PROTOZOOLOGY - ORIGINAL PAPER

Establishment of a model of *Neospora caninum* infection in pregnant mice

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Abstract

The aim of this study was to establish an animal model of *Neospora caninum* infection in pregnant BALB/c mice infected with different doses of *N. caninum* tachyzoites. After infection, the female BALB/c mice were housed with male BALB/c mice. The aim of this study was to observe clinical signs and pathological changes, detect *Nc5* gene expression in the main organs, and measure the wet weight and coefficient of the placenta of the pregnant mice. In addition, the level of cytokines and placental hormones in the serum was measured in pregnant mice. Our results showed that the optimal dose of the mice in the infected model was 10^5 tachyzoites. The infected pregnant mice presented with various clinical signs, including depression, ataxia, and variable mortality. Pathological observations of the brain, liver, and spleen in the mice exhibited hyperemia, bleeding, and swelling. Moreover, *N. caninum* tissue cysts or tachyzoites were observed in the brain, liver, and spleen tissues by hematoxylin-eosin (HE). The *Nc5* gene was detected in the brain, liver, spleen, and placental tissues of the mice. With the increase in infection days, the weight of the placenta in the model mice increased, and the placenta ratio decreased gradually. Compared with the control group, the placenta weight and placental ratio were significantly different ($P < 0.05$). Furthermore, the levels of the placental hormones, corticotropin-releasing hormone (CRH), chorionic gonadotropin (CG), prolactin (PRL), and estriol (E3), and cytokines IFN-γ, IL-4, and TGF-β were differentially expressed between the model and the control group ($P < 0.05$ or $P < 0.01$), which indicated that infection with *N. caninum* caused an imbalance in the regulatory function of the placental hormones and cytokines in pregnant mice. A pregnant mouse model of *N. caninum* infection was successfully established in this study, providing a foundation for the study of the pathogenic mechanisms of *N. caninum*.

Keywords *Neospora caninum* · Pregnant mice · Placenta · Hormone · Model

Introduction

Neospora caninum is an obligate intracellular protozoan that is parasitic in many mammalian cells (e.g., cattle, sheep, and dogs). *N. caninum* primarily causes abortion, stillbirth, or

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neonatal dyskinesia, and nervous system diseases in female animals (e.g., dogs and cattle) (Dubey et al. 1988; Klauck et al. 2016; Wilson et al. 2016). *N. caninum* can be vertically transmitted, which causes the most serious damage to cattle, and has become one of the main factors challenging the healthy development of the cattle industry (Hao et al. 2014; Japa et al. 2019; Lagomarsino et al. 2019). Statistics have shown that the global economic losses of cattle caused by *N. caninum* infection or abortion can be as high as $2.38 billion annually (Reichel et al. 2013). At present, although research on *N. caninum* has progressed in China and other countries, the pathogenic effect of *N. caninum* in causing abortion and stillbirth in pregnant animals remains unclear. However, an animal model of *N. caninum*–induced placenta damage has not been established. BALB/c mice, C57BL/6 mice, and gerbils are commonly used as model animals. *N. caninum* can artificially infect mice, rats, dogs, cats, gerbils, and rabbits. BALB/c mice have historically been used as

experimental animals to test the extent of *N. caninum* infection in pregnant mice, confirming that BALB/c mice can be used as both a congenital infection and vertical propagation model of *N. caninum* (Long and Baszler 1996).

During pregnancy, the placenta is a multi-functional organ that plays an extremely important role between the mother and fetus, including gas exchange, nutrition supply, metabolism, and disease prevention (Dorsch et al. 2019). The role of hormones and cytokines secreted by the placenta during pregnancy is extremely important. In particular, the placenta secretes a variety of hormonal substances to maintain a normal pregnancy, facilitate placental and fetal growth, and transfer material between the mother and the fetus (Rosbottom et al. 2008). The cytokines secreted by the placenta can promote and inhibit the growth of the placenta (García-Ispierto et al. 2015). Placental dysfunction is highly likely to affect the normal development and healthy growth of the fetus.

In the present study, the clinical signs, morbidity, and pathological changes in the mice were observed following an intraperitoneal infection with *N. caninum*, which were cultured in Vero cells in vitro. The *N. caninum* Nc5 gene was subsequently detected in the brain, liver, spleen, and placenta of pregnant mice by PCR. The wet weight of the placenta, placental coefficient, placental hormone, and cytokine levels in the pregnant mice were measured. The model of *N. caninum* infection in pregnant mice was established to provide a foundation for the study of the pathogenic mechanisms of *N. caninum*.

Materials and methods

Experimental animals

Both female and male BALB/c mice aged 8 weeks and weighing approximately 20 g were purchased from the Liaoning Changsheng Experimental Animal Co. Ltd., China (Certificate No. SCXK (Liao) 2015-0001). The mice were maintained under controlled temperature (25 °C ± 1 °C) and humidity (50% ± 5%) and had free access to food and water. All animal experimental procedures were approved by the Ethical Committee for the Experimental Use of Animals at Yanbian University (Yanji, China) in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China (Approval No.: 20180301).

