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RESEARCH

A preliminary study on placental damage associated to experimental neosporosis in BALB/c mice

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Abstract

Neospora caninum is a protozoan parasite which can infect a range of animals, including dogs, cattle, and sheep. Bovine neosporosis, which mainly causes abortion in cattle, results in substantial economic losses worldwide. To study the effects of *N. caninum* infection on the placenta, a pregnant mouse model for *N. caninum* infection was established. The litter size (8.6 ± 1.5) and the number of live pups (6.4 ± 1.8) of infected dams were significantly lower compared with those of non-infected dams. Trophoblast cell shrinkage and a large number of apoptosomes were detected in the placentas of the infected group. The parasite load in the placental tissue was significantly higher with time after infection. Likewise, apoptosis of placental trophoblast cells significantly increased with time after infection. Among the 66 apoptotic genes detected in this study, eight genes, including *Bcl-2*, were significantly differentially expressed by about > tenfold in infected and uninfected mice. The expression of BAX and tumor necrosis factor-alpha (TNF-α) was upregulated in the placental cells of the infected mice, whereas the expression of BCL-2 was downregulated. Enzyme-linked immunosorbent assays (ELISAs) showed that apoptotic protease caspase-3 level was significantly increased in placental cell suspension, and insulin-like growth factor (IGF)-2 level was significantly reduced. Acetylcholine (ACH) and placental prolactin (PL) levels were initially decreased but eventually increased. In summary, infection of mice with *N. caninum* caused apoptotic damage to the placental tissues, cells, and genes and affected the normal physiological functions of placenta, which may largely explain the adverse pregnancy outcomes caused by *N. caninum* infection in mice.

Keywords *Neospora caninum* · BALB/c mouse · Placenta · Pathology · Apoptosis

Introduction

Neosporosis is a protozoan disease caused by *Neospora caninum*. The disease was first described in puppies suffering from encephalitis and myositis in 1984 by the Norwegian veterinarian Bjerkås (Bjerkås et al. 1984). *Neospora caninum* has three infective stages: tachyzoites (rapidly multiplying stage), bradyzoites (slowly multiplying stage, tissue cysts), and sporozoites (oocysts) (Donahoe et al. 2015). The tachyzoites multiply and spread to different tissues and organs. The placenta is the most severely damaged tissue and organ by tachyzoites. The dog is the definitive host of *N. caninum*, and cattle, sheep, horse, deer, mouse, and birds all act as intermediate hosts (Hemphill et al. 2016; Bartley et al. 2019; Nardoni et al. 2019). The harm to cattle is particularly serious, predominantly causing abortion and stillbirth in pregnant animals and diseases of the motor nervous system in newborn calves (Lagomarsino et al. 2019; Lefkaditis et al.

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2020). Neosporosis can be transmitted vertically through infected cows by directly transmitting the parasite to newborn calves (Bartley et al. 2019; Nardoni et al. 2019).

The placenta is responsible for the functions of gas exchange, nutrient supply, metabolism, and disease prevention between the mother and the fetus (Collantes-Fernandez et al. 2012; Jia et al. 2020). The structural integrity and function of the placenta are the basis of a normal pregnancy, and apoptosis is a routine physiological phenomenon of the placental tissues. During pregnancy, spatiotemporally regulated apoptosis plays an important role in the development of villi and the immune tolerance of the mother to the fetus. When placental injury occurs, it affects the fetus' normal development and growth (Caspe et al. 2012; González-Warleta et al. 2018). The immunopathological response of the bovine and ovine placenta to *Neospora* infection have already been widely studied (Cantón et al. 2014; Almería et al. 2016; Arranz-Solís et al. 2016; Botta et al. 2019). However, the mechanisms underlying the placental damage caused by *N. caninum* is unclear. Therefore, in this study, a pregnant mouse model of *N. caninum* Yanbian strain infection was used to study this phenomenon. Litter size and the number of live pups was counted; and the parasite load, trophoblast cell apoptosis, apoptotic genes, apoptotic proteins, and placental hormones were measured to clarify the impact of *N. caninum* on the placenta of pregnant mice. Our findings lay the foundation for studying the damaging effects of *N. caninum* on the placenta by developing a pregnant mouse model.

