nc1-GFP [a] (±SD)	HFF cells [b] (±SD)	
Artemisia judaica L.	Asteraceae	leaves	8.6 (3.2)	316.8 (88.8)	36.8
Calotropis procera (Aiton) Dryand.	Apocynaceae	flowers	1.8 (1.6)	41.5 (22.6)	23.0
aq. M80%		flowers	0.9 (0.7)	2.9 (1.5)	3.0
Cleome droserifolia (Forssk.) Delile	Cleomaceae	leaves	1.0 (0.3)	370.9 (95.3)	370.9
Pulicaria undulata (L.) C.A.Mey.	Asteraceae	flowers	3.0 (3.0)	55.5 (10.7)	18.5
(E70%)					
Trichodesma africanum (L.) Sm.	Boraginaceae	leaves	3.1 (1.6)	413.0 (96.9)	133.2
Vachellia tortilis subsp. raddiana (Savi) Kyal. & Boatwr.	Fabaceae	seeds	17.2 (9.2)	554.5 (110.5)	32.2
Sulfadiazine			99.4 µg/ml*	>1,000	N.D

[a] The mean IC_{50} and standard deviation values against *N. caninum* (Nc1-GFP) were calculated from the average of three independent experiments after a 72-hour culture of the parasites with a plant extract.

[b] The mean IC_{50} against HFF cells was calculated from the average of three independent experiments after a 72-hour treatment. Except for those indicated to have an ethanolic or aqueous extract, all the plant extracts were methanolic. IC_{50}: half maximal inhibitory concentration 50; SD: standard deviation; SI: selectivity index; Nc1-GFP: a green fluorescent protein expressing-Nc1 strain of *N. caninum*; HFF: human foreskin fibroblast; M80%: 80% methanol; E70%: 70% ethanol; aq.: aqueous, * data from (Leesombun et al., 2019)

Table S2. Reported medicinal uses of the wild plant extracts used in this study

Plant Extract	**Plant family**	**Medicinal use** (References)
Aerva javanica (Burm.f.) Juss. ex Schult.	Amaranthacea	Anthelmintic, diuretic, demulcent (Movaliya and Zaveri, 2014)
Anabasis setifera Moq.	*Amaranthaceae*	Anti-inflammatory (Abdou et al., 2013).
Artemisia judaica L.	*Asteraceae*	Treatment of stomach ache, cardiac diseases, sexual weakness, diabetes, gastro-intestinal disorders and external wounds (Abu-Darwish et al., 2016).
Calotropis procera (Aiton) Dryand.	*Apocynaceae*	Antischistosomal activity (Seif el-Din et al., 2014), antioxidant, antimicrobial and cytostatic properties (Kumar and Arya, 2006)
Carthamus tinctorius L.	*Asteraceae*	Laxatives, poison antidotes, antioxidant, and neuroprotective activities (Delshad et al., 2018).
Citrullus colocynthis (L.) Schrad.	*Cucurbitaceae*	Antimicrobial (Najafi et al., 2010), hypolipidemic (Rahbar and Nabipour, 2010), and hypoglycemic (Abdel-Hassan et al., 2000)
Cleome droserifolia (Forssk.) Delile	*Cleomaceae*	Hypoglycemic action (Yang et al., 1990)
Forsskaolea tenacissima L.	*Urticaceae*	antibacterial, antinociceptive, antifungal (Sher et al., 2017) antioxidant ,and larvicidal activity (El-Hela et al., 2013)
Ochradenus baccatus Delile	*Resedaceae*	Antimicrobial, antiallergic (Al-Shabib et al., 2018)
Ocimum basilicum L.	*Lamiaceae*	Antioxidant, antibacterial, and antifungal (Elansary et al., 2020)
Pulicaria undulata (L.) C.A.Mey.	*Asteraceae*	Antioxidant (Mustafa et al., 2020), anti-liver cancer (Emam et al., 2019)
Trichodesma africanum (L.) Sm.	*Boraginaceae*	Antifungal (Ahmed et al., 2015)
Vachellia tortilis subsp. raddiana (Savi) Kyal. & Boatwr.	*Fabaceae*	Antiparasitic (leishmania), and antimalarial (Kigondu et al., 2009)

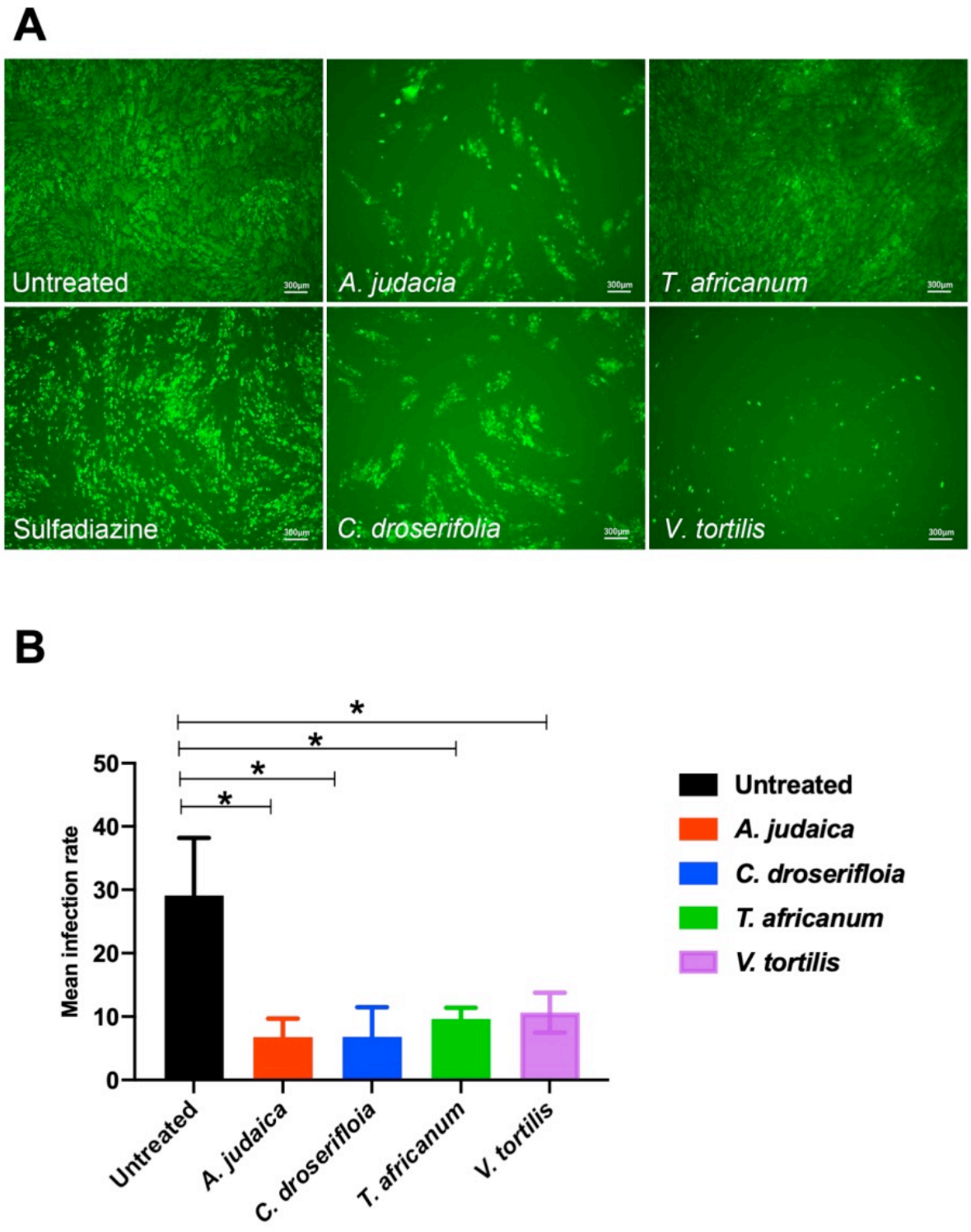


Fig. 12. Effect of plant extracts against *T. gondii* growth in vitro (A) Representative images of a green fluorescent protein-expressing RH strain of *T. gondii* (RH-GFP)-infected human foreskin fibroblast cells after treatment with 100 µg/ml of each of the following plant extracts: *Artemisia judaica*, *Trichodesma africanum*, *Cleome droserifolia,* or *Vachellia. tortilis*, and sulfadiazine (1 mg/ml), or medium alone, Scale bar = 300 µm. **(B)** Effects of the selected potent plant extracts at 100 µg/mL on extracellular *T. gondii* in vitro. RH-GFP was pretreated with either one of the four different extracts (*A. judaica*, *T. africanum*, *C. droserifolia*, or *V. tortilis*) (100 µg/mL/plant extract), or MEM with 8% FBS (1 mL/tube) and Sulfadiazine (1 mg/ml) and the infection rate for RH-GFP was measured. Each bar

represents the mean ± SD of three wells per plant sample. The results were representative of two independent experiments of similar data. *Statistically significant differences were determined by a one-way ANOVA and a Tukey-Kramer post hoc analysis ($P < 0.05$).

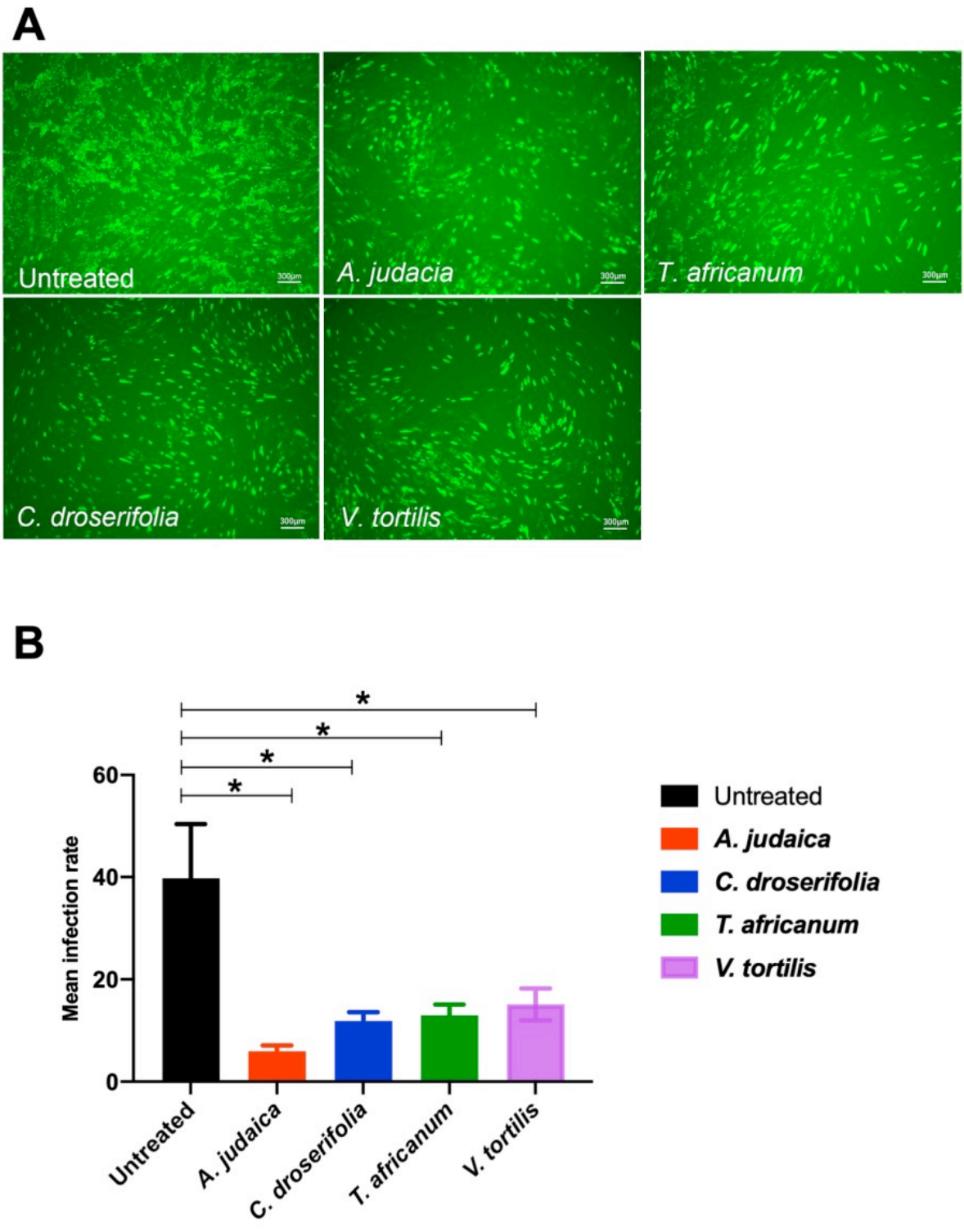


Fig. 13. Effect of plant extracts against *N. caninum* growth in vitro (A) Representative images of a green fluorescent protein-expressing Nc1 strain of *N. caninum* (Nc1-GFP)-infected human foreskin fibroblast cells after treatment of 100 µg/ml of either one of the plant extracts (*Artemisia judaica*,

Trichodesma africanum, *Cleome droserifolia,* or *Vachellia. tortilis*) or medium alone, Scale bar = 300 μm **(B)** Effects of the selected potent plant extracts at 100 μg/mL on extracellular *N. caninum* in vitro. The Nc1-GFP line was pre-treated with one of the four extracts (*A. judaica*, *T. africanum*, *C. droserifolia*, *V. tortilis*), and the infection rate for Nc1-GFP was measured. Each bar represents the mean ± SD of three wells per plant sample. The results were representative of two independent experiments of similar data. Statistically significant differences were determined by a one-way ANOVA and a Tukey-Kramer post hoc analysis ($P < 0.05$).

