Establishment of a cerebral schistosomiasis experimental model in rabbits

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Abstract: Objective The present study aimed to establish a cerebral schistosomiasis model in rabbits, to provide a valuable tool for morphological analysis, clinical manifestation observation, as well as investigations into immunological reactions and pathogenesis of focal inflammatory reaction in neuroschistosomiasis (NS). **Methods** Sixty New Zealand rabbits were randomly assigned into operation, sham-operation and normal groups. Rabbits in the operation group received direct injection of dead schistosome eggs into the brain, while their counterparts in the sham-operation group received saline injection. Rabbits in the normal group received no treatment. Base on the clinical manifestations, rabbits were sacrificed on days 3, 5, 7, 10, 20, and 30 post injection, and brain samples were sectioned and stained with hematoxylin-eosin. Sections were observed under the microscope. **Results** The rabbits in the operation group exhibited various neurological symptoms, including anorexy, partial and general seizures, and paralysis. The morphological analysis showed several schistosome eggs in the nervous tissue on day 3 post operation, with very mild inflammation. On days 7–10 post operation, several schistosome eggs were localized in proximity to red blood cells with many neutrophilic granulocytes and eosinophilic granulocytes around them. The schistosome eggs developed into the productive granuloma stage on days 14–20 post operation. On day 30, the schistosome eggs were found to be in the healing-by-fibrosis stage, and the granuloma area was replaced by fibrillary glia through astrocytosis. The sham-operation group and the normal group showed negative results. **Conclusion** This method might be used to establish the cerebral schistosomiasis experimental model. Several factors need to be considered in establishing this model, such as the antigenic property of eggs, the time of scarification, and the clinical manifestations.

Keywords: experimental model; schistosomiasis; neuroschistosomiasis; granuloma

1 Introduction

Schistosomes are tropical parasitic helminths that infect approximately 210 million people in 76 countries^[1].

Schistosoma japonicum (*S. japonicum*) is prevalent in Southeast Asia and is one of the major schistosome species worldwide^[2]. The involvement of the central nervous system (CNS) is considered as one of the most severe symptoms, and the pathogenesis of CNS lesions is obscure. However, it is proposed that the pathogenesis of neuroschistosomiasis (NS) depends on both the schistosomiasis egg granulomas in the nervous tissue and the host immune response^[3]. *S. japonicum* eggs are smaller and have a

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round size, compared with *S. mansoni* or *S. haematobium* eggs, and they can easily reach the brain. It has been reported that the incidence of cerebral NS by *S. japonicum* is about 2%–4%^[4–6]. However, few NS animal models, with either acute or chronic granulomatous lesion, have been reported, and only a few incidental observations of the cerebral schistosome granulomas in chronically infected mice have been reported^[7–10]. The aim of the present work was to investigate the association among the morphological analysis, the clinical manifestations, and the pathogenesis of schistosome egg granuloma formation in an animal model of cerebral NS, established through direct injection of schistosome eggs into the rabbit brain.

2 Materials and methods

2.1 NS model and animal grouping Sixty New Zealand rabbits (weighing approximately 2 kg, of either sex) were purchased from Wuhan Animal Center in China. The dead *S. japonicum* eggs were purchased from Hubei Provincial Center for Disease Control and Prevention, China, and were used as antigens to induce the formation of granulomas. The dead *S. japonicum* eggs were prepared in normal saline at the concentration of 1 mg/mL. The rabbits were randomized into operation ($n=30$), sham-operation ($n=20$) and normal ($n=10$) groups.

In the operation group, rabbits were anesthetized with urethane (4 mL/kg). The skin of the parietal part of the head was exposed, and sterilized with iodine liquid. A 2.5-cm-long incision was carefully made at the scalp in the middle, to avoid the sagittal sinus bleeding. A volume of 1 mL egg suspension (1 mg/mL) was injected slowly into the brain cortex by a needle inserted into the skull (at a depth of no more than 1 cm). After that, the needle was withdrawn, followed by careful hemostasis. Finally, the scalp was sutured to complete the operation.