Materials and methods

Experimental animals

Eighty ($n = 80$) (SPF) female BALB/c mice, aged 8 weeks and weighing approximately 20 g, were assigned to either control group or infected group. BALB/c mice were purchased from the Liaoning Changsheng Experimental Animal Co. Ltd (Liaoning, China; certificate no. SCXK [Liao] 2015–0001). The mice were domesticated under controlled temperature (25 ± 1 ℃) and humidity ($50 \pm 5\%$) 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 of the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China (approval no.: 20180301).

Neospora caninum cultures and collection in vitro

The *N. caninum* Chinese Yanbian strain used in the present study, was previously isolated from bovine brain tissue from an aborted fetus and propagated in African green monkey kidney (Vero) cells in the Yanbian University Preventive Veterinary Laboratory (Yanji, China) (Jia et al. 2014). The parasite numbers were counted with a hemocytometer.

Animal model and group processing

An *N. caninum* infection was established in a pregnant mouse model according to the methods of Regidor-Cerrillo et al. (2010) and Jia et al. (2020). After confirmation of the vaginal plug, 40 female mice were intraperitoneally inoculated with *N. caninum* tachyzoites at a dose of 10^5. Likewise, a control group comprising of 40 normal pregnant mice was also established. On days 12, 14, 16, and 18 after infection, ten mice from each of the infected and the control groups were euthanized. The following tests were performed after the aseptic separation of the fetus and the placenta.

Survival test of fetal mice

Mice in the infected and control groups were sacrificed on the 12th, 14th, 16th, and 18th days post infection, and the fetuses and placenta were isolated. The litter size and the number of live pups were counted to determine the impact of *N. caninum* infection on fetal survival.

Histopathological examination

Placentas were dissected, then fixed with 10% neutral buffered formalin for 12 h. After fixation, each placenta was processed routinely and embedded in paraffin wax. The Sections (4 μm) were stained with hematoxylin and eosin (H&E), sealed with neutral gum, and observed microscopically (Amini et al. 2020).

Quantitative PCR (qPCR) detection of the *Nc-5* gene

DNA was extracted from 50 ng of placenta tissue and purified with the FastPure Tissue DNA Isolation Mini Kit (Vazyme Biotech Co., Ltd, China), according to the instructions of the manufacturer. The *N. caninum Nc-5* gene (GenBank accession no. X84238) was quantified by qPCR based on the previously published procedures by Nishikawa et al. (2018), which amplifies a a 76-bp DNA fragment. Primers for the *Nc-5* gene (Forward: 5′-ACTGGAGGCACGCTGAACAC-3′, Reverse: 5′-AACAATGCTTCGCAAGAGGAA-3′) were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Detection of trophoblast apoptosis with flow cytometry

A suspension of placental trophoblasts was prepared and labeled with the Annexin V-FITC Apoptosis Detection Kit (Vazyme Biotech Co., Ltd), according to manufacturer's

instructions. Apoptosis was detected with flow cytometry (Accuri C6, BD Co., Ltd, USA).

Quantification of apoptotic gene expression in placentas with qPCR

Total RNA was extracted from 50 ng of placental tissues with the E.Z.N.A. Total RNA Kit I (R6834-01; Omega Bio-Tek). First-strand cDNA was synthesized with Transcriptor First Strand cDNA Synthesis Kit (lot no. 04897030001; Roche Life Science). qPCR was performed with FastStart Universal SYBR Green Master (lot no. 04913914001; Roche Life Science), according to the manufacturer's instructions. A panel of 86 apoptosis-related genes was run on a 96-well format including endogenous controls (Bioscience Corporation, USA) to detect the transcript levels of apoptosis-related genes, including BAX, BCL-2, and TNF-α. Differential expression in placental tissues was depicted in a heatmap using GraphPad Prism 9 (GraphPad Software, USA).

Detection of apoptotic protein expression in placentas with immunohistochemistry (IHC)

Placental samples were fixed in 10% neutral buffered formalin, processed with standard methods, embedded in paraffin blocks, and sectioned. A rabbit anti-mouse BAX antibody, rabbit anti-mouse BCL-2 antibody, and rabbit anti-mouse TNF-α antibody (1:1500 dilution) were used as the primary antibodies, and a horseradish-peroxidase-conjugated goat anti-rabbit IgG antibody (1:2000 dilution) was used as the secondary antibody. The expression of apoptotic proteins was detected with Vectastain® ABC-Peroxidase Kit (Xinbosheng Biotechnology Co., Ltd, China), according to the manufacturer's instructions. The primary antibody was replaced with PBS in the negative control group.

