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Research paper

Pathological and ultrastructural observations and liver function analysis of *Eimeria stiedai*-infected rabbits

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ABSTRACT

To study the pathogenicity of *Eimeria stiedai*, sporulated oocysts were given orally to coccidian-free two-month-old New Zealand rabbits(1000 ± 20 g). After 30 days, blood samples from the rabbit hearts were collected for routine blood tests, liver functions and four characteristics of blood coagulation. Additionally, specimens of the liver, bile duct and duodenum were collected to observe the changes in pathology and ultrastructure. *E. stiedai* severely restricted the growth and development of rabbits. Blood tests showed that glutamine transferase (GGT) and serum cholinesterase (ChE) were significantly different from the non-infected controls. Other extremely significant differences were observed in the biochemical indices of routine blood tests, liver function and four blood coagulation characteristics, indicating that the liver functions were significantly affected. Staining showed that, compared with the negative control group, the liver, bile duct and duodenum contained significant numbers of lesions, and organs and cell structures suffered severe damage in ultrastructure, which greatly affecting bodily functions. *E. stiedai*-infected rabbits model was successfully established, which might provide a theoretical basis for research on the pathogenesis of rabbit coccidia, and the diagnosis and prevention of coccidiosis in rabbits.

1. Introduction

Rabbit was an early experimental model that has been widely used in immunological and microbiological studies, as well as cardiovascular disease. In recent years, with increasing research needs, the production of rabbits is also growing. However, according to the literature, coccidiosis infections are common in rabbits and multiple coccidian species are involved in the response to coccidian infection (Duszynski and Couch, 2013). Song et al. (2012) epidemiologically investigated rabbit fields and showed that the coccidia infection rate of the surveyed rabbit fields was 100%, with mixed infections. Duszynski and Couch (2013), who reviewed the world's literature, pointed out that infection rate as high as 64% to 100% in the world. This indicates that the current quality of rabbits is worrisome and may bias rabbit-related teaching and research work.

Rabbit coccidiosis is a common and harmful parasitic diseases, which is termed the "young rabbit killer", with a mortality rate as high as 80% after infection. Rabbit coccidiosis infections are widespread in many parts of China, with infection rates raising up to 100% for weaning to three-month-old rabbits, which causes serious harm to the rabbit industry and is classified as a Level II animal disease by the Ministry of Agriculture. *Eimeria stiedai* has the strongest pathogenicity and is the most hazardous of the coccidiosis, which mainly infect rabbit liver and bile duct epithelial cells and cause severe liver coccidiosis. Sporulated oocysts invade rabbits and rupture, then sporozoites invade the liver and bile duct epithelial cells where merogony occurs. Merozoites continue to increase until they complete four generations. At the gametogony stage, macrogametes and microgametes combine into zygotes and eventually develop into oocysts. During *E. stiedai* reproduction, the liver cells are extremely compressed, resulting in large areas of damage and necrosis. The structures of the hepatic lobules are severely damaged, which causes functional disturbances in liver synthesis, decomposition, biotransformation and detoxification. A variety of metabolic processes become extremely disordered, increasing the secretion of toxins, diarrhea, slow growth and weight loss, and even causing the death of a large number of rabbits (Oliveira et al., 2011; Li and Ooi, 2009).

In this study, routine blood tests, liver functions and four characteristics of blood coagulation analyses between normal rabbits and *E. stiedai*-infected rabbits were performed and compared, and

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the pathology and ultrastructure of their liver, bile duct and duodenum were observed. This study provided a theoretical basis for research on the pathogenesis of rabbit coccidia, and the diagnosis and prevention of coccidiosis in rabbits.

2. Materials and methods

2.1. Animals

In total, 16 coccidian-free two-month-old rabbits (New Zealand rabbits) were randomly divided into two groups: the negetive control group and the infected group. All of the animals were allowed free access to sterile water and animal feed, and handled according to the guidelines of the Laboratory Animal Monitoring Committee of Jiangsu Province. Infection groups were infected with 5×10^5 sporulated *E. stiedai*.

2.2. Blood indexes

After 30 days, the abdominal perimeter was tested and body weights recorded. Additionally, 2 ml blood samples from the hearts of the 16 rabbits were collected using EDTAK2 as the anticoagulant to perform routine blood tests on a 950FS Automatic Hematology Analyzer. Then, as the above mentioned, using heparin as the anticoagulant to separate serum and test for alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), glutamine transferase (GGT) and serum cholinesterase (ChE) levels on a Hitachi 7600 Automatic Biochemistry Analyzer. Finally, as the above mentioned, using sodium citrate as the anticoagulant to test the prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), and fibrinogen (FIB) level on a MC-4000 Automatic Coagulation Analyzer.