Parasites, strains, and main reagents

Bovine *N. caninum* was isolated and cultured from the Yanbian University Preventive Veterinary Laboratory, Yanji, China (Jia et al. 2014). Vero cells and *E. coli* DH5α strains were preserved at the Preventive Veterinary Laboratory of Yanbian University.

Primer design and synthesis

Primers were designed using the *Nc5* gene sequences of *N. caninum* (AF061249) available from GenBank. P1 and P2 (Table 1) primers were designed using Oligo 6.0 software and synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China).

N. caninum cultures and collection in vitro

N. caninum parasites were propagated in African green monkey kidney (Vero, Procell, China) cells and cultured in Dulbecco's Modified Eagle's medium (DMEM, Sigma-Aldrich, USA). Subsequently, the cells were supplemented with 8% heat-inactivated fetal bovine serum (FBS, Gibco, USA), 100 μg/mL penicillin, and 10 mg/mL streptomycin (Solarbio, Beijing) at 37 °C in a 5% CO_2 atmosphere. Tachyzoites were purified from the infected Vero cells by washing the cells in ice-cold phosphate-buffered saline (PBS, Solarbio, Beijing), and then passaged three times through a 27-gauge needle syringe. The tachyzoites were subjected to filtration through a 5.0-μm pore filter to remove the host cell debris and centrifuged at 600 g for 10 min. The parasite numbers were counted with a hemocytometer.

Animal inoculation

A total of 40 female BALB/c mice aged 8 weeks were randomly selected and divided into four groups (10 mice per group). Tachyzoites (100 μL/mouse) suspended in ice-cold PBS were inoculated intraperitoneally at different doses to all but the normal group (group IV). The normal group received an inoculation of 100 μL ice-cold PBS per mouse. A total of 10^4, 10^5, and 10^6 tachyzoites inoculated into groups I, II, and III, respectively. After 24-h inoculation, the female and male BALB/c mice were caged at a ratio of 2:1 based on the detection of a vaginal plug. The diet, drinking water, mental state, and morbidity of the mice in each group were observed daily. This was used as an indicator of the test subject when infected pregnant mice presented with various clinical signs, including depression and ataxia, but did not die.

Table 1 The common PCR primer sets used for amplification of Nc5

Primers	Oligonucleotide sequences
P1	5′- CGGGATCC GTCGTTTTAGCAGTTGGGGTGTC -3′
P2	5′- CGGAATTC CTTAGGAATGGTCAGTGTCGC -3′

Pathological observations of the tissue

The mice in the survival model with clinical signs were selected and sacrificed. The organs (i.e., brain, liver, and spleen) were collected aseptically, and changes in the appearance were observed by pathological analysis. Hematoxylin-eosin (HE) staining was used to confirm the histopathological changes and infection of the tissue.

Detection of *N. caninum* in the tissues and placenta

PCR was performed to detect expression of the Nc5 gene in the brain, liver, spleen, and placenta, and the amplified gene fragment was cloned and sequenced. The amplification conditions consisted of an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 45 s, an annealing step at 55 °C for 1 min, an extension step at 72 °C for 1 min, and a final extension at 72 °C for 7 min.

Determination of the placental weight and placental coefficient

The mouse model of *N. caninum* infection was established based on the optimal parasite dose. The animals were randomly divided into pregnant mice infected with *N. caninum* group ($n = 40$), and a control group consisting of normal pregnant mice ($n = 40$). On days 12, 14, 16, and 18 post-infection, 10 pregnant mice from each group were humanely euthanized, and the blood was collected. The placenta was separated from each fetus and weighed. The placenta coefficient (placenta coefficient = placenta weight/fetal weight) was calculated.

Determination of the levels of serum placental hormones and cytokines in placental cell suspensions

On days 12, 14, 16, and 18 post-infection, blood samples were collected in tubes without anticoagulant. To obtain the serum, blood samples were centrifuged (2500*g* for 5 min at 37 °C). The serum samples from both the model and control groups were aseptically collected and tested in accordance with the manufacturer instructions of the CRH, CG, PRL, and E3 kits (ADI Company). The placenta was isolated aseptically from the pregnant mice and washed with ice-cold phosphate-buffered saline (PBS). The placenta was grounded with a lysis solution (1:10) into cell suspensions, which were centrifuged (12000 rpm for 15 min at 4 °C) and the supernatants were collected in tubes. The supernatants were assessed according to the manufacturer instructions in the IFN-γ, IL-4, and TGF-β detection kits (ADI Company). Depending on the concentration and OD value of the standard, a straight line regression equation of the standard curve was drawn according to the OD values of the tested samples to calculate the concentration of each sample.