Detection of caspase-3, IGF-2, acetylcholine (ACH), and placental prolactin (PL) levels in placental cell suspensions

Placental single-cell suspensions were prepared and analyzed with an ELISA to detect caspase-3, IGF-2, ACH, and PL levels (Enzyme Linked Immunosorbent assay kit, ADI, USA), according to manufacturer's instructions. Briefly, the assay buffer was serially diluted to different concentrations and a standard curve was made. Placental single-cell pellet from 25-mg tissue was homogenized in extraction buffer and centrifuged at 10,000 × g for 5 min. The supernatant were collected and used as samples. The supernatant (5 µl per well) was added onto a 96-well plate and the volume was adjusted to 50 µl using assay buffer. The plates were incubated at 37 °C for 30 min. The absorbance values (OD 450 nm) were measured and converted by extrapolation using a standard curve.

Statistical analysis

All values are expressed as means ± standard deviation (SD). One-way ANOVA test of variance was used to evaluate the significance of differences using GraphPad Prism version 9.0 for Windows (GraphPad Software Inc., USA). For all tests, a P value < 0.05 was considered statistically significant.

Results

N. caninum infection decreased litter size and live pups and caused damage to mouse placenta

In the *N. caninum*-infected group, the average litter size and the number of live pups decreased to varying degrees with the increasing days of infection (Fig. 1A). The average litter size of *N. caninum*-infected dams (8.6 ± 1.5) was significantly different compared to the control group (11.2 ± 2.1) on 18 days post infection ($P < 0.05$), while the number of live pups began to decrease at 14 days post infection (8.8 ± 1.1). Likewise, the number of live pups was significantly different at 16 days post infection (7.9 ± 1.4) ($P < 0.05$), and the difference was significant at 18 days post infection (6.4 ± 1.8) ($P < 0.01$, Fig. 1A). H&E staining of the placental tissues was conducted to further compare the differences of the placental tissues between the control group and infected group. On 14th day of the infection, the sponge trophoblast cells appeared atrophic and vacuolated, the labyrinth area became hyperemic and hemorrhagic, and a large number of apoptotic bodies were observed (Supplementary Fig. 1C). On day 18, the sponge trophoblast cells were severely vacuolated, and the labyrinth area was infiltrated by inflammatory cells (Supplementary Fig. 1D). Atrophy and vacuolisation of spongy trophoblast cells, hyperemia, and hemorrhage in the labyrinth area; the presence of a large number of apoptotic bodies; and inflammatory cell infiltration were not detected in the control group (Supplementary Fig. 1A and 1B). The number of parasites in the placenta at different times after the infection was also investigated, and the parasite load in the placenta showed a significant upward trend and differed significantly with time ($P < 0.01$). This clearly confirms that the number of parasites in the placental tissue followed a time-dependent process (Fig. 1B). In order to further reveal the damaging effects of *N. caninum* infection on the placenta of pregnant mice, the number of apoptotic cells in the placenta was counted. Flow cytometry results showed that in the infected group, the number of apoptotic cells in the placenta increased with time since infection and differed significantly from that in the control group at 12 days post infection