Fig. S15. Sampling map of the Egyptian plant samples collected. Plant taxa were collected from May 14-19, 2019, in a field survey of two locations along the southeastern desert roads around Qena Governorate. The first site was Qena-Safaga desert road **(A)**, and the second site was Qena-Sohag desert road **(B)**. Map designed by DIVA-GIS 7.5.0 software to show all country roads and Governorates; the sample collection sites were approximately determined and are shown in the magnified box and by the blue lines on the Google map.

Chapter 4

Wild Egyptian medicinal plants show in vitro and in vivo cytotoxicity and antimalarial activities

4-1. Introduction

Malaria is caused by parasites belonging to the phylum Apicomplexa, genus *Plasmodium*. In 2019, approximately 229 million cases of malaria and 409,000 associated deaths were reported across 87 malaria-endemic countries (WHO, 2020). Human malaria cases are caused by four different *Plasmodium* species—*Plasmodium ovale*, *Plasmodium vivax*, *Plasmodium malariae, Plasmodium falciparum*—of which *P. falciparum* is considered to be the most lethal (White et al., 2014; Garrido-Cardenas et al., 2019). *Plasmodium* parasites are typically transmitted by the bite of an infected female *Anopheles* mosquito, although malaria can also be transmitted through exposure to blood products from an infected individual (transfusion malaria) or congenitally (Trampuz et al., 2003). Multidrug-resistant *Plasmodium* parasites are the biggest challenge to health care in most malaria-endemic areas. Thus, research to develop new antimalarial drugs is critical (Kremsner et al., 1987).

The two main malaria species that are responsible for most human malaria cases, *P. falciparum* and *P. vivax*, have developed resistance against chloroquine. This drug resistance first emerged in the late 1950s in both Colombia and at the Cambodia–Thailand border and may stem from the great success of chloroquine and its multiple large-scale usages over the decades (Payne, 1987). Great efforts have been made to develop the novel active agent, artemisinin, as an alternative drug to chloroquine. However, presently, there is no single drug effective for treating multi-drug resistant malaria, and effective combination treatment includes artemisinin derivatives, such as artesunate, or mixtures with previously developed drugs, such as an atovaquone-proguanil combination (Taylor and White, 2004).

From ancient times, medical plants have been used for various pharmacological purposes because they contain many useful biological compounds (Dias et al., 2012). More than 1,277 plant species have been traditionally used for the treatment of malaria (Willcox et al., 2011; Rasoanaivo et

al., 2011). Natural products still have an effective role in disease treatment. Finding anti-parasitic compounds produced by natural products, especially traditional medicinal plants from Asia, Africa, or the Americas, which have been reported as being successfully used to treat many diseases, could be an initial step toward controlling an array of diseases (Wink, 2012). Currently, the WHO recommends widespread use of the RTS,S/AS01 (RTS,S) malaria vaccine among children at risk in sub-Saharan Africa (WHO, 2021). Egypt has multiple aromatic and medicinal plants owing to its favorable geographical position, climate, and soil condition; thus, it is a useful site for exploring herbal and medicinal plants (Mostafa and Singab, 2018). Previous studies illustrated the use of plant extracts in inhibition of *P. falciparum* in vitro (Hout et al., 2006; Bagavan et al., 2011; Rufin Marie et al., 2018; Kwansa-Bentum et al., 2019) and in vivo using various doses of *Ficus platyphylla* plant extract ranging from 100-300 mg/kg/day against *Plasmodium berghei* infection (Shittu et al., 2011). In addition, other studies evaluated the effect of plant extracts by oral treatment in a murine model by chemotherapeutic test against different murine *Plasmodium* species in BALB/c mice (Chandel et al., 2012; Tepongning et al., 2013; Chutoam et al., 2015). Furthermore, the combination of the plant extracts with the reference drug artemisinin in treatment of *Plasmodium yoelii* was also reported (Mohd Ridzuan et al., 2007). Other reported study evaluated febrifugine and isofebrifugine mixture prepared from the dried leaves of *H. macrophylla var. Otaksa* against three rodent *Plasmodia* species; *P. yoelii* 17XL, *P. berghei* NK65, and *P. chabaudi* AS in Institute of Cancer Research (ICR) mice (Ishih et al., 2003). The previous reported experimental models support the use of our in vitro assay and in vivo model in treatment of *P. falciparum* in vitro and *P. yoelii*-infected mice in murine malaria model. Therefore, the present study aimed to evaluate the effectiveness of extracts from Egyptian medicinal plants randomly selected from the desert roads against human malaria, first via an in vitro assay and then with a murine malaria model.

4-2 Methods

Ethical statement

This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan (permit numbers 19-185, 20-157, 21-32). Mouse work, such as injection with parasites or extracts, and euthanasia was implemented under general inhalation anesthesia induced with isoflurane (2%) to minimize animal suffering. Mice were euthanized by cervical dislocation at 30 days after parasite infection.

Plasmodium falciparum culture and maintenance

Plasmodium falciparum parasites were transferred to previously washed human O^+ red blood cells (RBCs) obtained from Hokkaido Red Cross Blood Center maintained in fresh complete RPMI-1640 medium (Sigma, St Louis, MO, USA), which was supplemented with a mixture of 6 g of HEPES (Sigma), 2 g of $NaHCO_3$, 25 mg of hypoxanthine, 5 g of albumax II (Gibco, Carlsbad, CA, USA), and 250 µl of gentamicin (stock concentration, 50 mg/ml) in dissolved in MilliQ water. The final prepared complete medium was filtered with a 0.20-µm membrane filter (IWAKI, Saitama, Japan). The parasite cultures were maintained at 37 °C in a 5% CO_2 atmosphere.

Plant material collection and extraction

The plants used in this study were obtained from a field survey conducted in two locations in Qena governorate (Latitude: 26° 09' 51.05" N, Longitude: 32° 43' 36.16" E), which is in the southern region of Egypt: Qena-Sohag and Qena-Safaga (after Km 85) desert roads, Eastern desert, Egypt. A map marking the collection sites is shown in Figure S16. Plants collection sites coordinates were shown in table S3. Material was collected from 13 different plant species in May 2019 (plant flowering season). Samples were collected between 4:00 AM and 12:00 PM. The plant samples were collected under the approval of South Valley University, Qena,

Egypt and were microscopically identified by Dr. Mohamed Owis Badry, in the herbarium of South Valley University at Faculty of Science, South Valley University, Egypt and voucher specimens were deposited in the same herbarium. Identification was performed according to the available literature (Boulos, 1999a;1999b; 2002; 2009), and an official identification letter was obtained. Images of the herbarium sheets of the identified plant species from which samples were collected are shown in Figure S17. Plant taxonomy and species were further updated in accordance with information from Plants of the World Online (POWO, 2019).

For plant collection from the study areas, although that there are no specific licenses were required for the field studies, permission for collection of plants was obtained from Faculty of Veterinary Medicine, South Valley University, Qena, Egypt. Collection was performed under the guidelines and rules of South Valley University, Qena. The surveyed locations were not protected or privately-owned in any way and the field studies did not include any protected or endangered Egyptian plant species. The Latin binomial names of all plant extracts studied in this study were shown in Table 9.

Plant samples were dried in the shade for 3–10 days, then a fine powder was obtained from the dried leaves, flowers, fruit, or seed parts by using a kitchen blender. The powdered plant material from each plant was dissolved at a 1:10 ratio in 80% methanol, 70% ethanol, or distilled water (100 g of plant powder/1 L of solvent) for a minimum of 1–3 days. The plant supernatant was further collected and filtrated by glass filtration apparatus and was collected in wide conical flask, and then it was dissolved in a wide petri dish at room temperature for 1–3 days. The final crude extract was collected in centrifuge tubes and stored in −30 °C until use. To test the antimalarial potential of the various plant extracts, they were solubilized individually in the solvent dimethyl sulfoxide (DMSO) to prepare stock solutions (100 mg/ml).

Determination of cytotoxicity of plant extracts

To determine the cytotoxic potential of the plant extracts, their cytotoxicity against human foreskin fibroblast (HFF) cells was evaluated. Cell suspensions (1×10^5 cells/ml) in Dulbecco's Modified Eagle medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Nichirei Bioscience, Tokyo, Japan) were plated at 100 µl/well in 96-well plates and incubated at 37 °C in a 5% CO_2 atmosphere for 48 h. The plant extracts were added to the cells at final concentrations of a two-fold serial dilution starting from 1000 µg/ml. To evaluate cell viability, cell proliferation inhibition (%) was calculated as described previously (Leesombun et al., 2019; Pagmadulam et al., 2020).

In vitro anti-plasmodial activity

Plasmodium falciparum (3D7 strain) was maintained in O^+ human erythrocytes (1% hematocrit) in complete RPMI medium (Sigma-Aldrich). *P. falciparum* was further synchronized to the ring stage with 5% sorbitol (>90%, as verified by light microscopy on Giemsa-stained blood smears [Giemsa stain for microscopy, Merck, Darmstadt, Germany]). Parasite solutions were prepared at 0.5% parasitemia and 2% hematocrit in complete RPMI medium. A 50-µl sample of the infected erythrocytes was added to each well of 96-well plates containing 50 µl of plant extract (concentrations ranging from 0.25–100 µg/ml). Medium only was used as a negative control, while chloroquine was used as a positive control. The plates were then incubated in an atmosphere of 5% CO_2, 5% O_2 at 37 °C for 72 h.

Parasite growth inhibition was determined by adding 100 µl of 0.02% of Syber Green I stain (SYBR® Green I Nucleic acid stain 10,000×, Lonza, Rockland, ME, USA) in lysis buffer (25 mM Tris, pH 7.5, containing 10 mM ethylenediamine tetraacetic acid, 0.01% saponin, and 0.1% Triton X-100) to each well, mixing gently, and incubating the plates for 1–2 h in the dark (Johnson et al., 2007; Leesombun et al., 2021). The relative fluorescent inhibition values were determined by using a fluorescent plate reader Fluoroskan Ascent (Thermo Labsystems, Waltham, MA, USA) with excitation and emission wavelengths of 485 nm and 518 nm, respectively (Leesombun et al., 2019; Leesombun et al., 2021; Arifeta et al., 2021). Parasite morphology was observed by examining Giemsa-stained blood smears with an all-in-one microscope BioRevo BZ-9000 (Keyence BioRevo, Tokyo, Japan). Parasite

growth inhibition percentages were calculated as described previously (Leesombun et al., 2021; Arifeta et al., 2021). The antiplasmodial activities of the natural plant extracts used in this study were classified as follows: IC_{50} <0.1 µg/ml: very good activity; IC_{50} between 0.1–1 µg/ml: good activity; IC_{50} between 1.1–10 µg/ml: good to moderate activity; IC_{50} between 11–50 µg/ml weak activity; IC_{50} >100 µg/ml: inactive according to the classification mentioned in the reported study (Rasoanaivo et al., 2004).

In vivo antimalarial efficacy of plant extracts

BALB/c mice, originally purchased from Clea Japan (Tokyo, Japan), were bred under specific pathogen-free conditions in the animal facility of the National Research Center for Protozoan Diseases at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. The animals were treated in accordance with the guiding principles for the care and use of research animals published by the Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. The animals were kept under standard laboratory conditions on a 12/12-h light/dark cycle at 21 °C under 40% relative humidity and fed with commercial food and water ad libitum.

The non-lethal strain *Plasmodium yoelii* 17XNL was recovered from a stock of frozen parasitized RBCs (pRBC) via passage in donor mice intraperitoneally inoculated. Parasitemia was monitored daily. When the parasitemia level reached 20%–30%, the donor mice were anesthetized, and blood was collected by cardiac puncture into a syringe containing 0.1 ml of ethylene diamine-N, N, N', N'- tetraacetic acid disodium salt (EDTA) (Djindo Kumamoto, Japan).