In the sham-operation group, rabbits received the same anesthesiological and surgical procedures as those in the operation group, but were injected with 1 mL saline instead of schistosome egg suspension.

In the normal group, rabbits received neither surgical treatment nor egg suspension injection.

2.2 Follow-up study of the rabbits After operation, rabbits in the operation and sham-operation groups received intramuscular injection of 150 000 U penicillin each day for one week. After these procedures, the clinical manifestations such as emergence of anorexia, hemiplegia, and other symptoms of the nervous system, were observed and recorded. Autopsy was carried out after the rabbits were sacrificed.

2.3 Morphological analysis On days 3, 5, 7, 10, 20, and 30 after egg injection, rabbits were sacrificed by overdose injection of urethane. The brain was removed and placed in formalin. The appropriate samples of tissue were cut into 20-mm-thick sections with a cryostat and stained with hematoxylin-eosin for histological observations. The granulomas present in coronal sections of samples were counted.

3 Results

S. japonicum appears to induce clinical cerebral schistosomiasis more easily than *S. mansoni* does. The neurological complications of acute NS include delirium and confusional state, loss of consciousness, headache, seizures, dysphasia, visual field impairment, focal motor deficits (hemiparesis, paraplegia), and cerebellar syndrome^[11,12]. Brain edema and multiple focal cerebral lesions in the different lobes or areas may affect different functions.

In the present study, the rabbits in the 3 groups were sacrificed on days 3, 5, 7, 10, 20 and 30 post injection. Their various clinical manifestations before sacrifice were also recorded.

3.1 The clinical symptoms In the first week, rabbits in the operation and sham-operation groups all recovered from anesthesiological unconsciousness at 6–24 h after operation. In the operation group, 4 rabbits remained anorexic for 3 d during the first week post operation, without any movement. They were then killed for pathological observation. Besides, 6 rabbits experienced the partial and general seizures for 3–5 d during the first week, among which 2 rabbits exhibited decerebrate rigidity of both limbs and bilateral pulmonary rales before death. All the 10 rabbits were then sacrificed for morphological observation (Fig. 1).

In the second week, most of the rabbits in the operation group exhibited normal behaviors, as compared with those in the sham and the normal groups. Only 6 rabbits suddenly changed to be anorexic and showed no interest in the surroundings for 2 d. Another 4 rabbits gradually exhibited loss of appetite and were motionless, paying no attention to its surroundings. These 4 rabbits were then sacrificed for morphological observation (Fig. 2).

Besides, the 6 rabbits were sacrificed by over-dose injection of urethane in the end of the second week (Fig. 3A–F) and on day 20 (Fig. 3G, H) post operation, and the brains were removed for histological observation.

On day 30, only 2 rabbits in the operation group began to exhibit paralysis, and had partial seizures. The 2 rabbits

were then sacrificed for histological observation (Fig. 4)

Animals in the sham and the normal groups showed no clinical symptoms.

3.2 Histological analysis As shown in Fig. 1A–D, on day 3 post operation, several schistosome eggs were observed in the nervous tissue, with very mild inflammation.

On days 7–10 post operation, several schistosome eggs were localized in proximity to red blood cells. There were many neutrophilic granulocytes and eosinophilic granulocytes around the eggs. Fragmented, deformed shells were also found to be infiltrated with lymphocytes and neutrophilic granulocytes in the subacute schistosome granuloma stage (Fig. 2A–F).

On days 14–20 post operation, the schistosome eggs

Fig. 1 A: Hematoxylin-eosin staining of schistosome eggs in the central nervous system of a representative rabbit that experienced anorexia followed by seizures. Several schistosome eggs (black arrows) could be found in the nervous tissue with very mild inflammation at day 3 post operation. B–D were local magnifications of A. Scale bar: A, 275 μm ; B and C, 46.3 μm ; D, 23.5 μm .