Statistical analysis

All values are expressed as the mean ± SD. A one-way analysis of variance (ANOVA) was used. All statistical analyses were performed using SPSS 20.0 statistical analysis software (SPSS Inc., Chicago, IL, USA). A *P* value < 0.05 was considered to be statistically significant.

Results

### *N. caninum* culture and collection

The *N. caninum*–infected Vero cells were observed to form parasitophorous vacuoles (PV) within 3–5 days. After the multiplication and division of the tachyzoites, a single tachyzoite could be obtained by isolation and purification with a 27-gauge needle and 5.0-μm pore filter. The tachyzoites were counted and diluted to 10^4, 10^5, and 10^6 tachyzoites per 100 μL.

Clinical symptoms and morbidity of *N. caninum*–infected BALB/c mice

BALB/c mice infected with tachyzoites exhibited clinical signs, including rebellion, depression, and ataxia. The mice in groups I, II, and III showed differential mortality after infection, with survival rates of 90%, 80%, and 50%, respectively, whereas no changes were observed in group IV (control group) (Fig. 1).

Pathological changes of the tissues in the *N. caninum*–infected BALB/c mice

The pathological analysis revealed the presence of meningeal congestion and hemorrhage, spleen and kidney enlargement, liver white lesions and punctate hemorrhage, and punctate hemorrhage of lung and heart compared with the control group. Analysis of the HE-stained tissue showed aggregation
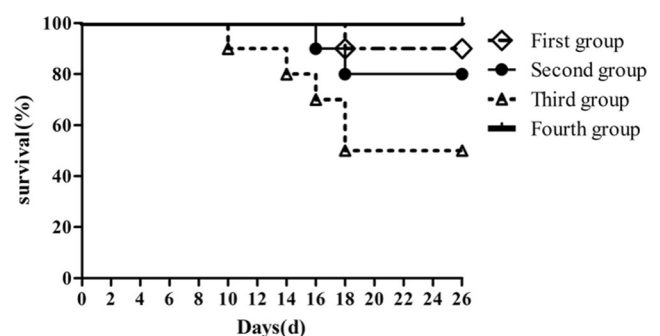
Fig. 1 Survival rate of parasite-infected mice. The number of tachyzoites inoculated into groups I, II, and III was 10^4, 10^5, and 10^6, respectively. Group IV was the control group, which was not inoculated with parasites

of the brain tissue cells, with horny nodules, phagocytic phenomena, and focal encephalitis. The hepatocytes were associated with edema, granular degeneration, local, and perivascular inflammation. The spleen exhibited congestion and hemorrhage. The pulmonary tissue displayed infiltrating lymphocytes, as well as altered tracheal and bronchial inflammation. The kidney contained localized congestion and hemorrhage. The heart showed myocardial granule degeneration, necrotic focus, and intravascular hemolysis. Moreover, *N. caninum* tissue cysts and tachyzoites were found in the brain, liver, and spleen (Fig. 2a, 2b, and 2c).

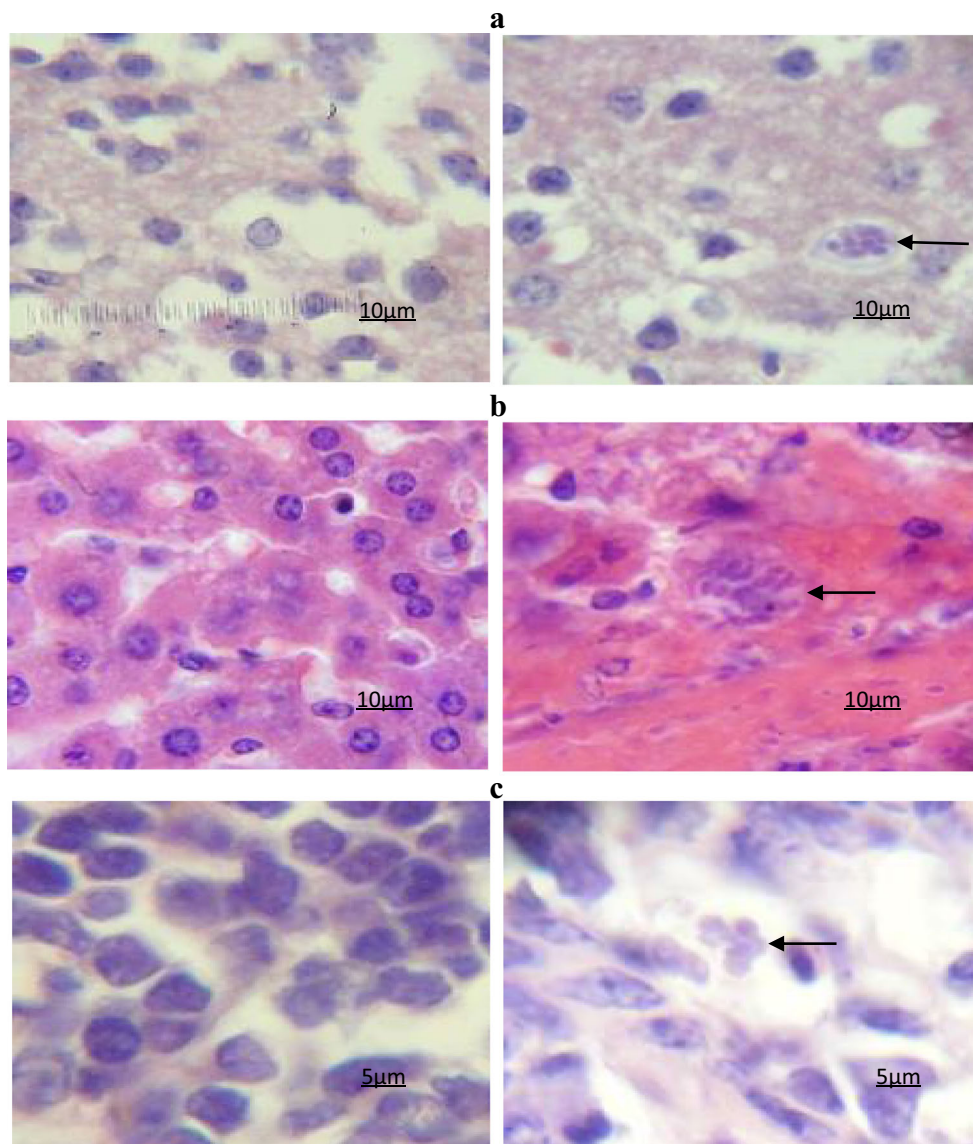### Detection of *N. caninum* in the tissues and placenta in the mouse model