Two male BALB/c mice aged 8–10 weeks and weighing 25–30 g, were infected with approximately 1×10^7 *P. yoelii*-infected erythrocytes in total volume of 0.5 ml of phosphate-buffered saline (PBS). For each independent experiment, the mice were randomly divided into three groups of five according to previous published studies. AMA was aware of the group allocation at the different stages of the experiment. Total 17 mice were used for one trial. When the level of parasitemia reached 1%, oral treatment using 100 mg/kg/day of plant extracts was begun and continued for 1 week from day 0 (3 h post-challenge) until day 6 post-infection. The negative-control animals received only PBS. The parasitemia was assessed daily until 30 days post-infection by examining thin blood films made from

mouse tail blood and stained with 10% Giemsa solution. The films were examined using a microscope to determine the parasite suppression activity of each extract. To measure the hematocrit percentage, 10 µl of blood was collected from the tail vein every other day until 30 days post-infection and measured by Celltac-α MEK-6550 (Nihon Kohden, Tokyo, Japan). A parasitemia suppression test (chemotherapeutic test) was performed daily for 1 week from challenge (day 0) until 6 days post-infection; the percentage of parasite growth suppression was calculated by using the following previously reported equation (Kweyamba et al., 2019), which is slightly modified from the study that originally reported it (Peters, 1975).

$$\%\ of\ parasite\ growth\ suppression\ =\ (A\ -\ B)/A\ \times 100 \quad \text{Eq. 1.}$$

Where *A* is the mean parasitemia of the untreated group and *B* is parasitemia of each individual mouse in the treated groups.

The parasitemia percentage, bodyweight, and survival rates were monitored daily, and the hematocrit was monitored every other day. The percentage of parasitemia of each mouse was calculated by counting the number of parasite-infected erythrocytes per 600–1000 erythrocytes visible under a light microscope in 4–5 randomly selected fields of methanol-fixed thin blood smears slides stained with 10% Giemsa solution.

$$\%\ Parasitemia\ =\ (number\ of\ infected\ RBCs)/(total\ Number\ of\ RBCs\ ) \times 100 \quad \text{Eq. 2}$$

Order of treatment starts from the control then the treated groups. Order of challenge infection starts from the control then the treated groups. Measurements of body weight, hematocrit, and parasitemia were randomly done in group starting from control then the treated groups. Cage location was not changed from the start of the experiment until the end.

Statistical analysis

Graph Pad Prism 8.4.3 software (Graph Pad Software Inc. La Jolla, CA, USA) was used for all statistical tests. For the in vitro data, the IC_{50} values for the inhibition percentage of parasites and host cells were determined. The final mean IC_{50} of anti-*P. falciparum* (3D7) activity was calculated

based on three independent experiments, and mean IC_{50} values against HFF cells were calculated based on three independent experiments. For the in vivo data (mean parasitemia %, mouse bodyweight and hematocrit changes), statistical analyses were performed using a two-way analysis of variance (ANOVA). Survival curves were generated with the Kaplan–Meier method, and survival rates were analyzed by a χ^2 test. Statistically significant differences (those with a *p*-value of <0.05) are marked in the figures by asterisks and defined in each figure legend. There were no any criteria used for including and excluding animals.

4-3 Results

In vitro antimalarial efficacy of plant extracts

The in vitro activities against *P. falciparum* 3D7 growth of 13 different types of Egyptian plant extracts were evaluated. Among the 13 tested plant extracts, four (extracts of *Trichodesma africanum*, *Artemisia judaica*, *Cleome droserifolia,* and *Vachellia tortilis*) showed low-to-moderate activity against *P. falciparum* 3D7; their mean IC_{50} values were 11.7 µg/ml, 20.0 µg/ml, 32.1 µg/ml, and 40.0 µg/ml, respectively, and their mean selectivity index values were 35.2, 15.8, 11.5, and 13.8, respectively, (Table 10). Despite the low-to-moderate activities of the previously mentioned plant extracts, they possess good selectivity index values (Table 10). The ethanolic extract of *Pulicaria undulata* and both the ethanolic and methanolic extracts of *Citrullus colocynthis* showed weak activity against *P. falciparum* 3D7 growth (mean IC_{50} values: 18.9 µg/ml, 51.7 µg/ml, 45.9 µg/ml, respectively) and had mean selectivity index values of 2.9, 1.7, and 1.4, respectively (Table 10).

All extracts of *Aerva javanica* and *Anabasis setifera,* both the aqueous and methanolic extracts of *Calotropis procera, Carthamus tinctorius, Forsskaolea tenacissima, Ochradenus baccatus*, and *Ocimum basilicum*, and the methanolic extract of *P. undulata* showed no efficacy against the growth of *P. falciparum* (3D7) in vitro, with IC_{50} values of >100 µg/ml (Table 10).

In vitro effect of plant extracts on *P. falciparum* growth stages and morphology

To confirm the in vitro antimalarial efficacy of the tested plant extracts, we observed thin blood smears from 72-h parasite culture (Fig. 14). Treatment with plant extracts for 72 h caused dose-dependent suppression of the parasite growth in the percentage of parasites (Fig. 14A). Among the four tested plant extracts, *T. africanum* crude extract showed the highest level of parasite growth inhibition at all parasite stages (Fig. 14A). Morphological alterations, such as cell shrinkage, and parasite fragmentation were observed following treatment with plant extract concentration of 50 µg/ml as well as after treatment with the positive control drug, chloroquine, in comparison with untreated parasites (Fig. 14B).

Cytotoxicity of plant extracts

The cytotoxic potential of all included plant extracts at concentrations ranging from 1,000–7.8 µg/ml (two-fold serial dilutions) were determined, and the mean IC_{50} values against HFF cells were calculated (Table 10). The methanolic and aqueous extracts from *C. procera*, the ethanolic extract from *P. undulata*, both the methanolic and ethanolic extracts from *C. colocynthis*, the methanolic extract from *P. undulata*, and the ethanolic extract from *O. basilicum* showed the highest cytotoxicity against HFF cells with mean IC_{50}s of 2.9, 41.5, 55.5, 65.6, 88.0, 197.5, and 252.6 µg/ml, respectively. Extracts from plants *A. judaica, C. droserifolia, A. javanica, T. africanum, F. tenacissima,* and *V. tortilis* showed moderate-to-weak toxicity against HFF cells with mean IC_{50}s of 316.8, 370.9, 378.1, 413.0, 519.0, and 554.5 µg/ml, respectively. Lastly, Extracts from *A. setifera* and *O. baccatus* were nontoxic or safe for HFF cells as cytotoxicity was not observed and their mean IC_{50}s were >1,000 µg/ml of 1263.6 µg/ml, and 1179.0 µg/ml, respectively (Table 10).

In vivo antimalarial activity of plant extracts

Their in-vitro results suggested that plant extracts from *T. africanum, C. droserifolia, A. judaica*, and *V. tortilis* have low or no cytotoxicity and might have activity against *Plasmodium* parasites. Therefore, we decided to evaluate their efficacy against *P. yoelii* in a murine malaria model. A chemotherapeutic test of each plant extract was performed beginning at the treatment start time of each extract (3 h post-challenge; day 0) and continuing through 6 days post-infection (the end of the course of the treatment). The four tested extracts each showed a time-dependent suppression of parasitemia through 7 days post-infection (Fig. 15). The mean level of parasite growth suppression observed after treatment with extracts of *A. judaica*, *C. droserifolia*, *T. africanum*, or *V. tortilis* ranged from 13.5%–60.6%, 17.1-61.9%, 35.2%–65.5%, and 36.3%–72.5%, respectively (Fig. 15, Table S4).

In the murine malaria model, a significantly reduced level of parasitemia was observed from 6–15 days post-infection after oral treatment with *T. africanum* extract (Fig. 16C), whereas there was no significant difference in the hematocrit, bodyweight change, or survival rate of *T. africanum* extract-treated mice as compared with mice in the untreated group (Figs. S18C, S19C, and S20C). Parasite growth suppression by *C. droserifolia* extract was not observed, except at day 14 post-infection (Fig. 16B). The hematocrit, bodyweight change, and survival rate of *C. droserifolia* extract-treated mice did not show any significant difference compared with untreated animals (Figs. S18B, S19B, and S20B). Although some parasite suppression efficacy was observed for *A. judaica* extract following treatment initiation through 6 days post-infection (Table S4; Fig. 15A), the level of parasite suppression at the peak of parasitemia was not significant, and *A. judaica* extract-treated mice took a similar length of time to recover as compared with the untreated mice (Fig. 16A); furthermore, the hematocrit, bodyweight percentage, and survival rate of these mice were not different from those of the untreated animals (Figs. S18A, S19A, and S20A). The in vivo parasite suppression induced by *V. tortilis* extract was only partial, but it was significant during the peak of parasitemia at days 10, 11, and 12 post-infection (Table S4, Fig. 16D); however, the hematocrit, bodyweight change, and survival rate of *V. tortilis* extract-treated mice were not significantly different from those of the untreated mice (Figs. S18D, S19D, and S20D).

4-4. Discussion

Trichodesma africanum has been reported to have multiple medicinal uses (Table S3). The antibacterial efficacy of an oil extract of *T. africanum* was evaluated against the growth of three bacterial strains obtained from the American type culture collection (ATCC) (*Staphylococcus aureus* [ATCC 25923], *Escherichia coli* [ATCC 25922], and *Pseudomonas aeruginosa* [ATCC 27853]) as well as against the growth of methicillin-resistant *S. aureus* (MRSA) isolates, and its antifungal activity was tested against *Candida albicans* (Jaradat et al., 2016). The chemical constituents of *T. africanum* include essential oils, steroids, coumarins, flavonoids, phenolics, alkaloids, and glycosides (El-Moaty, 2009). Its chemical constituents might be involved in its biological activity, as flavonoids had been reported to have antiprotozoal activity, specifically anti-leishmanial and anti-trypanosomal activities (Tasdemir et al., 2006).

Trichodesma africanum collected from Saudi Arabia was reported to have weak antimalarial efficacy in vitro against a chloroquine-sensitive strain of *P. falciparum* with an IC_{50} value of 32.0 µg/ml (SI of 2) (Abdel-Sattar et al., 2010). Here, *T. africanum* from an Egyptian desert was found to possess a moderate-to-weak activity in vitro against *P. falciparum* with a mean IC_{50} value of 11.7 µg/ml (SI of 35.2; Table 10). These results suggest that the efficacy of plant extracts can vary owing to differences in the area, time of day, and season of plant collection, extraction procedures used in collection, and cell lines used for the determination of its cytotoxic potential. Although the antimalarial activity of *T. africanum* was previously examined in vitro, our study is the first to illustrate the antimalarial efficacy of a *T. africanum* extract in a mouse model of malaria.

Cleome droserifolia, which is facing extinction, is found in tropical and subtropical areas, such as North Africa and India (Boulos, 1999a;Batanouny et al., 1999; Kamel et al., 2010). *C. droserifolia* is an important plant species owing to its historical use in traditional medicine in Egypt (Rahman et al., 2004; Aparadh et al., 2012; Moustafa et al., 2019). Regarding its medicinal uses, it has an immediate effect on abdominal and rheumatic pain, is anti-inflammatory, and is also effective for improving wound healing and treating snake bites and scorpion stings (Rahman et al., 2004; Aparadh

et al., 2012; Sarhan et al., 2016; Moustafa et al., 2019). These effects are attributed to the rubefacient, antimicrobial, analgesic, antipyretic, antioxidant, and anti-inflammatory activities of its components (El-Ghazali et al., 2010; Sarhan et al., 2016; Abd el-Gawad et al., 2018; Moustafa et al., 2019; Paniker et al., 2020), which include flavonoids, glycosides, carbohydrates, buchariol, teucladiol, daucosterol, cardenolides, saponins, sterols, tannins, catechins, triterpenes and sesquiterpenes as well as a newly described alkaloid found in its aerial parts (Aboushoer et al., 2010; Hussain et al., 2015; Abdullah et al., 2016; Singh et al., 2018). *C. droserifolia* was previously reported to have an antibacterial effect (Muhaidat et al., 2015), but little is known about its antiprotozoal efficacy. Although there is no literature on the effect of *C. droserifolia* extract on malaria, another species in the *Cleome* genus (e.g., *Cleome rutidosperma*) was reported to have moderate anti-plasmodial activity against *P. falciparum* CQS D10 strain in vitro (IC_{50} value: 34.4 µg/ml) (Bose et al., 2010). In the present study, *C. droserifolia* was evaluated with both in vitro and in vivo assays. It had a moderate-to–weak inhibitory effect, with an IC_{50} value of 32.1 µg/ml and an SI of 12.9 in vitro against *P. falciparum*, and partially inhibited murine malaria in a short-term treatment of 100 mg/kg/day.