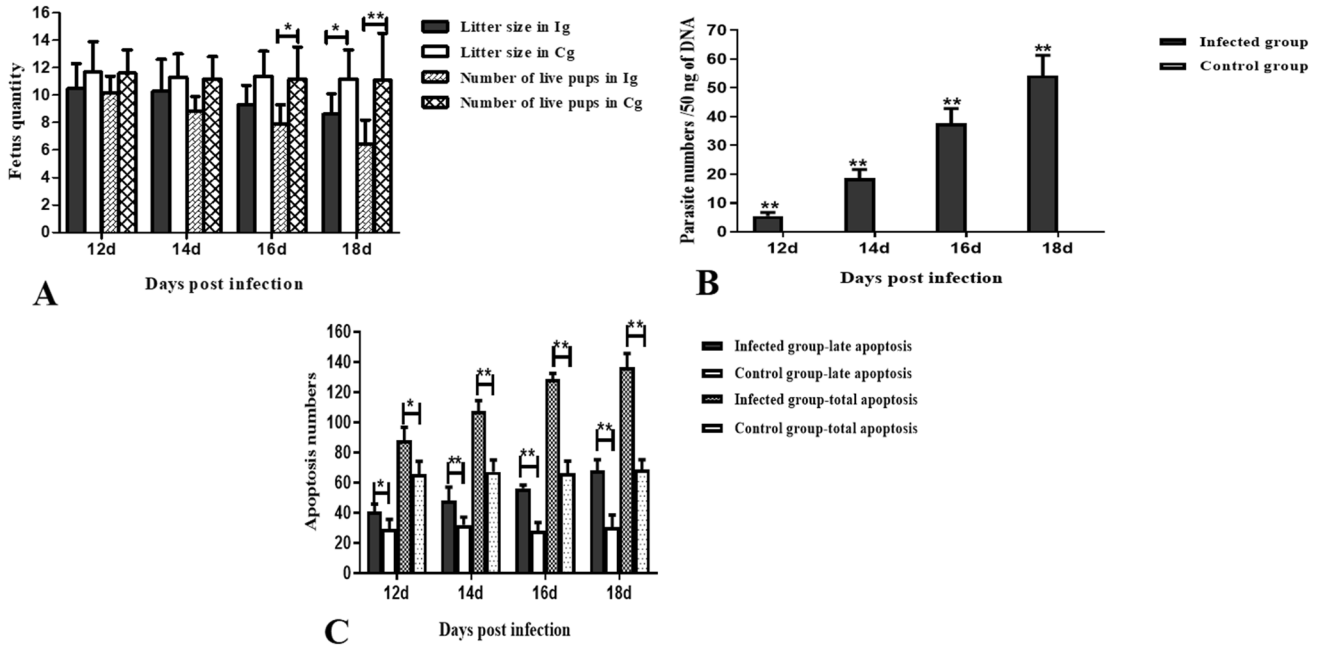

Fig. 1 **A** The litter size and the number of live pups. *Ig*, infected group; *Cg*, control group. **B** Parasite load in the placental tissues. Parasite load in the placenta was detected with qPCR. Values are the numbers of parasites in 50 ng of tissue DNA. No parasite was detected in the DNA of the control group (data not shown in the figure). (**C**) Trophoblast apoptosis in placental tissues. Number of late apoptosis and total apoptosis in placental trophoblast cells. Results are expressed as means ± SD. Asterisks indicate statistical significance (*$P < 0.05$; **$P < 0.01$)

($P < 0.05$), and even more significantly at 14–18 days post infection ($P < 0.01$, Fig. 1C), which proves that trophoblast apoptosis may be the main cause of abortion in mice.

N. caninum infection influenced the expression of apoptotic gene and proteins

To determine if there were global differences in *N. caninum*-infected placental cells, an apoptosis-associated gene data heatmap was produced. Of the 86 apoptosis-related genes detectable with qPCR, 66 of them were differentially expressed in the placentas of the infected and control mice, accounting for 76.7% of the total genes screened. qPCR showed that 51 genes of these differentially expressed genes were downregulated while 15 genes were upregulated in the infected group relative to their expression in the control group; 27 of these genes showed a difference in expression of > twofold whereas eight genes showed a difference of > tenfold, including *Bcl-2* (Fig. 2A). IHC analysis of placental tissues with a rabbit anti-mouse BAX antibody showed that BAX was widely expressed in trophoblasts and chorionic tissues. The numbers of BAX-positive cells in the infected group increased with days post infection, but the numbers differed significantly at different timepoints ($P < 0.05$ or $P < 0.01$). The number of BAX-positive cells on 18 days post infection differed significantly from the numbers on all other days tested in infected mice ($P < 0.05$, Fig. 2B). Meanwhile, IHC analysis of placental tissues with a rabbit anti-mouse BCL-2 antibody showed that the BCL-2 protein was expressed in the placental villi and decidua. The numbers of BCL-2-positive cells in the infected group decreased with days post infection and was less than those of the control group on different days ($P < 0.01$). The number of BCL-2-positive cells on day 18 differed significantly from the numbers on all other days tested ($P < 0.01$, Fig. 2C). BCL-2 inhibits apoptosis and BAX promotes it. An imbalance in their expression could lead to apoptosis, which means that placental damage had occurred. Moreover, a similar analysis of placental tissues using a rabbit anti-mouse TNF-α antibody showed that TNF-α was only slightly expressed in the placenta. Compared with the control group, the numbers of TNF-α-positive cells in the infected group increased with days post infection and differed significantly at different timepoints ($P < 0.05$ or $P < 0.01$). The difference between the infected group and the control group was significant on 12 and 14 days after infection with the parasites ($P < 0.01$, Fig. 2D). These results clearly suggest that *N. caninum* infection promoted TNF-α secretion, which induced apoptosis, and thus, led to abortion.