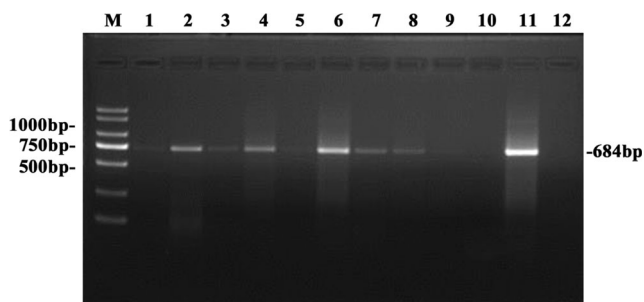The *N. caninum* Nc5 gene was detected in the main organs of the brain, liver, and spleen by PCR, and the Nc5 gene of

N. caninum was detected in the placenta tissues of the model on days 12, 14, and 16 post-infection (Fig. 3). Additionally, the *N. caninum* Nc5 gene detected in the main organ tissues and placenta of the model mice was 100% homologous with the GenBank sequence (AF061249).

The wet weight and placenta coefficient results

After aseptically separating the fetus from the placenta, the placenta coefficients were weighed and calculated. The results showed that the placental and fetal weights of both the model and the control groups increased continuously, and the placental coefficient gradually decreased with increased time post-infection. On days 12, 14, 16, and 18, the placental weight and placental coefficient between the model and the control groups were significantly different ($P < 0.05$).

Fig. 2 Pathological changes in the major organs in dying BABL/c mice 18 days post-infection with isolated parasites. The left side is the control group, and the right side is the model group (*N. caninum*–infected mice; the arrows indicate the *N. caninum* tissue cyst or tachyzoites). **a** Brain, *N. caninum* tissue cyst. **b** Liver, *N. caninum* tachyzoites. **c** Spleen, *N. caninum* tachyzoites

Fig. 3 PCR detection of *N. caninum* Nc5 gene expression in the main organs and placenta of mice in a 1% agarose gel. Lane M, DL2000 DNA marker; lanes 1–6, DNA extracted from infected mice heart, liver, spleen, lung, kidney, and brain; lanes 7–9, DNA extracted from the placenta of infected pregnant mice on days 16, 14, and 12 post-infection; lane 10, DNA extracted from infected mice blood; lane 11, DNA extracted from *N. caninum* tachyzoites (Nc5 gene positive control); lane 12, PCR water control

However, the fetal weight was not significantly different between the model and the control groups (*P* > 0.05) (Fig. 4).

Detection of placental hormone levels

The CRH results showed that the concentration in the control group was continuously upregulated with time post-infection, whereas that of the pregnant mice in the model group was continuously downregulated. Compared with the control group, the level of CRH in pregnant mice was significantly different on day 12 (*P* < 0.05) and on day 18 post-infection (*P*

< 0.01) (Fig. 5a). The CG results showed that during pregnancy and after *N. caninum* infection, the CG level increased and subsequently decreased in the control group. However, in the test group, the CG concentration continuously increased. Serum CG levels were significantly different between the model and control groups on day 12 (*P* < 0.01) and day 16 (*P* < 0.05) (Fig. 5b). While the levels of serum PRL increased and subsequently decreased in both groups, the overall level of PRL in the model group was lower than that in the control group. The levels of PRL significantly differed between the two groups on day 18 (*P* < 0.05) (Fig. 5c). Serum levels of E3 showed increased-and-decreased fluctuations during pregnancy and following *N. caninum* infection. However, the E3 levels significantly fluctuated in the model group. Compared with the control group, the levels of serum E3 in the model group increased significantly on days 12 and decreased significantly on 16 post-infection (*P* < 0.05) (Fig. 5d).