Recently, *A. tortilis* plant name was changed and become well known as *V. tortilis* according to (POWO, 2019), therefore, all information about *V. tortilis* in this discussion section is reported under its old name, *A. tortilis*. This plant is one of 1,200 species of *Acacia*. It grows in tropical and subtropical areas with temperatures in the range of 40–45 °C in summer and <5 °C in winter, such as locations in African, Australian, and Arabian countries (Ibrahim and Aref, 2000). *A. tortilis* possesses multiple medicinal and pharmacological properties, e.g., antidiabetic, antifungal, antidiarrheal, antitussive, and anti-inflammatory (Yadav et al., 2013), but little is known about its antiprotozoal potential. Despite of the potent effectiveness of the methanolic extract of *A. tortilis* collected from Kenya as an anti-parasitic treatment, there are conflicting reports regarding its antimalarial activity. Some studies found that it had moderate efficacy against *P. falciparum* (Kigondu et al., 2009; Yadav et al., 2013). In contrast, although the first in a pair of studies initially showed that *A. tortilis* root bark showed antimalarial activity, its follow-up study did not consider it to be an antimalarial candidate (Yadav et al., 2013; Nguta and Mbaria, 2013). In the present work, the methanolic extract from *A. tortilis* seeds collected from an Egyptian

desert showed moderate-to-weak activity in vitro against *P. falciparum* (3D7). These results suggest that the efficacy of *V. tortilis* extract against malaria may be correlated to its medicinal uses (i.e., as an antimicrobial or other treatment) or to its chemical constituents and may vary depending on the extracted plant part and area from which the plant was collected. In vivo parasite suppression against *P. yoelii* in mice was observed until 6 days post-infection, and this extract significantly suppressed the parasitemia during its peak from days 10–12 post-infection, suggesting that it has partial efficacy against murine malaria.

Artemisia judaica belongs to the family *Asteraceae*, which is one of the largest families of angiosperms and contains 1,600 to 1,700 genera and about 24,000 species distributed worldwide (Hussain et al., 2017). Known as shih in the Middle-East, *A. judaica* is an aromatic shrub found mainly in the deserts of the Middle-East, Egypt, and serval North African countries and is traditionally used as an anthelmintic drug (Wyk and Wink, 2004). Although *A. judaica* has not been previously reported to have antimalarial efficacy, numerous other *Artemisia* species have been found to have antimalarial activity, including *Artemisia nilagirica* (Petrus and Seetharaman, 2005), *Artemisia maciverae* (chloroform extract) (Ene et al., 2009), *Artemisia maritima* (ethanolic and petroleum extracts), *Artemisia nilegarica*, *Artemisia japonica* (Valecha et al., 1994), *Artemisia ciniformis*, *Artemisia biennis*, and *Artemisia turanica* (Mojarrab et al., 2015). Artemisinin, the well-known conventional drug discovered by Chinese scientists obtained from *Artemisia annua* (Covello, 2008), has also been found in several other species of *Artemisia*, including *Artemisia lancea*, *Artemisia apiacea* (Qian et al., 2005), *Artemisia vulgaris* (Numonov et al., 2019), *A. japonica* (Rashmi et al., 2014), *Artemisia sieberi* (Arab et al., 2006), *Artemisia absinthium* (Zia et al., 2007), *Artemisia dubia*, and *Artemisia indica* (Mannan et al., 2008). There is no available information about the presence of artemisinin in *A. judaica*.

Plasmodium often develops drug resistance, and there is now evidence of resistance to artemisinin drugs (Dondrop et al., 2009). Therefore, there is an urgent need to find novel candidates for the development of drugs to treat *Plasmodium*; other *Artemisia* plants, such as *A. judaica*, and other plant species may be useful sources. Several *Artemisia* species have been evaluated for their antimalarial activity in rodent malaria models. *A. vulgaris* showed potent activity without toxicity when

administrated orally to mice infected with *P. yoelii*, according to the results of a 4-day suppressive test performed following treatment with high doses (500 mg/kg and 1,000 mg/kg) of *A. vulgaris* extract (Kodippili et al., 2011). Furthermore, the efficacy of an ethanolic extract of *A. vulgaris* leaves was confirmed against *Plasmodium berghei* ANKA strain in ICR mice. Treatment with doses of 500, 750, and 1,000 mg/kg of this extract significantly reduced parasitemia by 79.3%, 79.6%, and 87.3%, respectively (Bamunuarachchi et al., 2013). *A. sieberi* from Iran showed antimalarial efficacy against *P. berghei* in NMRI mice, reducing some pathophysiological signs of malaria (Nahrevanian et al., 2012). An infusion of *A. annua* (tea) failed to cause any reduction to the parasitemia caused by *Plasmodium chabaudi* in OF1 mice (Atemnkeng et al., 2009), whereas the oral administration of dried whole *A. annua* leaves killed these parasites more effectively than did a comparable dose of the pure drug artemisinin in C57BL/6 mice (Elfawal et al., 2012).

Here, a methanolic leaf extract of *A. judaica* was found to possess moderate-to-weak antimalarial activity against *P. falciparum* with no apparent cytotoxicity along with a moderate efficacy against murine malaria at a lower dose (100 mg/kg/day) than used in previous studies. However, it did not cause a significant reduction in the parasitemia of *P. yoelii* during the peak of infection in mice.

Calotropis procera has been reported to have multiple biological and medicinal uses (Meena et al., 2010). An ethanolic extract of this plant was reported to have a schizonticidal effect in vitro (Sharma and Sharma, 2000). Furthermore, fractions from the leaf extract show anti-plasmodial activity (Mudi and Bukar, 2011). However, in my study, neither the methanolic nor the aqueous extract of *C. procera* flowers showed any efficacy in vitro against *P. falciparum* (3D7) when administered at a dose of 100 µg/ml.

Three rodent-specific *Plasmodium* species, *P. berghei*, *P. yoelii*, and *P. chabaudi*, are commonly used in animal models of malaria; these models exhibit different manifestations of the human disease. In vitro cultures of these parasites are not well established; thus, they require maintenance in mice (Huang et al., 2015). Here, I used a *P. yoelii* mouse model to evaluate the antimalarial efficacy of four plant extracts. In previous plant extract treatment trials of *P. yoelii* in Swiss albino mice, treatment

with aqueous or ethanolic extracts of *Phyllanthus amarus* at doses of 200, 400, 800, and 1,600 mg/kg/day was performed until 6 days post-infection; respectively, the aqueous extract induced 56.0%, 68.0%, 77.9%, and 81.2% parasite suppression, and the ethanolic extract induced 51.7%, 67.9%, 74.2%, and 52.3% parasite suppression (Ajala et al., 2011). Another study used 1.25 g/kg of methanolic extract from *Nigella sativa* seeds. Parasite suppression of 84.6%, 89.2%, and 94% was observed at days 6, 7, and 8 post-infection with *P. yoelii* nigeriensis, respectively (Okeola et al., 2011). Furthermore, the efficacies of methanolic-chloroform (MC) and methanolic-aqueous (MA) extracts from *Brucei mollis* collected from India were evaluated against *P. yoelii* N-67 (chloroquine-resistant strain [CQR]); they had respective median effective doses 50 (ED_{50}s) of 30 mg/kg/day and 72 mg/kg/day at 4 days post-infection and of 66 mg/kg/day and 79 mg/kg/day at 6 days post-infection (Prakash et al., 2013). In the present study, treatment with 100 mg/kg/day methanolic extracts of *A. judaica, C. droserifolia, T. africanum*, or *V. tortilis* from 0–6 days post-infection each caused significant parasite suppression, with mean suppression percentages ranges of 13.5%–60.6%, 17.1-61.9%, 35.2%–65.5%, and 36.3%–72.5%, respectively, despite the extract dose being lower compared with previously reported studies (Table S4).

This study showed that crude extracts of four wild plants collected from Egypt had antimalarial efficacy against the human malaria-causing parasite *P. falciparum* in vitro and against the murine malaria-causing parasite *P. yoelii* in a mouse model. Although the administration of these extracts at a dose of 100 mg/kg/day for a 7-day course of treatment did not achieve 100% inhibition of *P. yoelii* growth in BALB/c mice, the parasite suppression data suggests that these extracts may have potent antimalarial activity. Their efficacies are likely correlated with their multiple medicinal uses and their chemical constituents. Among the four tested candidates, the *T. africanum* crude extract possessed the highest parasite suppression ability in a short-term treatment course in vivo and had the highest IC_{50} in vitro against the human malaria-causing parasite *P. falciparum*, whereas *V. tortilis* extract showed moderate-to-weak effect against *P. falciparum* in vitro and induced partial inhibition against *P. yoelii* in vivo. These data support the use of these extracts in the future development of an antimalarial therapeutic. Further study will be needed to understand the mechanism of action and identify the main biological components of these crude extracts.

4-5. Summary

Medicinal plants have been successfully used as an alternative source of drugs for the treatment of microbial diseases. Finding a novel treatment for malaria is still challenging, and various extracts from different wild desert plants have been reported to have multiple medicinal uses for human public health, this study evaluated the antimalarial efficacy of several Egyptian plant extracts. I assessed the cytotoxic potential of 13 plant extracts and their abilities ~~of~~ to inhibit the in vitro growth of *Plasmodium falciparum* (3D7), and to treat infection with non-lethal *Plasmodium yoelii* 17XNL in an in vivo malaria model in BALB/c mice. In vitro screening identified four promising candidates, *Trichodesma africanum, Artemisia judaica, Cleome droserifolia*, and *Vachellia tortilis*, with weak-to-moderate activity against *P. falciparum* erythrocytic blood stages with mean half-maximal inhibitory concentration 50 (IC_{50}) of 11.7 µg/ml, 20.0 µg/ml, 32.1 µg/ml, and 40.0 µg/ml, respectively. Their selectivity index values were 35.2, 15.8, 11.5, and 13.8, respectively. Among these four candidates, *T. africanum* crude extract exhibited the highest parasite suppression in a murine malaria model against *P. yoelii*. My study identified novel natural antimalarial agents of plant origin that have potential for development into therapeutics for treating malaria.

Table 9. Latin binomial name of all plant extracts used in this study

Plant Extract	Family	Latin binomial name
Aerva javanica	Amaranthaceae	Aerva javanica (Burm.f.) Juss. ex Schult.
Anabasis setifera	Amaranthaceae	Anabasis setifera Moq.
Artemisia judaica	Asteraceae	Artemisia judaica L.
Calotropis procera	Apocynaceae	Calotropis procera (Aiton) W.T.Aiton
Carthamus tinctorius	Asteraceae	Carthamus tinctorius L.
Citrullus colocynthis	Cucurbitaceae	Citrullus colocynthis (L.) Schrad.
Cleome droserifolia	Cleomaceae	Cleome droserifolia (Forssk.) Delile
Forsskaolea tenacissima	Urticaceae	Forsskaolea tenacissima L.
Ochradenus baccatus	Resedaceae	Ochradenus baccatus Delile
Ocimum basilicum	Lamiaceae	Ocimum basilicum L.
Pulicaria undulata	Asteraceae	Pulicaria undulata (L.) C.A.Mey.
Trichodesma africanum	Boraginaceae	Trichodesma africanum (L.) Sm.
Vachellia tortilis	Fabaceae	Vachellia tortilis subsp. raddiana (Savi) Kyal. & Boatwr.

Plants used in this study was collected from the wild survey from the desert roads around Qena Governorate and were identified microscopically in South Valley University herbarium, Faculty of science, South Valley university, Qena, Egypt. Latin names were provided in the identification letter.

Table 10. Mean IC_{50} of Egyptian plant extracts against *Plasmodium falciparum* (3D7) and HFF cells in vit

Plant Extract	Plant family	Plant part	Mean IC_{50} (µg/ml)		Mean Selectivity index (SI)
			P. falciparum (3D7) [a] (±SD)	HFF cells [b] (±SD)	
Aerva javanica (Burm.f.) Juss. ex Schult.	Amaranthaceae	leaves	>100 (43.4)	378.1 (134.0)	>3.7
Anabasis setifera Moq.	Amaranthaceae	leaves	>100	1263.6 (194.9)	>12.6
Artemisia judaica L.	Asteraceae	leaves	20.0 (3.5)	316.8 (88.8)	15.8
Calotropis procera (Aiton) Dryand.	Apocynaceae	flowers	>100	41.5 (22.6)	>0.4
		flowers	>100	2.9 (1.5)	>0.02
Carthamus tinctorius L.	Asteraceae	flowers	>100	444.7 (169.5)	>4.4
Citrullus colocynthis (L.) Schrad.	Cucurbitaceae	seeds	51.7 (10.8)	88.0 (14.3)	1.7
		seeds	45.9 (23.3)	65.6 (7.2)	1.4
Cleome droserifolia (Forssk.) Delile	Cleomaceae	leaves	32.1 (3.8)	370.9 (95.3)	11.5
Forsskaolea tenacissima L.	Urticaceae	leaves	>100	519.0 (141.9)	>5.1
Ochradenus baccatus Delile	Resedaceae	fruit	>100	1179. 0 (245.4)	>11.7
Ocimum basilicum L. (E70%)	Lamiaceae	leaves	>100	252.6 (13.9)	>2.5
Pulicaria undulata (L.) C.A.Mey.	Asteraceae	flowers	18.9 (2.8)	55.5 (10.7)	2.9
		flowers	>100 (38.2)	197.5 (61.3)	>1.9

Trichodesma africanum (L.) Sm.	Boraginaceae	leaves	11.7 (4.7)	413.0 (96.9)	35.2
Vachellia tortilis subsp. raddiana (Savi) Kyal. & Boatwr.	Fabaceae	seeds	40.0 (2.8)	554.5 (110.5)	13.8
Chloroquine			0.009		

[a] The mean IC_{50} and standard deviation values against *P. falciparum* were calculated from the average of three independent experiments after a 72-hour culture of the parasites with a plant extract. [b] the mean IC_{50} against HFF cells was calculated from two independent experiments after a 72-hour culture. Except those that are indicated to have an ethanolic or aqueous extract, all the plant extractions are methanolic. IC_{50}: half maximal inhibitory concentration 50; SD: standard deviation; SI: selectivity index; HFF: human foreskin fibroblast; M80%; 80% methanol; E70%; 70% ethanol; aq.; aqueous.