N. caninum infection affected caspase-3 and IGF-2 levels in placental cell suspensions

Results showed that caspase-3 was upregulated in the infected group, whereas those in the control group were downregulated. The difference in caspase-3 levels between

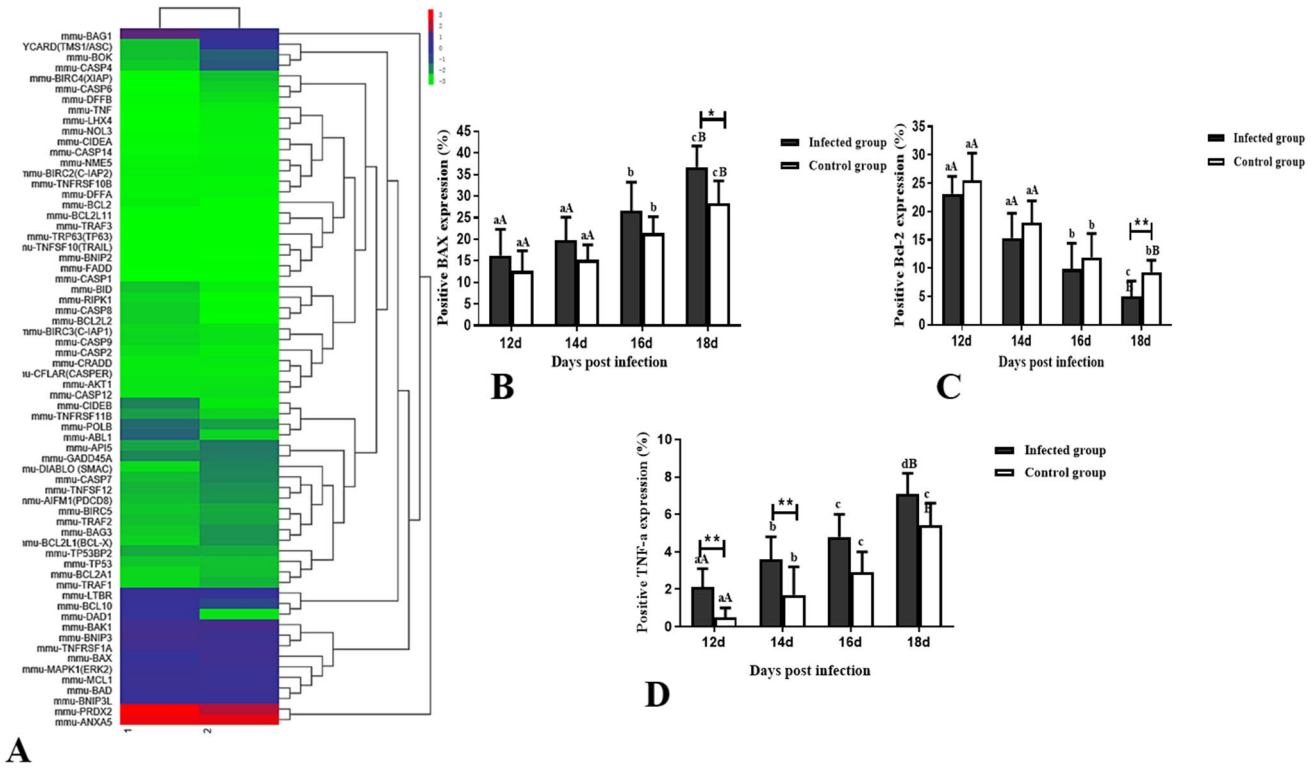

Fig. 2 **A** Cluster map of differentially expressed apoptosis-associated genes in the placenta. Using the qPCR technology, a cluster analysis of apoptotic gene expression was performed on representative samples from the infected group and control group. Sixty-six genes were differentially expressed in the placentas of the infected and control mice. Red represents upregulation of gene expression; green represents the downregulation of gene expression; blue represents no difference in gene expression**.** Among them, the expression of *Bax*, a gene associated with apoptosis, was significantly upregulated, whereas the expression of the *Bcl-2* gene was significantly downregulated. (1): control group, (2): infected group. Expression of apoptosis-related proteins BAX, BCL-2 and TNF-α. **B** BAX expression, **C** BCL-2 expression, and **D** TNF-α expression. *Y* axis values are the number of positive cells of target protein detected by IHC. Results are expressed as means ± SD. Asterisks indicate statistical significance of differences between the infected group and control group (*$P < 0.05$; **$P < 0.01$). Letters indicate statistically significant differences within the same group (same letter means no significant difference; different uppercase letter means $P < 0.01$; different lowercase means $P < 0.05$)