Determination of cytokine levels

The results showed that the level of IFN-γ was upregulated and subsequently downregulated with increased time post-infection; however, the levels of IFN-γ in the model group were higher than those of the control group. Compared with the control group, the levels of IFN-γ in the placental cell suspension of pregnant mice were significantly different on day 18 post-infection (*P* < 0.05) (Fig. 6a). The IL-4 results
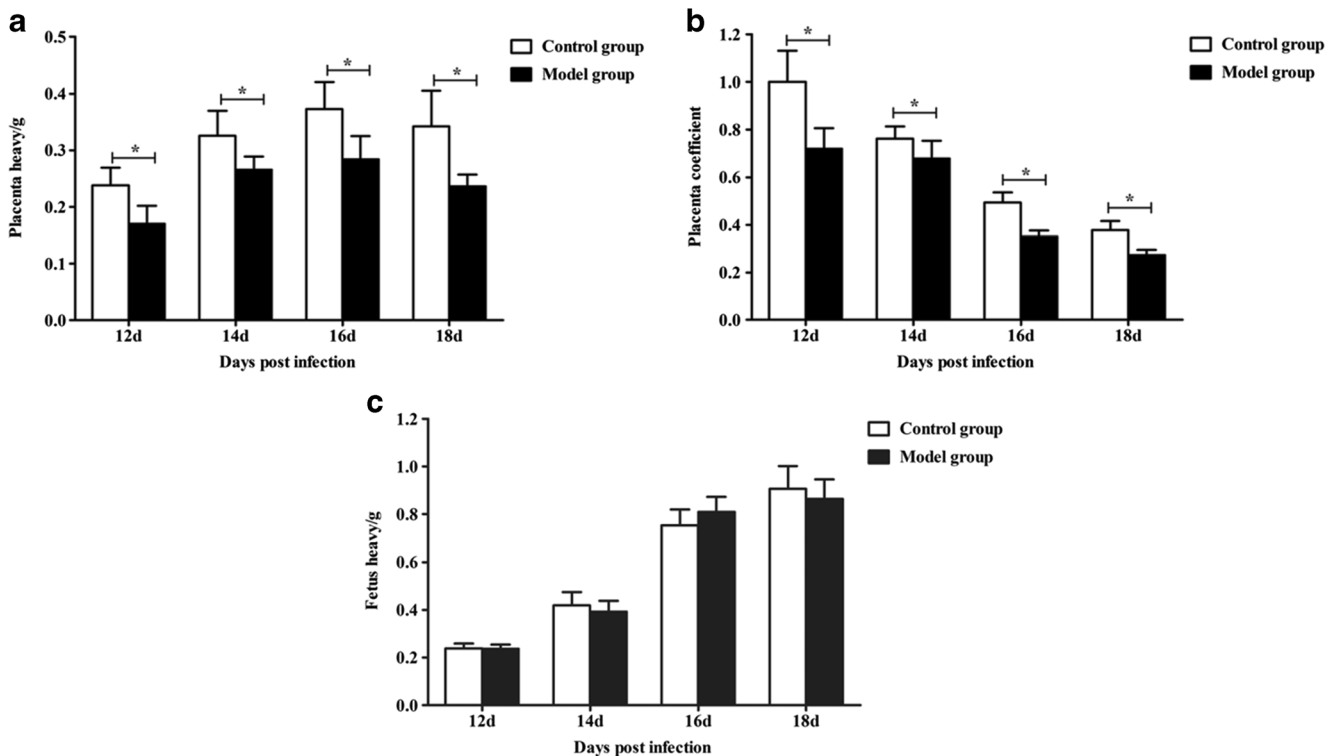