Table S3. The plants used in this study and their medicinal uses

Plant Extract	**Coordinates**		**Plant family**	**Medicinal use** (References)
	Latitude)N(	**Longitude (E)**		
Aerva javanica (Burm.f.) Juss. ex Schult.	26°29'11.5"	33°16'19.6"	Amaranthaceae	Antifungal, antimicrobial, nematocidal, used in diuretic, and urinary trouble (Movaliya and Zaveri, 2014).
Anabasis setifera Moq.	26°28'44.1"	33°14'42.4"	Amaranthaceae	Anti-inflammatory (Abdou et al., 2013).
Artemisia judaica L.	26°28'31.7"	33°14'09.6"	Asteraceae	Antihelmintic, expectorant, and analgesic (Mahmoud and Gairola, 2013).
Calotropis procera (Aiton) Dryand.	26°28'16.1"	33°13'39.6"	Apocynaceae	Antirheumatic, antifungal, molluscicides, leprosy treatment, bronchial asthma and skin affection (Meena et al., 2010), anti-scorpion bite (Kubmarawa et al., 2007), antimalarial (Sharma and Sharma, 2000;Mudi and Bukar, 2011; Al-Snafi, 2016).
Carthamus tinctorius L.	26°23'13.7"	32°47'56.0"	Asteraceae	Anti-inflammatory, antioxidant, anti-hypoxic, anti-coagulant and anti-tumor, analgesic (Zhou et al., 2014).
Citrullus colocynthis (L.) Schrad.	26°25'55.1"	32°47'59.1"	Cucurbitaceae	Antimicrobial, antimicrobial and anticancer (Gurudeeban et al., 2010).
Cleome droserifolia (Forssk.) Delile	26°17'54.5"	32°46'26.3"	Cleomaceae	For treatment for urinary tract pains, diabetes, wounds, dermatitis, and antimicrobial (Mahmoud and Gairola, 2013).
Forsskaolea tenacissima L.	26°17'59.1"	32°46'24.6"	Urticaceae	Hepatoprotective, antioxidant (Assaf et al., 2017), antibacterial, antinociceptive, and antipyretic (Sher et al., 2017).
Ochradenus baccatus Delile	26°18'05.1"	32°46'31.6"	Resedaceae	Diuretic, antiseptic, antitussive (Mothana et al., 2014).
Ocimum basilicum L.	26°18'03.0"	32°46'42.9"	Lamiaceae	Antimicrobial (Adigüzel et al., 2005).
Pulicaria undulata (L.) C.A.Mey.	26°18'44.5"	32°46'31.8"	Asteraceae	Antiulcerogenic (Fahmi et al., 2019), and anti-inflammatory (Rav et al., 2011).
Trichodesma africanum (L.) Sm.	26°19'40.2"	32°46'47.9"	Boraginaceae	Antitumor (Moustafa et al., 2014), antimicrobial, and antifungal (Jaradat et al., 2016).
Vachellia tortilis subsp. raddiana (Savi) Kyal. & Boatwr.	26°20'27.9"	32°47'01.9"	Fabaceae	Antitussive (Kubmarawa et al., 2007), ocular infection treatment, and jaundice

				(Mahmoud and Gairola, 2013), antifungal (Maregesi et al., 2008).

The name, coordinates of the collection sites, family as well as the reported medicinal uses of each plant used in this s
indicated.

Table S4. Chemotherapeutic test of four plant extracts against the growth of *Plasmodium yoelii* in mice

Dpi	Plant extract	Dose of the extract (mg/kg/day)	Mice daily parasitemia of treated animals					Mean parasitemia of untreated animals	Mice daily parasite growth suppression (%)					Mean suppression/group (%)	survival rate (%)
			M1	M2	M3	M4	M5		M1	M2	M3	M4	M5		
1	*Artemisia judaica L.*	100	1.4	1.3	2.5	1.8	2.0	3.6	61.3	64.1	30.9	50.3	44.8	50.3	100
2			2.7	5.3	5.2	4.8	3.1	4.9	44.7	-8.6	-6.6	1.6	36.5	13.5	100
3			1.4	8.2	3.0	6.2	4.2	8.2	82.8	-0.2	63.3	24.2	48.7	43.8	100
4			6.1	10.3	7.7	8.9	11.6	20.5	70.2	49.7	62.4	56.5	43.3	56.4	100
5			7.7	12.3	5.9	12.3	10.1	24.6	68.6	49.9	76.0	49.9	58.9	60.7	100
6			10.0	24.8	19.3	6.0	6.4	24.6	59.3	-1.0	21.4	75.6	73.9	45.8	100
7			22.5	11.4	19.0	19.8	22.3	41.0	45.1	72.2	53.6	51.7	45.6	53.6	100
1	*Cleome droserifolla (Forssk.) Delile*	100	3.1	2.8	1.7	2.9	4.5	3.6	14.4	22.7	53.0	19.9	-24.3	17.1	100
2			3.6	3.5	4.9	4.7	3.1	4.9	26.2	28.3	-0.4	3.7	36.5	18.9	100
3			4.7	7.4	2	4.0	6.4	8.2	42.5	9.5	75.6	51.1	21.8	40.1	100
4			4.7	9.6	6.6	13.0	17.6	20.5	77.0	53.1	67.7	36.5	14.0	49.7	100
5			11.9	25.4	13.2	15.2	9.6	24.6	51.5	-3.4	46.3	38.1	60.9	38.7	100
6			8.6	25.9	18.8	26.2	13.5	24.6	65.0	-5.5	23.5	-6.7	45.0	24.3	100

7			14.2	17.8	10.4	22.2	13.5	41.0	65.3	56.6	74.6	45.8	67.1	61.9	100
1	*Trichodesma africanum (L.) sm.*	100	1.7	2.2	1.3	0.6	1.7	4.4	61.0	49.5	70.2	86.2	61.0	65.6	100
2			9.2	12.9	4.5	2.6	14.0	18.4	50.0	29.9	75.5	85.8	23.9	53.0	100
3			6.9	7.4	9.2	4.5	7.7	18.8	63.4	60.7	51.2	76.1	59.1	62.1	100
4			24.2	23.1	14.5	13.2	13.9	27.5	11.9	15.9	47.2	51.9	49.4	35.3	100
5			11.3	9.3	23.9	6.6	8.7	27.8	59.4	66.6	14.2	76.3	68.8	57.0	100
6			10.7	6.7	9.2	28.4	5.8	31.9	66.4	79.0	71.1	10.9	81.8	61.8	100
7			22.7	22.0	16.5	11.8	11.1	40.5	44.0	45.7	59.3	70.9	72.6	58.5	100
1	*Vachellia toritilis subsp. raddiana (Savi) Brenan*	100	2.0	2.7	2.6	2.8	2.3	4.4	54.1	38.1	40.4	35.8	47.2	43.1	100
2			9.4	2.8	4.8	4.0	4.3	18.4	48.9	84.8	73.9	78.3	76.6	72.5	100
3			10.2	10.5	10.6	7.7	21.0	18.8	45.9	44.3	43.7	59.1	-11.5	36.3	100
4			10.9	16.4	11.4	15.4	12.7	27.5	60.3	40.3	58.5	43.9	53.8	51.3	100
5			15.1	13.1	12.7	26.8	18.7	27.8	45.8	52.9	54.4	3.7	32.8	37.9	100
6			17.1	12.9	5.3	21.1	22.3	31.9	46.3	59.5	83.4	33.8	30.0	50.6	100
7			32.0	34.5	28.3	22.6	13.5	40.5	21.0	14.9	30.2	44.2	64.2	34.9	100

Estimated daily suppression of *P. yoelii* growth in mice. Parasite growth inhibition percentages were calculated from 3 h post-challenge until the end of the course of treatment at 6 days post-infection for each individual animal against the untreated group mice. All mice were challenged with an intraperitoneal injection of 1×10^7 *P. yoelii*-infected erythrocytes and then treated with 100 mg/kg/day of each plant extract, administered orally, for 1 week (N = 5 per group). Giemsa-stained thin blood smears were made daily from each mouse, and 600–1,000 RBCs were counted per blood smear. *A.*

judaica and *C. droserifolia* extracts were tested in one independent experiment with the same control, and *T. africanum* and *V. tortilis* extracts were tested in another independent experiment sharing the same control. Mean parasite suppression values for all tested plant extracts are illustrated in Figure 2. The percentage of parasitemia was calculated by using the following equation: parasitemia % = [(number of infected RBCs) / (total number of RBCs) ×100]. Daily parasite suppression was calculated by using the following equation: [(mean parasitemia of the untreated group − the parasitemia of the treated group) / (mean parasitemia of the untreated group) × 100]. Dpi, days post-infection.

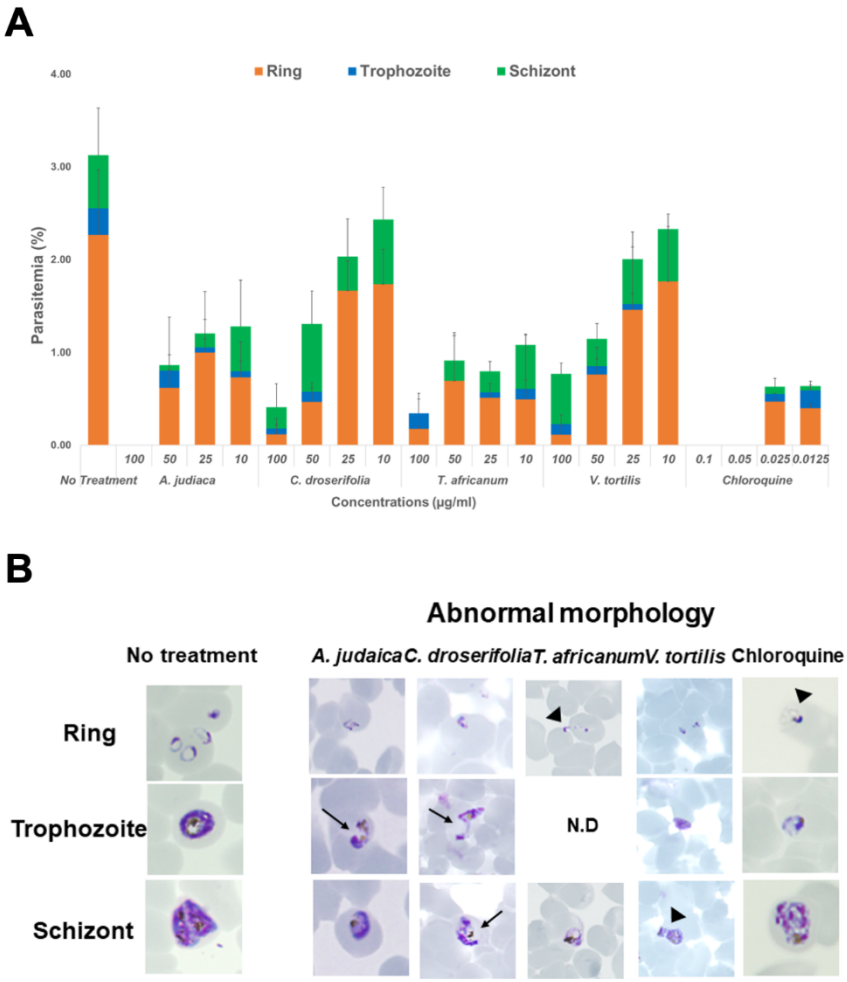


Fig. 14. Effect of plant extracts on stage-specific *P. falciparum* (3D7) morphology in vitro. (A) Percentage of parasites at each stage (i.e., ring, trophozoite, or schizont) after treatment with 10, 25, 50, or 100 µg/ml of extracts of *Artemisia judaica, Trichodesma africanum, Cleome droserifolia*, or *Vachellia toritilis* plants, chloroquine, or medium alone. The number of parasites at each stage was determined from a total of 600–900 erythrocytes. Data are representative of two independent experiments with similar results. **(B)** *P. falciparum* parasites were treated with 50 µg/ml of an extract of *A. judaica*, *T. africanum, C. droserifolia*, or *V. tortilis*. Chloroquine (0.025 µg/ml) was used as a positive control, and medium alone was used as a negative control. Three wells were used for each plant or drug concentration. After 72 h, the parasite morphology was observed via microscopy (×100 magnification) on Giemsa-stained thin blood smears. Line arrow indicate the fragmented parasites, while arrow head indicate the shrinkage parasites. Data shown here are representative of two independent experiments that produced similar results.