the infected group and the control group was significant on 18 days post infection ($P < 0.01$, Fig. 3A). This means that the risk of abortion increased with time since infection. Meanwhile, IGF-2 levels decreased in the pregnant infected mice but increased in the control mice. The difference in IGF-2 levels between the infected group and the control group was significant at 18 days post infection ($P < 0.05$, Fig. 3B), indicating that growth of fetal mice was stunted over time in infected mice.

N. caninum infection affected ACH and PL levels in placental cell suspensions

Results indicated that the ACH level in *N. caninum*-infected group was initially downregulated until 14 days after infection, then was upregulated consistent with longer duration of infection, while the opposite was noted in the control group. The ACH level in the control group was initially upregulated, then starting at 16 days, was downregulated consistent with longer duration of pregnancy. There was a notable difference between the ACH level in the infected group and the control group at 14 days post infection ($P < 0.05$, Fig. 4A). On the other hand, the PL level was first downregulated, then upregulated in the infected group, but continued to be downregulated in the control group. At 18 days post infection, there was a significant difference between the PL level of the infected group and the control group ($P < 0.05$, Fig. 4B). Altogether, our results show that *N. caninum* infection seriously affected the nutrient absorption, growth, and development of the fetus.

Discussion

The placenta is a temporary, multifunctional organ; and its structural integrity and normal functions, including gas exchange between the mother and fetus, fetal metabolism, and nutrient supply, are the basis of pregnancy (Ferro et al. 2002;

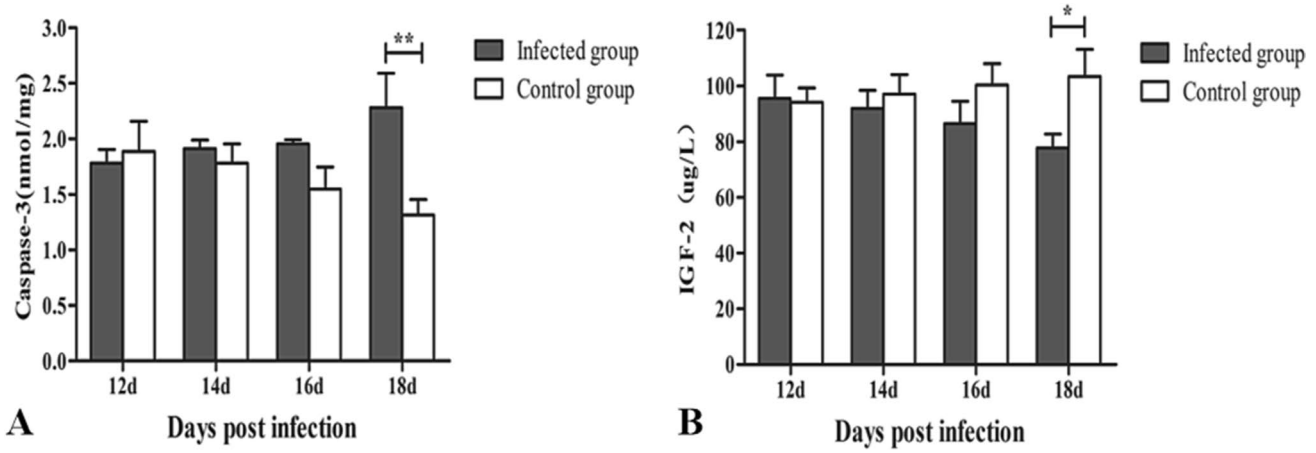

Fig. 3 Caspase-3 and IGF-2 levels in placental cell suspensions. **A** Caspase-3 and **B** IGF-2 were detected by ELISA. Results are expressed as means ± SD. Asterisks indicate statistical significance (*$P < 0.05$; **$P < 0.01$)