Fig. 4 Placenta weight and placenta coefficient of pregnant mice. **a** Placental weight. **b** Placental coefficient. **c** Fetal weight. The results show the mean values ± SD. The asterisks indicate statistical significance (**P* < 0.05)
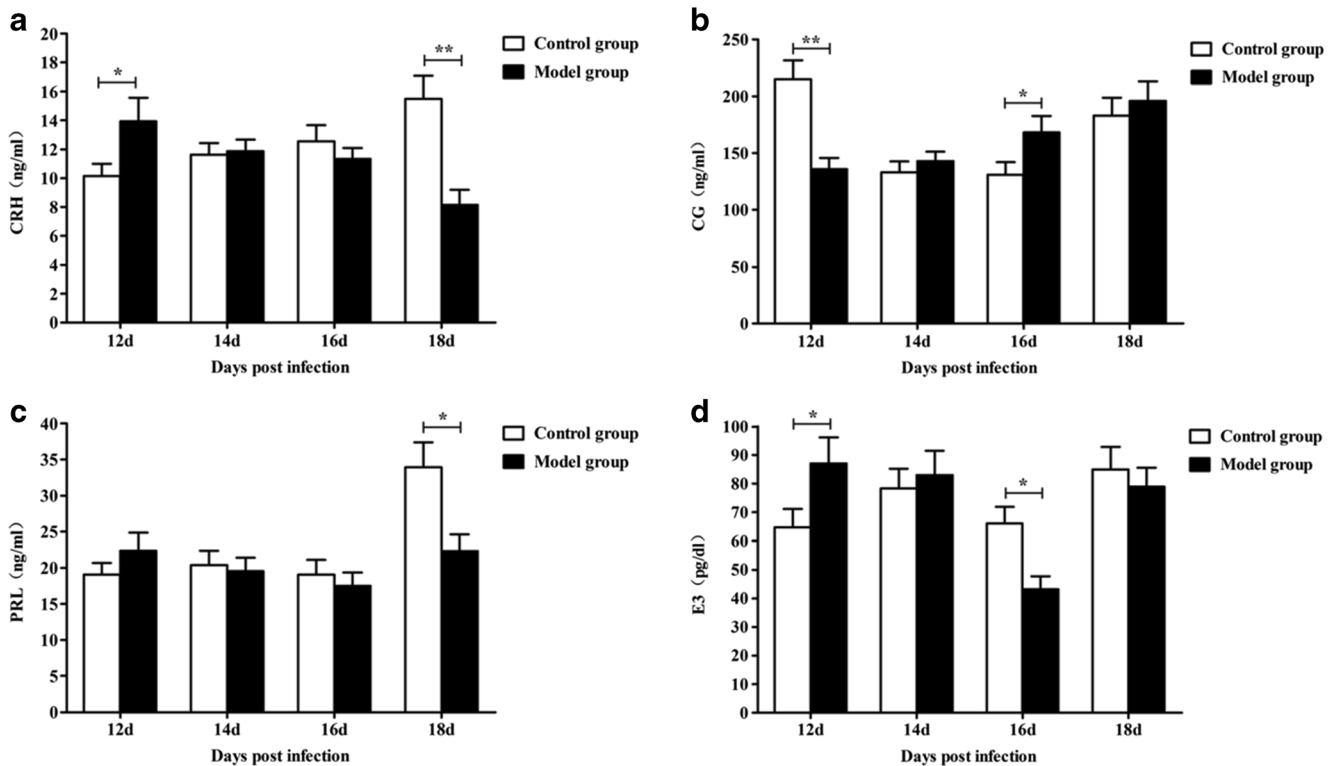
Fig. 5 Determination of placental hormone levels. Serum levels of the placental hormone CRH (**a**), CG (**b**), PRL (**c**), and E3 (**d**) in *N. caninum*–infected pregnant mice on days 12, 14, 16, and 18 post-inoculation. Placental hormones were analyzed in triplicate. The results show the mean ± SD. The asterisks indicate statistical significance (*P < 0.05)
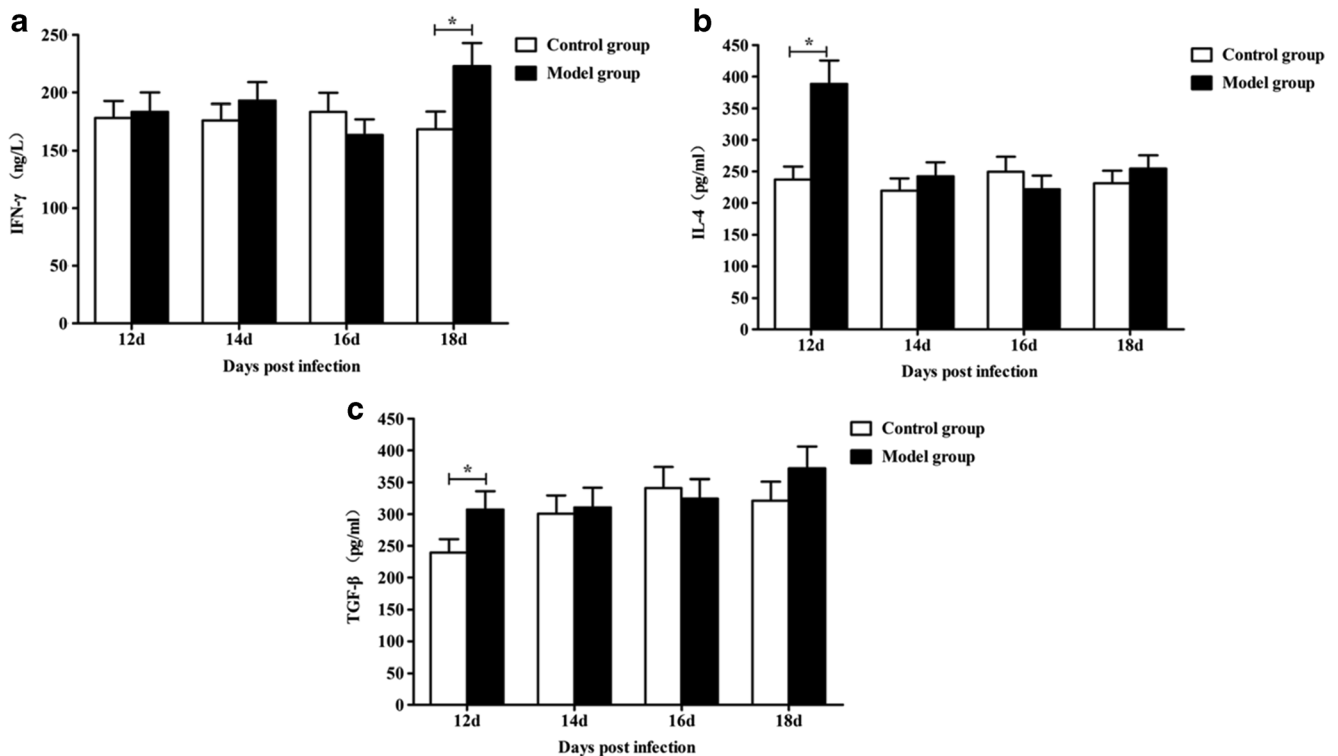
Fig. 6 Determination of cytokine levels. Cytokine levels in the placental cell suspensions IFN-γ (**a**), IL-4 (**b**), and TGF-β (**c**) of *N. caninum*–infected pregnant mice on days 12, 14, 16, and 18 post-inoculation. The level of cytokine production was analyzed in triplicate. The results show the mean ± SD. The asterisks indicate statistical significance (*P < 0.05)