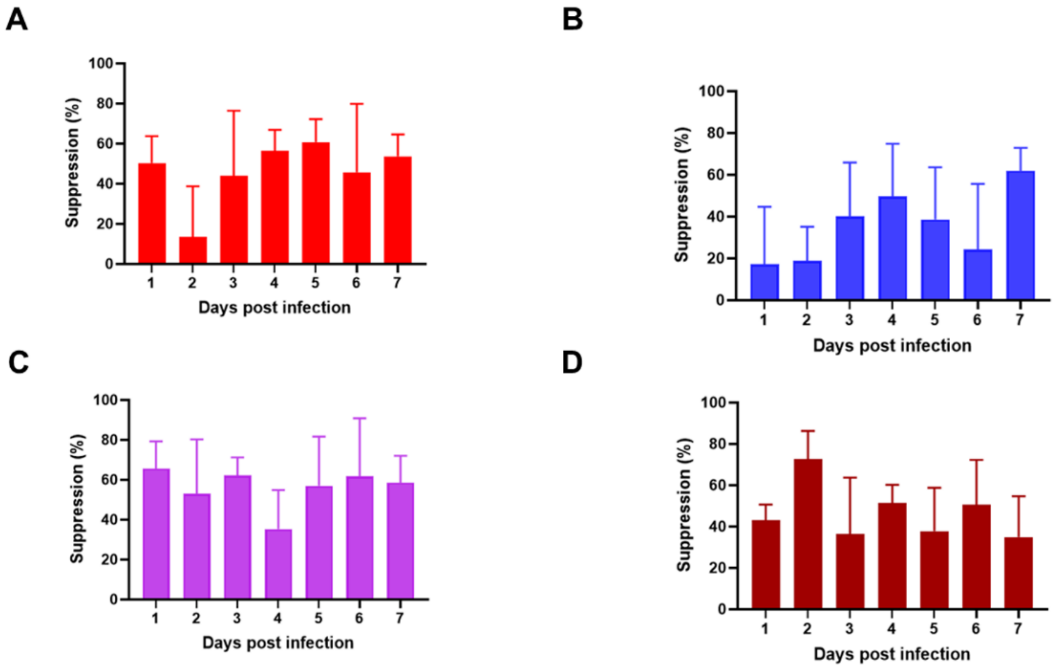


Fig. 15. Suppression percentage of *Plasmodium yoelii* in mice induced by plant extracts at 1-week post-infection. Five mice were used per group. *Artemisia judaica* and *Cleome droserifolia* plant extracts were tested in one independent experiment that shared the same control, and *Trichodesma africanum* and *Vachellia tortilis* plant extracts were tested in another independent experiment that shared the same control. All mice were challenged by an intraperitoneal injection of approximately 1×10^7 *P. yoelii*-infected erythrocytes and then were treated orally with 100 mg/kg/day of each plant extract for 1 week. The mean and standard deviation of the parasite growth inhibition percentages were calculated against the untreated group mice from 24 hours after challenge (dpi = 1) until 7 days post-infection (24 hours after end course of treatment) **(A–D)** Parasite growth inhibition percentages in mice treated with *A. judaica* **(A)**, *C. droserifolia* **(B)**, *T. africanum* **(C)**, or *V. tortilis* **(D)** plant extract. N.D. not detected.

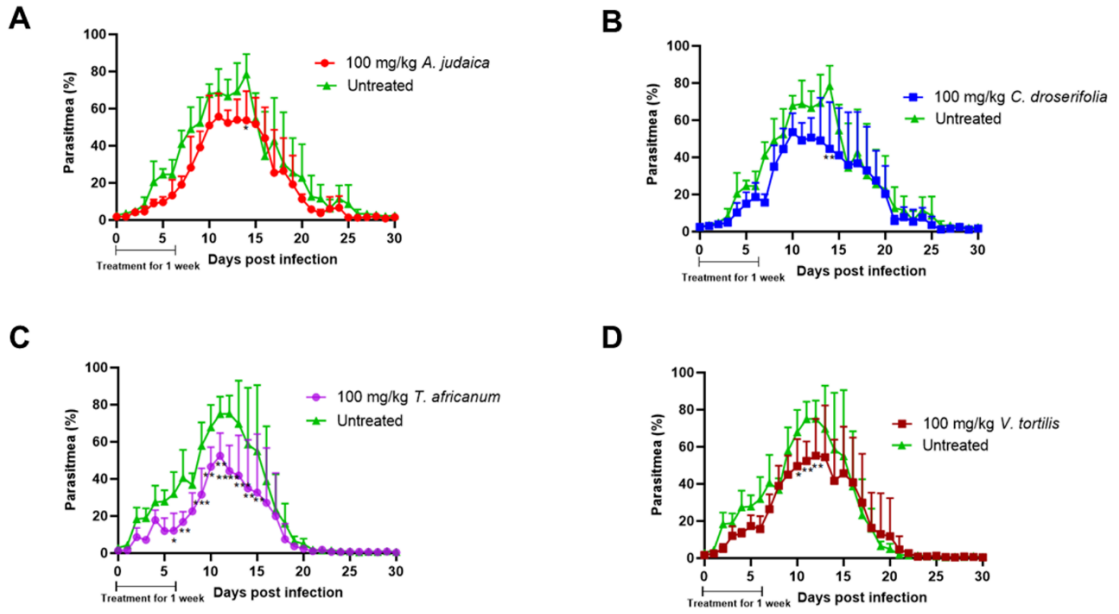


Fig.16. Effect of wild plant extracts on *Plasmodium yoelii* growth in mice through 30 days post-infection. Five mice were used in each group. *Artemisia judaica* and *Cleome droserifolia* plant extracts were tested in one independent experiment that shared the same control, and *Trichodesma africanum* and *Vachellia tortilis* plant extracts were tested in another independent experiment that shared the same control. Mean parasitemia % was monitored daily from day 0 (challenge day) until 30 days post-infection. All mice were challenged by an intraperitoneal injection of approximately 1×10^7 *Plasmodium yoelii*-infected erythrocytes and then treated orally with 100 mg/kg/day of plant extract for 1 week. The untreated group received only PBS. **(A–D)** The mean parasitemia % of *P. yoelii*-infected mice treated with *A. judaica* **(A)**, *C. droserifolia* **(B)**, *T. africanum* **(C)**, or *V. tortilis* **(D)** extract. Data were analyzed by a two-way ANOVA followed by a Bonferroni test against the untreated group (*$p < 0.05$).

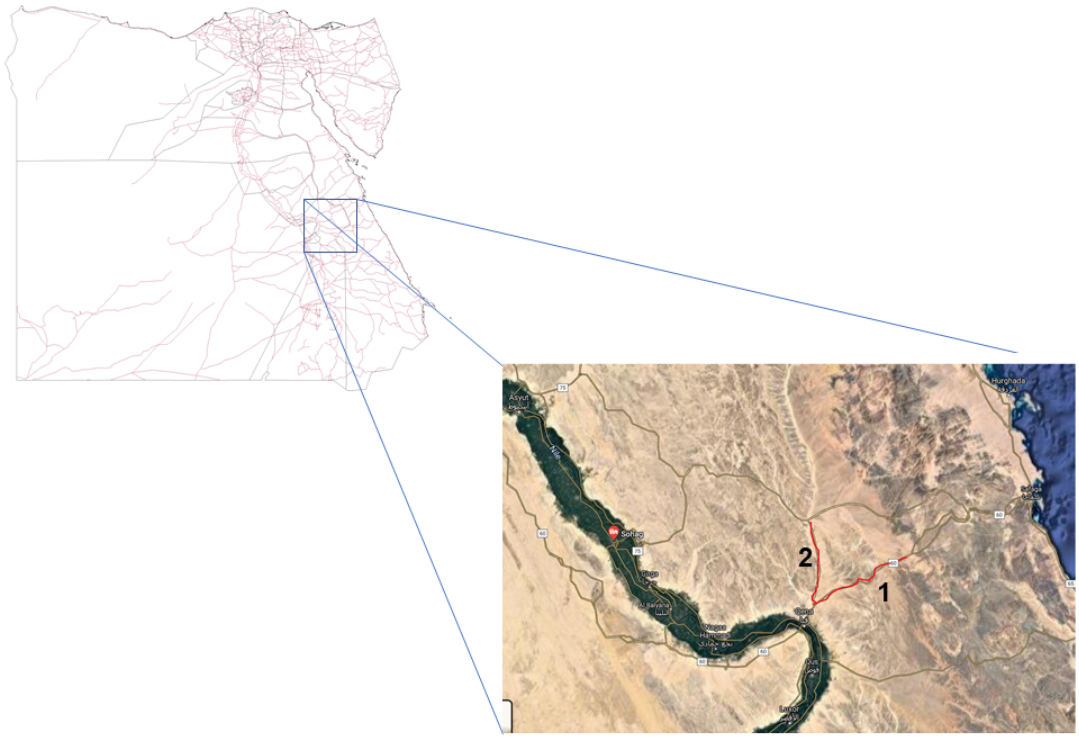


Fig. S16. Sampling map of the plant samples collected in Egypt. Plant materials were collected during the period from 14–19 May, 2019 in a field survey from two sites in the southern part of Egypt in Qena Governorate. The first site was Qena-Safaga desert road **(1)**, and the second site was Qena-Sohag desert road **(2)**. Plants collection sites coordinates were shown in table S1. Map designed by DIVA-GIS 7.5.0 software to show all country roads and Governorates; the sample collection sites were approximately determined and are shown in the magnified box as highlighted red lines on a Google map.

Fig. S17. Images of the collected plant materials. Plants were collected during the period from 14–19 May, 2019. Images of *Calotropis procera* (Aiton) Dryand. **(A)**, Citrullus *colocynthis* (L.) Schrad. **(B)**, *Trichodesma africanum (L.) Sm*. **(C)**, *Vachellia tortilis* subsp. *raddiana* (Savi) Brenan (Aiton) Dryand. **(D)**, *Forsskaolea tenacissima L.* **(E)**, *Aerva javanica* (*Burm.f.*) *Juss. ex Schult.* **(F)**, *Cleome droserifolia* (Forssk.) Delile. **(G)**, *Ocimum basilicum* L. **(H)**, *Pulicaria undulata* (*L.*) *C.A.Mey.* **(I)**, *Ochradenus baccatus* Delile. **(J)**, *Carthamus tinctorius* L. **(K)**, *Artemisia judaica* L. **(L)**, and *Anabasis setifera* Moq. **(M)**. A sample from each collected sample was transferred to the herbarium of the Faculty of Science, South Valley University, Qena, Egypt for identification via microscopy.