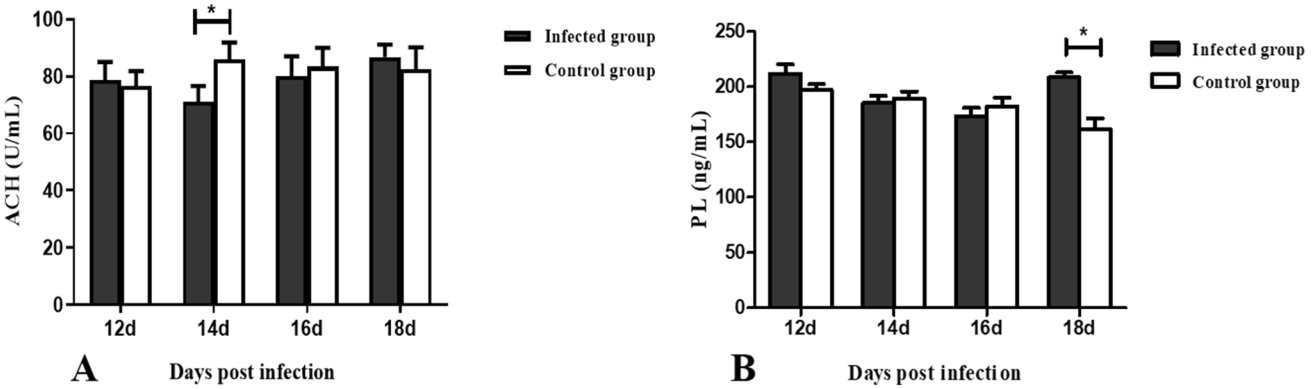

Fig. 4 ACH and PL levels in placental cell suspensions. **A** ACH and **B** PL were detected by ELISA. Results are expressed as means ± SD. Asterisks indicate statistical significance (*$P < 0.05$)

Juliano et al. 2006). In the study by López-Pérez et al. which investigated *N. caninum* infection in the placentas of pregnant mice, histopathological analysis detected multifocal necrotic areas on the maternal and fetal sides of the placenta, with non-suppurative infiltration, and necrosis (López-Pérez et al 2008). In the present study, atrophy and vacuolization of the sponge trophoblast cells, inflammation, and a large number of apoptotic bodies were detected in the infected placentas with histopathological analysis, and a large number of parasites were detected in the placenta with qPCR at different times after the infection, which indicate that the invasion of the placenta by *N. caninum* caused extensive damage to the placental cells. Nevertheless, no tachyzoites were found in the placentas, which may be due to narrow observation fields, atypical observation sites, or the qPCR detection being more sensitive than histopathological observation. In a previous study, *N. caninum* DNA was detected in the placenta of pregnant mice at 12 days post infection (Jia et al. 2020); therefore, in the present study, days 12, 14, 16, and 18 post infection were selected for analysis. The reason why there were no tachyzoites observed in the placenta remains to be elucidated.

The increased apoptosis of syncytiotrophoblasts can cause the stagnation of placental development, cell proliferation, apoptotic disorders, and the inhibition of placental hormone synthesis, which can lead to fetal miscarriage and stillbirth (Nelson 1996). In the present study, the number of apoptotic placental trophoblast cells increased with time after infection. We speculate that the parasite invades the placental tissue after reproducing in the host body, which may cause damage to the placental tissues, destruction of placental trophoblast cells, and increased numbers of apoptotic placental trophoblast cells.

We also examined the expression of 86 apoptosis-related genes in infected placental tissues, and detected the differential expression of 66, accounting for 76.7% of the total genes screened. An analysis of the differentially expressed genes showed that *Bcl-2*, an apoptosis-related gene, was significantly downregulated. An imbalance in their expression reduces the formation of BCL-2–BAX heterodimers in cells,

which increases the permeability of the mitochondria and leads to apoptosis (Shamas-Din et al. 2013). Results showed that the downregulation of BCL-2, an apoptosis suppressor protein, increased apoptosis in the infected mice, leading to a large number of apoptotic placental cells which meant that placental damage had occurred. During infection, macrophages secrete large amounts of TNF-α, which induces apoptosis, and thus leads to abortion and premature delivery (Abrahams et al. 2004). The results in our study indicate that apoptosis was induced by *N. caninum* infection as evidenced by the increase in apoptotic cells and may have been associated with increased TNF-α-positive cells, particularly at 12 and 14 days post infection.