showed that the levels of IL-4 were balanced in the control group, which increased and subsequently decreased during pregnancy and following *N. caninum* infection in the test group. Moreover, the IL-4 levels in the model group were higher than those in the control group. IL-4 levels in the placenta cell suspension were significantly different ($P < 0.05$) between the two groups on day 12 post-infection (Fig. 6b). The concentration of TGF-β was upregulated and then downregulated in the control group, whereas that in the model group continuously increased. The level of TGF-β in the placental cell suspensions of the pregnant mice was significantly different ($P < 0.05$) between the two groups on day 12 post-infection (Fig. 6c).

Discussion

The study by Ramamoorthy et al. used C57BL/6 mice to test the efficacy of a vaccine against *N. caninum*, and confirmed that C57BL/6 mice could be used as a vertical propagation model of *N. caninum* infection (Ramamoorthy et al. 2007). Similarly, Hůrková-Hofmannnová et al. used gerbils as experimental animals to observe the clinical symptoms and histopathological changes (Cuddon et al. 1992; Gondim et al. 1999; Dubey and Lindsay 2000; Hůrková-Hofmannnová et al. 2007). Moreover, Quinn et al. found that the response of pregnant mice with tachyzoites inoculated during different gestational periods varied greatly depending on the gestational period in a gestational mouse model (Quinn et al. 2002; Regidor-Cerrillo et al. 2010; Dellarupe et al. 2014; Aguado-Martínez et al. 2017). It was confirmed that gerbils are highly sensitive to *N. caninum* and can be used as an animal model. In the present study, we used BALB/c mice as experimental animals to establish an *N. caninum*–infected pregnant mouse model. After infection, the BALB/c mice exhibited the typical clinical signs of *N. caninum*, including coat reversal and ataxia. At 20 days post-infection, groups I, II, and III exhibited variable mortality, with survival rates of 90%, 80%, and 50%, respectively. Although the survival rate was higher in group I (10^4), the onset of clinical signs was late. The clinical signs of the mice in group III (10^6) were observed early; however, because the survival rate was lower, the mice inoculated with 10^4 and 10^6 tachyzoites were not considered to be suitable animal models. Therefore, 10^5 tachyzoites are the optimal dose for establishing the mouse model.

The *N. caninum* Nc5 gene bands were detected in the brain, liver, and spleens of the infected pregnant mice. In addition, the Nc5 gene was also detected on days 12, 14, and 16 post-infection in the placenta, with 100% homology in GenBank (AF061249) by sequencing. According to the observations of the amplification band, the number of parasites in the placental tissues gradually increased with time post-infection, indicating that the parasite-induced damage to the placenta was also increased.

The common index of placental function is the placenta coefficient, which can be used to determine the functional status of the placenta (Rao 2009). The results showed that both the placental and fetal weight in the model and the control groups continuously increased, while the placental coefficient decreased gradually. The placenta weight and the placenta coefficient of the model group were significantly different from the control group ($P < 0.05$). Therefore, we believe that infection with *N. caninum* had a great influence on the embryos of pregnant mice. Such infection can cause pathophysiological reactions in the fetus but may also cause pathological damage to the reproductive system. However, the extent of specific damage remains to be further studied.