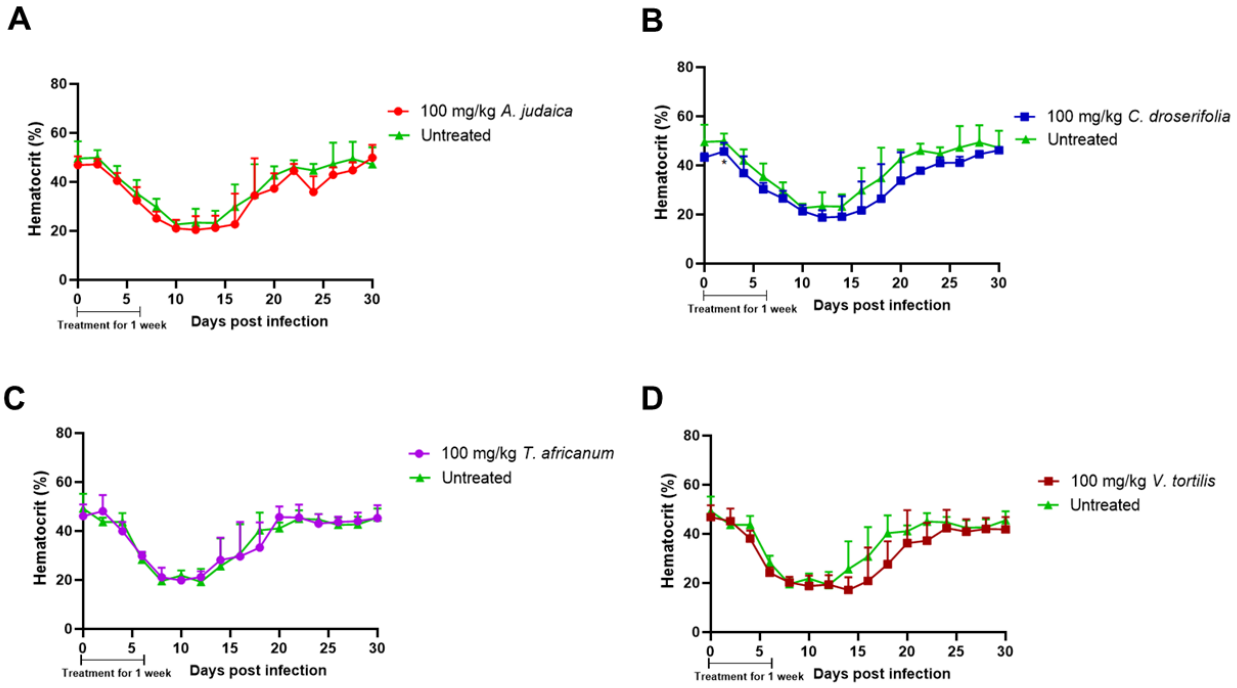


Fig. S18. Effect of wild plant extracts on the growth of *Plasmodium yoelii* in male BALB/c mice. Five mice were used per group. *Artemisia judaica* and *Cleome droserifolia* plant extracts were tested in one independent experiment that shared the same control, and *Trichodesma africanum* and *Vachellia tortilis* plant extracts were tested in another independent experiment that shared the same control. Hematocrit (%) was monitored every other day from day 0 (challenge infection day) until 30 days post-infection. All mice were challenged by an intraperitoneal injection of 1×10^7 *P. yoelii*-infected erythrocytes and then treated orally with 100 mg/kg/day of one plant extract for 1 week. The untreated group received only PBS. **(A–D)** Hematocrit (%) for the *P. yoelii*-infected mice treated with *A. judaica* **(A)**, C. *droserifolia* **(B)**, *T. africanum* **(C)**, or *V. tortilis* **(D)** extract. Data were analyzed by performing a two-way ANOVA followed by a Bonferroni test against the untreated group (*$p < 0.05$).

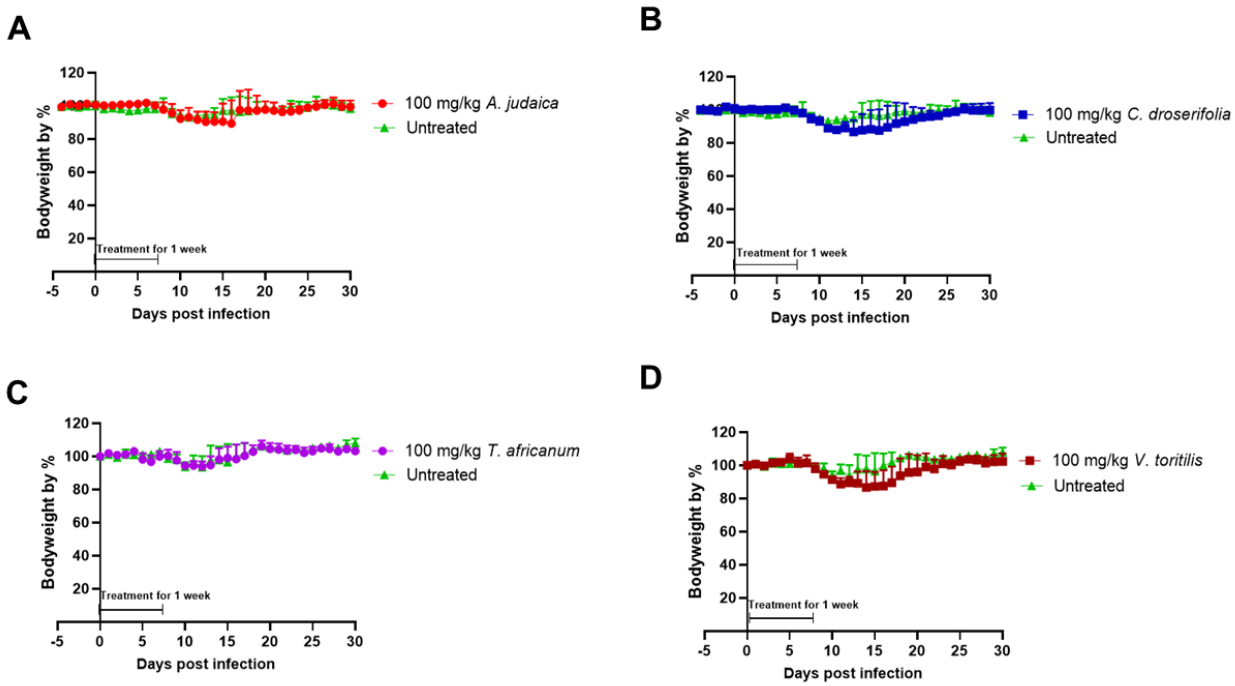


Fig. S19. Effect of wild plant extracts on bodyweight change in *Plasmodium*-infected mice. Five male BALB/c mice were used per group. *Artemisia judaica* and *Cleome droserifolia* plant extracts were tested in one independent experiment that shared the same control, and *Trichodesma africanum* and *Vachellia tortilis* plant extracts were tested in another independent experiment that shared the same control. Bodyweight change (%) was monitored daily from day 0 (challenge infection day) until 30 days post-infection. All mice were challenged by an intraperitoneal injection of 1×10^7 *P. yoelii*-infected erythrocytes and then treated orally with 100 mg/kg/day of one plant extract for 1 week. The untreated group received only PBS. **(A–D)** Bodyweight changes of the *P. yoelii*-infected mice treated with *A. judaica* **(A)**, C. *droserifolia* **(B)**, *T. africanum* **(C)**, or *V. tortilis* **(D)** extract. Data were analyzed by performing a two-way ANOVA followed by a Bonferroni test against the untreated group (*$p < 0.05$).

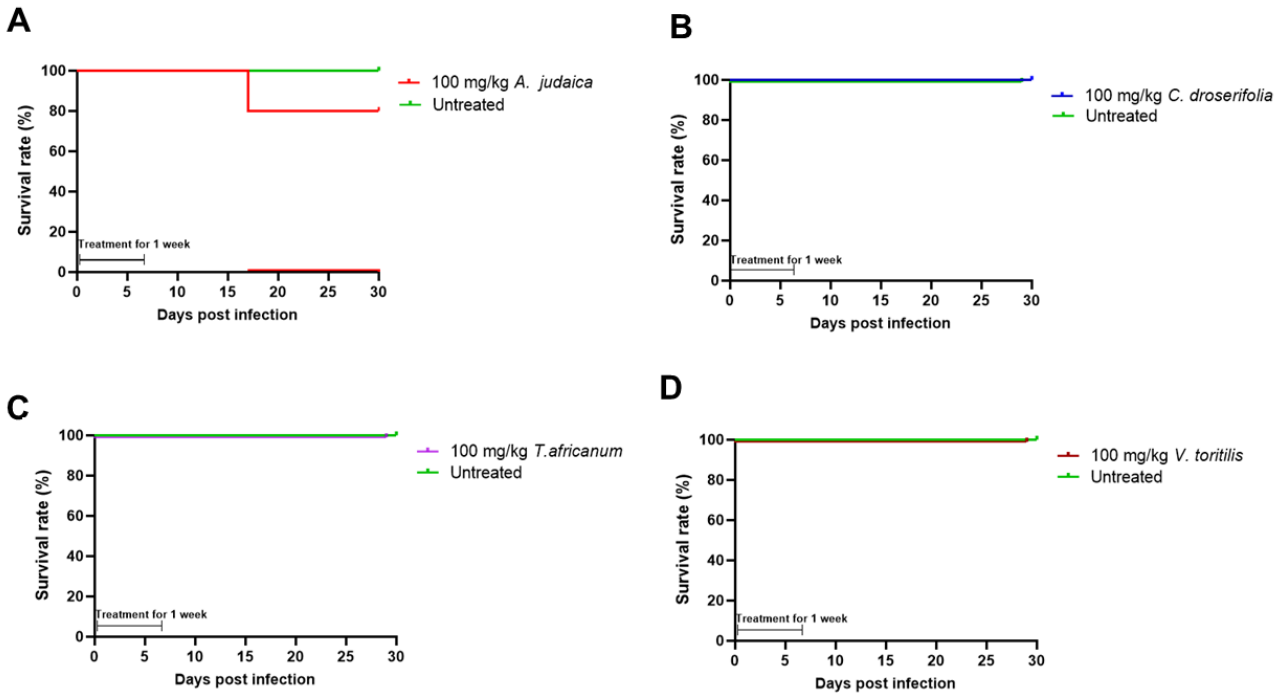


Fig. S20. Effect of wild plant extracts on the survival rate of *Plasmodium*-infected mice. Five male BALB/c mice were used per group. *Artemisia judaica* and *Cleome droserifolia* plant extracts were tested in one independent experiment that shared the same control, and *Trichodesma africanum* and *Vachellia tortilis* plant extracts were tested in another independent experiment that shared the same control. Survival rates of mice were monitored every day from day 0 (challenge infection day) until 30 days post-infection. All mice were challenged by an intraperitoneal injection of 1×10^7 *P. yoelii*-infected erythrocytes and then treated orally with 100 mg/kg/day of one plant extract for 1 week. The untreated group received only PBS. **(A–D)** Survival rates of the *P. yoelii*-infected mice treated with *A. judaica* (4/5, 80%) **(A)**, C. *droserifolia* (5/5,100%) **(B)**, *T. africanum* (5/5, 100%) **(C)**, or *V. tortilis* (5/5, 100%) **(D)** extract. In both experiments, the uninfected mice had a 100% survival rate (5/5). The significance of each difference was analyzed with a χ^2 test, but none of the differences were significantly different.

General discussion

In my study, in chapter 1, I evaluated the role of NcGRA7 during pregnancy. Reported studies illustrate the function of dense granule antigens during pregnancy were limited. The reported optimized mice to assess the vertical transmission of *N. caninum* infections was BALB/c mice (Liddel et al., 1999; 2003; Omata et al., 2004), while in my study, I used C57BL/6 mice which were reported once that it could be used in vaccine and vertical transmission studies (Ramamoorthy et al., 2007a). Ramamoorthy et al., 2007a infect C57BL/6 during mid-pregnancy and before mating, and the vertical transmission rates were 100.0% and 90.5%, respectively. While in my study, the vertical transmission rate ranges from 95.2%-100.0% after challenge infection with high (1×10^6) and low (1×10^5) doses of Nc1 at an early stage of gestation, the vertical transmission rates of NcGRA7KO show significant lower PCR positive offsprings (66.67%-70.0%) as compared to the Nc1 (95.2%-100.0%). Collectively, my study shows novel findings in the role of NcGRA7 during pregnancy and opens the field for using the NcGRA7KO parasite as a live attenuated vaccine candidate against *N. caninum* infection.

The summary of the reported vaccines using live tachyzoites, different parasite antigens against *N. caninum* in mice was summarized by (Hemphill and Müller, 2015), but none of them was commercially licensed. A previous commercial inactivated vaccine (Neoguard®, Intervet International B.V., Boxmeer, The Netherlands) was developed and was withdrawn from the market, as only moderate protection against abortions was observed in field trials (Romero et al., 2004; Weston et al., 2012). This explains that the development of a novel type of vaccine from the most protective method (live tachyzoites) is now challenging. Although, the results achieved by (Penarete-Vargas et al., 2010) suggest a correlation between the development of cellular-specific immune responses and the protective efficacy, but the data for the protection against chronic and vertical transmission was missing. In my study, I had undergone a protective study using the NcGRA7KO-deficient line in non-pregnant and pregnant mouse models in BALB/c mice. The vaccination doses were high (1×10^5) and low (1×10^4) of live tachyzoites, the study aims is to evaluate the rate of vertical transmission after the challenge as

well as the survival of both dam and offsprings until 30 days post-partum. A significant protective effect was observed with (75.0%) in survival in the offsprings from NcGRA7KO-vaccinated dams than the unvaccinated ones (6.9%). Additionally, lower vertical transmission rate and parasite DNA in the pups' brains from NcGRA7KO-vaccinated dams than the unvaccinated ones were observed.

Although multiple chemotherapeutic compounds were used for the treatment of *N. caninum* infection in farm ruminants, no safe drug was reported until the present time (Sánchez Sánchez et al., 2018), therefore, finding a novel treatment from the natural resources which might have fewer or no side effects is urgently needed. Wild medicinal plants from the desert were traditionally used as antimicrobial agents. Here, I evaluated the anti-*T. gondii* and anti-*N. caninum* activities of the extracts of wild Egyptian plants randomly collected from the desert in vitro. Twelve Extracts were initially screened for their anti-*toxoplasma* activities, extracts with potent activities against *T. gondii* growth were evaluated against *N. caninum in vitro.* *C. droserifolia*, ethanolic extract of *P. undulata*, *T. africanum*, *A. judaica*, and *V. tortilis*, showed potent efficacy against the growth of *N. caninum* in vitro. The evaluation of the effectiveness of the plant extract against malaria shows promising results. It shows moderate-to-weak activities against human malaria (*plasmodium falciparum*) in vitro and *P. yoelii* in vivo. These extracts were used in a much lower dose (100 mg/kg/day) than the reported studies of malaria treatment. In conclusion, my data support using of these extracts in the future development of an antiprotozoal therapeutic.