The occurrence of apoptosis is mediated by a variety of pathways, including the apoptosis effector enzyme caspase-3, a unique enzyme in the process of apoptosis. The activation of caspase-3 means that the cell will undergo apoptosis (Debierre-Grockiego et al. 2007). The significant upregulation of caspase-3 expression indicates that these placental cells would inevitably undergo apoptosis, which is consistent with the results of our study. IGF is a multifunctional cell regulator of the processes of fetal growth, and is involved in the regulation of cell proliferation, differentiation, and apoptosis, and the development of the organism. Fetal dysplasia caused by abnormal placental function manifests first as a reduction in IGF levels, which leads to a decline in the ability of various cell types to grow, the extension of the cell cycle, and a reduction in the number of cells, ultimately leading to growth retardation in the fetus. Our results show that the downregulation of IGF-2 levels may lead to fetal retardation and affect the normal development of the fetus.

Acetylcholine (ACH) has a regulatory effect on the placental material transport, and the ACH synthesized and secreted by placental tissue is closely related to the transport of placental amino acids. ACH mainly exists in the syncytiotrophoblast cells of the chorionic lobules. The placenta can synthesize and secrete ACH in the first trimester of pregnancy, and it reaches its peak level in the second trimester as well as decline subsequently (Yue 2012). Our results showed that the level of ACH in the infected group was decreased at first, and then increased with the increasing of days of infection, and the fluctuation was obvious. Especially at 14 days of gestation (2nd trimester of pregnancy), there was a significant difference between the infected and the control group. This result denotes that the intake of amino acids in the infected mice changes constantly during pregnancy, which may affect the growth and development of the fetus in the mother's womb. Placental prolactin (PL) has significant role in the differentiation and proliferation of placental cells, fetal nutrient metabolism, and energy metabolism. Yue showed that the placental hormone PL in the serum of pregnant mice infected with *Toxoplasma gondii* fluctuated significantly with the prolonged gestation, especially at 18 days (Yue 2012). In the present study, the PL level of the infected group was first downregulated and then upregulated with longer infection, and it was significantly different from the control group on the 18th day. This suggests that the invasion of the parasites into the placenta may have led to the decrease in placental function, affecting the energy metabolism and nutrient uptake of the fetus, which then led to the occurrence of fetal malnutrition or growth retardation.

The *N. caninum* strain used in the present study was isolated from the brain tissue of an aborted fetal calf (Yanbian, Chinese) (Jia et al. 2014). Based on the establishment of an animal model of pregnant mice infected with *N. caninum* (Collantes-Fernandez et al. 2012), the number of live pups, the pathological damage to the placenta and parasite load, trophoblast cell apoptosis, apoptotic genes, apoptotic proteins, placental hormones, and other indicators were measured, and the damaging effects of *N. caninum* isolates on the tissue cells and protein genes of the mouse placenta at a molecular level were analyzed.

Conclusions

Neospora caninum infection affects the litter size and survival of pups in pregnant mice and damages the tissues and cells of the placenta. It simultaneously increases the expression of apoptosis-associated genes, proteins, and proteases; reduces the levels of IGF; and affects the secretion of placental hormones. These changes may be associated with fetal dysplasia, impaired placental function, and impaired pregnancy. In the future, further research on the mechanism of placental injury by *N. caninum* is warranted.

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Author contribution Conceptualization: Lijun Jia, Zeyu Tang, Hang Li, Suzhu Xie, Shaowei Zhao; methodology: Lijun Jia, Zeyu Tang; investigation: Lijun Jia, Hang Li, Suzhu Xie, Shaowei Zha; data analysis: Shuang Zhang, Hao Wang, Nanli Li, Xuancheng Zhang, Fanglin Zhao; writing-—original draft: Zeyu Tang, Hang Li, Suzhu Xie, Shaowei Zhao; writing—review and editing: All authors; project administration and supervision: Lijun Jia

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval 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 of the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China (approval no.: 20180301).

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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