The placenta is a temporary multi-functional organ and is extremely important for secreting hormones during pregnancy. CRH, CG, PRL, and E3 are hormones secreted by the placenta that directly affect the pregnancy, fetal growth and development, maternal-to-fetal material transport, and maternal-fetal placenta cell proliferation and differentiation. Moreover, CRH is a peptide hormone. During pregnancy, the placental synthesis of CRH can act on the uterus and has important paracrine and autocrine functions, which are conducive to immune tolerance and the protection of blastocyst implantation. Other important physiological functions of CRH include the dilation of placental blood vessels, which can protect the blood perfusion between mother and fetus, fetal metabolism, and fetal hypoxia. CG is a glycoprotein hormone secreted by placental trophoblastic cells, which is high in both the plasma and urine during pregnancy. CG regulates the synthesis of prostaglandins and steroids, but also has an immunosuppressive effect. The changes in plasma CG can be used to predict and diagnose pregnancy-induced hypertension, miscarriage, fetal hypoxia, intrauterine growth retardation, and other adverse pregnancy outcomes (Alsat et al. 1996; Esterman et al. 1996). PRL, a protein hormone, is synthesized by placental syncytiotrophoblast cells, and can promote the proliferation and differentiation of cells in the placenta, nutrient metabolism, and energy metabolism in the fetus. This may lead to an adverse pregnancy outcome following abnormal secretion. Serum E3 is primarily synthesized in the placenta during pregnancy, and the changes in maternal plasma E3 levels can reflect the function of the fetus and placenta. When the E3 content is reduced, fetal malformation or intrauterine development can occur. The results of the present study show that with increased time post-infection, the CRH levels in the test group continued to decrease compared with the control group. On day 18 post-infection, the CRH levels were significantly different from those in the control group ($P < 0.01$), suggesting that the fetus may exist in an ischemic and hypoxic state, and may damage both vascular and placental cells. The levels of CG in the pregnant mice in the model group were continuously increased, with extremely significant difference observed on day 12 post-infection ($P <$

0.01), and a significant difference was found on day 16 post-infection ($P < 0.05$), suggesting that the continuous increase in the CG concentration may lead to a miscarriage in pregnant mice, stillbirth, and intrauterine growth retardation. The level of PRL in pregnant mice in the model group decreased and subsequently increased, with an overall level that was significantly lower than that of the control group. On day 18 post-infection, the PRL level was significantly different ($P < 0.05$). These results suggested that the invasion of *N. caninum* led to placental damage and placental dysfunction, which seriously affected fetal energy metabolism and nutrient uptake. The E3 levels in the model group significantly fluctuated compared with the control group, and significantly differed on days 12 and 16 ($P < 0.05$). This suggested that changes in the level of E3 prior to delivery could lead to fetal malformation or intra-uterine growth retardation.

There is a close relationship between cytokines and pregnancy. Normally, the placenta secretes a variety of cytokines, including IFN-γ, IL-4, and TGF-β. The study by Rosbottom et al. showed that cytokines play a role in *N. caninum* infection, which may be related to fetal death (Rosbottom et al. 2008)). Another study performed by Lopez-Perez et al. showed that the level of IFN-γ gene expression in the spleens of mice infected with *N. caninum* was significantly higher than that in the placenta, and the level of IL-4 in the placenta was increased, which may enhance the chance of transmission from the placenta to the fetus (Lopez-Perez et al. 2010). The study by Almeria et al. inoculated tachyzoites into cattle at 110 days of gestation, and confirmed that the upregulation of IFN-γ expression and downregulation of TGF-β expression in the placental tissues of *N. caninum* are beneficial to fetal survival, indicating that cytokines play an important role in the placental transmission of *N. caninum* (Almeria et al. 2011). In this study, the level of IFN-γ and IL-4 in the placental cell suspension of the pregnant mice in the model group was significantly higher than the control group, which may be the main factor leading to the vertical transmission of *N. caninum*. However, the level of TGF-β in the model group continuously increased, which will be detrimental to fetal survival, and may represent an important factor leading to abortion and stillbirth, which is consistent with the findings of Almeria et al.

Conclusions

In this study, a *N. caninum*–infected pregnant mouse model was successfully established. The optimal dose of *N. caninum* infection was 10^5 parasites. Moreover, our results showed that the brain, liver, spleen, and placenta of the pregnant mice were damaged by an infection with *N. caninum*. Furthermore, the regulation of placental hormones and cytokines was unbalanced in pregnant mice. This experiment laid the foundation for the study of *N. caninum* pathogenesis.

Authors' contributions LJJ and SZX conceived and designed the experiments. SZX, JXL, HL, HW, and SZ performed the experiments. SWZ carried out the data analysis. LJJ and SZX wrote the manuscript.

Compliance with ethical standards

All animal experimental procedures were approved by the Ethical Committee for the Experimental Use of Animals at Yanbian University (Yanji, China) in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China (Approval No.: 20180301).

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

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