General summary

Neosporosis is a parasitic disease affecting the health of dogs and cattle worldwide. It is caused by *Neospora caninum*, an obligate intracellular apicomplexan parasite. Dogs are its definitive host, it mostly infects livestock animals, especially cattle that acts as an intermediate host. It is necessary to have well-established models of abortion and vertical transmission in experimental animals, in order to determine basic control measures for the *N. caninum* infection. I evaluated the role of *N. caninum* dense granule antigen 7 (NcGRA7) in the vertical transmission of *N. caninum* using the C57BL/6 pregnant mouse model. I inoculated mice on day 3.5 of pregnancy with parental Nc1 or NcGRA7-deficient parasites (NcGRA7KO). Post-mortem analyses were performed on day 30 after birth and the surviving pups were kept until day 30 postpartum. The number of parasites in brain tissues of offspring from NcGRA7KO-infected dams was significantly lower than that of the Nc1-infected dams under two infection doses (1×10^6 and 1×10^5 tachyzoites/mouse). The vertical transmission rates in the NcGRA7KO-infected group were significantly lower than those of the Nc1-infected group. To understand the mechanism by which the lack of NcGRA7 decreased the vertical transmission, pregnant mice were sacrificed on day 13.5 of pregnancy (10 days after infection). Although parasite DNA was detected in the placentas, no significant difference was found between the two parasite lines. Histopathological analysis revealed a greater inflammatory response in the placentas from NcGRA7KO-infected dams than in those from the parental strain. This finding correlated with upregulated chemokine mRNA expression for CCL2, CCL8, and CXCL9 in the placentas from the NcGRA7KO-infected mice. In conclusion, these results suggest that loss of NcGRA7 triggers an inflammatory response in the placenta, resulting in decreased vertical transmission of *N. caninum*.

Live vaccination is the most protective method against bovine neosporosis, which is the major cause of bovine abortion globally. Here, parenteral stain Nc1 and less virulent NcGRA7KO line were evaluated as potential live vaccines. Pregnant and non-pregnant BALB/c mice were subcutaneously inoculated with high (1×10^5) or low (1×10^4) doses of tachyzoites. Non-pregnant mice were challenged

intraperitoneally with 2×10^6 Nc1-GFP tachyzoites at 45 days post-vaccination (dpv), and postmortem examination was performed at 30 days post-challenge infection (76 dpv). At both doses, NcGRA7KO-vaccinated animals showed higher bodyweight gain and fewer clinical signs than unvaccinated animals. Although there was no significant difference in the survival rate among experimental groups, the parasite burden in the brain of vaccinated animals was lower than that of unvaccinated animals. For pregnancy conditions, mating was started at 3 weeks post-vaccination, and challenge was performed with 1×10^5 Nc1-GFP at day 10.5 of pregnancy. Postmortem examination was performed at 30 days postpartum. For high-dose vaccination, there was no significant difference in the survival rates of offspring between vaccinated (27.8%) and unvaccinated dams (39.1%), whereas for low-dose vaccination, the offspring from vaccinated dams had a significantly higher survival rate (75.0%) than those from unvaccinated dams (6.9%). The amount of challenge (Nc1-GFP) parasite DNA in the brain of offspring from vaccinated dams was significantly lower than that of offspring from unvaccinated dams. These results suggest that NcGRA7KO parasites have potential for use as a live attenuated vaccine against *N. caninum* infection.

Since there is no available chemotherapy against different protozoan infections as toxoplasmosis, Neosporosis and malaria, treatment with medicinal plants will help to overcome the drug toxicity and parasite resistance compared with the commercially used drugs. Wild medicinal plants from the desert have been traditionally used as antimicrobial agents in Egypt. However, little is known about the antiprotozoal efficacy of these plants. Here, I evaluated the in vitro activity of extracts from certain wild Egyptian desert plants against *Toxoplasma gondii* and *N. caninum*. Of the 12 plant extracts tested, the methanolic extracts from *Artemisia judaica*, *Cleome droserifolia*, *Trichodesma africanum*, and *Vachellia tortilis* demonstrated potent activity against the growth of *T. gondii*, with half-maximal inhibitory concentrations 50 (IC_{50}) of 2.1 µg/ml, 12.5 µg/ml, 21.8 µg/ml, and 24.5 µg/ml, respectively. Their mean selectivity index (SI) values were 150.8, 29.6, 18.9, and 22.6, respectively. These extracts, along with an ethanolic extract of *P. undulata*, were then evaluated against *N. caninum*. *C. droserifolia*, an ethanolic extract of *P. undulata*, *T. africanum*, *A. judaica*, and *V. tortilis* demonstrated potent efficacy against the growth of *N. caninum* in vitro, with mean IC_{50}s of 1.0 µg/ml, 3.0 µg/ml, 3.1 µg/ml, 8.6

µg/ml, and 17.2 µg/ml, respectively. Their mean SI values were 370.9, 18.5, 133.2, 36.8, and 32.2, respectively. These findings indicate that *A. judaica* is most potent against *T. gondii*, while both *C. droserifolia* and *T. africanum* extracts were more effective to *N. caninum*. Our data suggest these extracts could provide an alternative treatment for *T. gondii* and *N. caninum* infections.

Finding a novel treatment for malaria is still challenging, and various extracts from different wild desert plants have been reported to have multiple medicinal uses for human public health, in my study, I had evaluated the antimalarial efficacy of several Egyptian plant extracts. I assessed the cytotoxic potential of 13 plant extracts and their abilities to inhibit the in vitro growth of *Plasmodium falciparum* (3D7), and to treat infection with non-lethal *Plasmodium yoelii* 17XNL in an in vivo malaria model in BALB/c mice. In vitro screening identified four promising candidates, *T. africanum*, *A. judaica*, *C. droserifolia*, and *V. tortilis*, with weak-to-moderate activity against *P. falciparum* erythrocytic blood stages with mean IC_{50} of 11.7 µg/ml, 20.0 µg/ml, 32.1 µg/ml, and 40.0 µg/ml, respectively. Their mean SI values were 35.2, 15.8, 11.5, and 13.8, respectively. Morphological alterations, such as cell shrinkage, and parasite fragmentation were observed following treatment with plant extract concentration of 50 µg/ml as well as after treatment with the positive control drug, chloroquine, in comparison with untreated parasites. Among these four candidates, *T. africanum* crude extract exhibited the highest parasite suppression in a murine malaria model against *P. yoelii*. As it causes the significantly reduced level of parasitemia observed from 6–15 days post-infection after oral treatment. My study identified novel natural antimalarial agents of plant origin that have the potential for development into therapeutics for treating malaria.

和文要約

ネオスポラ症はイヌとウシに症状をもたらす寄生虫感染症である。本症は細胞内寄生原虫ネオスポラ・カニナムの感染により起こる。イヌはその終宿主であり、ウシなどの家畜動物を中間宿主とする。ネオスポラ感染を制御するためには、実験動物を用いた流産、垂直感染の実験モデルが必要となる。第一章では、C57BL/6 妊娠マウスを用いてネオスポラの垂直感染におけるデンスグラニュルタンパク質７（NcGRA7）の役割を解析した。妊娠 3.5 日のマウスに親株原虫 Nc1 および NcGRA7 欠損原虫株（NcGRA7KO）を感染させた。出生後 30 日間新生マウスの生存を観察した。Nc1 感染と比較して、NcGRA7KO 感染母マウス由来の子マウスにおける脳内原虫数は少なく、この結果は２つの条件の感染量（マウス１匹あたり 1×10^6 あるいは 1×10^5 タキゾイト）で確認できた。NcGRA7KO 感染群の垂直感染率は、Nc1 感染群よりも有意に低かった。NcGRA7 欠損による垂直感染の低下の原因を明らかにするため、妊娠マウスを妊娠 13.5 日（感染後 10 日）で解剖し解析した。原虫 DNA は胎盤で検出されたが、両実験群で顕著な差は認められなかった。病理組織学的な解析により、Nc1 感染マウスの胎盤に比べて NcGRA7KO 感染マウスの胎盤では過剰な炎症反応が認められた。さらに、NcGRA7KO 感染マウスの胎盤ではケモカイン CCL2、CCL8、CXCL9 の mRNA 発現が増加していた。以上より、NcGRA7 の欠損により胎盤で炎症反応が誘導され、その結果原虫の垂直感染が低下することが示唆された。

ウシのネオスポラ症の防御方法には、生ワクチンが最も効果的である。第二章では、親株原虫 Nc1 と低病原性株 NcGRA7KO を用いて生ワクチンとしての有効性を評価した。妊娠と非妊娠の BALB/c マウスに高容量（1×10^5）と低容量（1×10^4）のタキゾイトをワクチンとして皮下接種した。非妊娠マウスに対して、ワクチン接種後 45 日目に 2×10^6 Nc1-GFP タキゾイトを腹腔内感染させ、その後 30 日間経過観察を行なった。ワクチン未接種マウスと比較して、NcGRA7KO 接種マウスは両接種量で体重が増加し臨床症状の程度も低く推移した。実験群間でマウスの生存率に有意差は認められなかったが、ワクチン未接種マウスよりワクチン接種マウスの脳内原虫数は少なかった。妊娠実験では、NcGRA7KO 接種後３週目に交配を開始し、妊娠 10.5 日目に 1×10^5 Nc1-GFP タキゾイトを腹腔内感染させた。高容量（1×10^5）NcGRA7KO 接種群ではワクチン未接種群との間に新生マウスの生存率に差は認められなかった（NcGRA7KO 接種群：27.8%、未接種群：39.1%）。一方で、低容量（1×10^4）NcGRA7KO 接種群では有意に新生マウスの生存率が上昇した（NcGRA7KO 接種群：75.0%、未接種群：6.9%）。ワクチン接種群由来子マウスの脳内 Nc1-GFP 原虫数はワクチン未接種群と比べて有意に少なかった。以上の結果は、NcGRA7KO はネオスポラ感染に対する有効な弱毒生ワクチン株になる可能性を示している。

トキソプラズマ症、ネオスポラ症、マラリアに対する有効な化学療法については、原虫の抵抗性株の出現や毒性の観点からも薬用植物の研究が重要となる。エジプトでは抗菌性物質として砂漠由来の野生薬用植物が伝統的に使用されてきた。しかしながら、これら野生薬用植物の抗原虫効果についてはあまり知られてない。第三章では、エジプトの砂漠由来野生薬用植物についてトキソプラズマとネオスポラに対する in vitro の阻害作用について解析した。調べた 12 種類の抽出物から、*Artemisia judaica*, *Cleome droserifolia*, *Trichodesma africanum*, *Vachellia tortilis* のメタノール抽出物にてトキソプラズマに対する増殖阻害活性を確認した（それぞれの 50%阻害濃度：2.1 μg/ml, 12.5 μg/ml, 21.8 μg/ml, 24.5 μg/ml、それぞれ

の選択毒性：150.8, 29.6, 18.9, 22.6）。加えて、*C. droserifolia* のメタノール抽出物、*P. undulata*, *T. africanum*, *A. judaica*, *V. tortilis* のエタノール抽出物にてネオスポラに対する増殖阻害活性を確認した（それぞれの 50%阻害濃度：1.0 μg/ml, 3.0 μg/ml, 3.1 μg/ml, 8.6 μg/ml, 17.2 μg/ml、それぞれの選択毒性：370.9, 18.5, 133.2, 36.8, 32.2）。以上の結果より、*A. judaica* はトキソプラズマに効果的で、*C. droserifolia* と *T. africanum* はネオスポラに対して有効であった。今回の結果は、これら植物抽出物がトキソプラズマやネオスポラ感染に対する代替治療法に使用できる可能性を示している。

第四章では、エジプトの砂漠由来野生薬用植物 13 種類について、in vitro における熱帯熱マラリア原虫 *Plasmodium falciparum*（3D7）の増殖と *Plasmodium yoelii*（17XNL）を用いた BALB/c マウス感染実験を指標に抗マラリア活性を解析した。In vitro の解析結果から、*T. africanum*, *A. judaica*, *C. droserifolia*, *V. tortilis* の抽出物で原虫増殖抑制効果が確認された（それぞれの 50%阻害濃度：11.7 μg/ml, 20.0 μg/ml, 32.1 μg/ml, 40.0 μg/ml、それぞれの選択毒性：35.2, 15.8, 11.5, 13.8）。これら抽出物の処理により原虫の収縮、断片化などの形態異常が観察された。マウス感染実験では、*T. africanum* の抽出物が最も原虫増殖の抑制効果が高かった。この結果により、マラリア治療の開発に有用な植物資源を同定することができた。

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