2.3. Hematoxylin and eosin (HE) stain

The rabbits were sacrificed, the abdominal cavity opened and the liver index (liver index = liver weight/body weight × 100%), relative growth rate (average weight gain in infection group/average weight gain in negative control group × 100%) and average weight gain (average body weight before sacrifice minus average body weight before infection) were measured. Specimens of liver, bile duct and duodenum were diced into 10 mm × 10 mm × 2 mm and fixed in 4% formalin in 0.1 M phosphate buffer at pH 7.4. Serial 4 μm sections of paraffin-embedded tissues were stained with HE staining and analyzed under a BX51 optical microscope (OLYMPUS, Japan).

2.4. Transmission electron microscopy

Specimens of liver, bile duct and duodenum were prefixed in 2.5% glutaraldehyde solution, diced into 1 mm^3, then rinsed three times for15 min each with 0.1 M phosphate buffer (pH 7.4). Postfixation was in a cold 1% aqueous osmium tetroxide for 2 h. After rinsing with phosphate buffer again, the specimens were dehydrated in a graded ethanol series of 50–100% and then embedded in Epon 812. Ultrathin sections were sliced with glass knives on a ECU6 ultramicrotome (LEICA, Germany), stained with uranyl acetate and lead citrate, and examined under a JEM-1230 electron microscope (Jeol, Japan).

2.5. Statistical analyses

The data obtained in this study were analyzed using GraphPad Prism version 5. An analysis of variance (ANOVA) was applied to compare the differences among experimental groups. P values < 0.05 were considered to indicate significant differences. P values < 0.01 were considered to indicate extremely significant differences.

Table 1
The pathogenicity of *E. stiedai* to New Zealand rabbits ($\bar{x} \pm s$, n = 8). Data are shown as the mean ± SE. There were extremely significant differences between the two groups. The negative control group was not infected by *E. stiedai* and the infected group was infected with 5×10^5 oocysts. (* $p < 0.05$, ** $p < 0.01$; Student's *t*-test).

Indexes	Negative control group	Infection group
Abdominal perimeter (cm)	27.33 ± 1.07	31.18 ± 1.42**
Liver index (%)	3.42 ± 0.64	14.27 ± 2.13**
Starting weight (g)	1000 ± 20	1000 ±20
Final weight (g)	1412 ± 67.24	1188 ± 78.31**
Gain (g)	412 ± 64.54	188 ± 76.79**
Relative growth rate (%)	100	45.63
Mortality (%)	0	37.5

Table 2
Routine blood tests in negative control and infected groups of rabbits($\bar{x} \pm s$, n = 8). Data are shown as the mean ± SE. There were extremely significant differences between the two groups. (* $p < 0.05$, ** $p < 0.01$; Student's *t*-test).

Indexes	Negative control group	Infection group
WBC ($10\hat{9}$/L)	6.37 ± 0.94	10.24 ± 1.89**
LY($10\hat{9}$/L)	4.29 ± 1.51	65.68 ± 16.30**
EOS($10\hat{9}$/L)	0.01 ± 0.01	0.05 ± 0.01**
PLT($10\hat{9}$/L)	475.75 ± 63.66	283.87 ± 38.73**

3. Results

3.1. The pathogenicity of E. stiedai to New Zealand rabbits

Obvious abnormalities in negative control group of rabbits were not observed in, but in the infected group obvious clinical symptoms, including loss of appetite, glazed eyes, often pronating and slowly moving, were observed. Additionally, the cony hair was sparse and dull, and the animals often had diarrhea and became weak. Three rabbits died and autopsies revealed serious liver infections. The results of the tested characteristics, including abdominal perimeter, liver index and relative growth rate, are shown in Table 1.

3.2. Blood indices

3.2.1. Routine blood test

Compared with the negative control group, the white blood cell, lymphocyte (LY) and eosinophil (EOS) contents in the blood of the infected group were extremely significantly increased ($p < 0.01$). The platelet content of blood samples from the infected group were significantly decreased ($p < 0.01$), as shown in Table 2.

3.2.2. Liver function

Compared with the negative control group, the ALT, AST and LDH levels in the infected group's samples were extremely significantly increased ($p < 0.01$). The GGT levels in the infected group were significantly increased ($p < 0.05$). The serum ChE level in the infected group's samples were significantly decreased ($p < 0.05$), as shown in Table 3.

3.2.3. Four characteristics of blood coagulation

Compared with the negative control group, the PT, APTT and TT of the infected group were extremely significantly increased ($p < 0.01$). The FIB level in the infected group's samples were significantly decreased ($p < 0.01$), as shown in Table 4.