Fig. 2 On days 7–10 post operation, several schistosome eggs (black arrows) were localized in proximity to red blood cells. There are many neutrophilic granulocytes and eosinophilic granulocytes around the eggs. Fragmented, deformed shells were found to be infiltrated with lymphocytes and neutrophilic granulocytes in the subacute exudative schistosome granuloma stage (A–E). Epithelioid and multinuclear giant cells were also found to be around the eggs and egg shells (F). Scale bar: A, 275 μm ; B–D, F, 46.3 μm ; E, 23.5 μm .

were in the productive granuloma stage. The egg immunocomplexes were formed by soluble egg antigen and some collagenic fibers with fewer lymphocytes, neutrophilic or eosinophilic granulocytes (Fig. 3A, C, E–H) and with epithelioid cells and mononuclear cells lying radially to the central zone of productive granulomas (Fig. 3B, D).

On day 30, the schistosome eggs were in the healing-by-fibrosis stage. The granuloma area was replaced by fibrillary glia through astrocytosis (Fig. 4).

Besides, rabbits in sham-operation and control groups showed normal results.

4 Discussion

Cerebral schistosomiasis is considered as one of the

most severe disorders caused by *S. japonicum*, although it was mostly reported in Chinese literatures. *S. japonicum* appears to induce clinical cerebral schistosomiasis more easily than *S. mansoni*. The pathogenesis of brain invasion is still unknown, although currently available evidence suggests the direct deposition theory (the adult worm migration) and the indirect deposition theory (the egg embolization). Details on the pathways of NS pathogenesis have been discussed previously^[11]. The real damage of NS depends on both the amount of eggs and the immune system of the host. The egg granulomas, although considered to be detrimental, can effectively protect the host through sequestering the antigen secretion, from uncontrolled inflammatory responses and the subsequent extensive damage to

Fig. 3 The schistosome eggs (black arrows) in the productive stage. The egg immunocomplexes were formed by soluble egg antigen and some collagenic fibers with fewer lymphocytes, neutrophilic or eosinophilic granulocytes (A, C, E–H). Some epithelioid cells and mononuclear cells were detected lying radially to the central zone of the productive granulomas (B, D). Scale bar: A and B, 275 μ m; C–F, 46.3 μ m; G and H, 23.5 μ m.

the affected tissues^[3]. The granulomas also have a mass effect in the brain, provoking a tumor-like syndrome due to slow-expanding cerebral lesions. The involvement of CNS

is more frequent in patients without systemic symptoms of infection, thus here the method of direct injection of dead schistosome eggs into the brain was employed.

Fig. 4 On day 30 post injection, granulomas were in the healing-by-fibrosis stage. The granuloma areas were replaced by fibrillary glia through astrogliosis. Scale bar: A, 275 μ m; B and C, 46.3 μ m; D, 23.5 μ m.

Currently, few proper animal models of NS have been reported. In the study of Aloe L *et al.*^[10], female albino CD-1 mice infected percutaneously with 60 cercariae of *S. mansoni* worms develop liver granulomas 7 weeks after schistosome infection. At 15 and 20 weeks, *S. mansoni* eggs and granulomas are found in different areas of brain, including the hippocampus, cerebellum, brain stem and cortex. Besides, this infection alters the level of nerve growth factor (NGF) and causes paw hyperalgesia. However, Silva LM *et al.*^[9] have reported different results and revealed the inadequacy of using mouse as an experimental model of NS. In fact, in as early as 1970, Jane JA *et al.*^[8] designed their experiments in different conditions, based on the aetiological possibilities of the direct or indirect

pathways for NS, but the condition in the experiment was unlikely to occur during the nature course of infection. The involvement of CNS by schistosomes is more frequent in patients without systemic symptoms of infection^[11,12].

In the present study, it is the first time to test the validity of direct injection of egg suspension into rabbit brain in establishing the NS model. Although different from the nature course of infection, this method has several advantages. Firstly, the experimental course is greatly shortened, compared with percutaneous infection course (5–7 weeks)^[8–10], since it only takes 1–2 weeks to induce the NS granulomas in the acute stage of infection. Secondly, this method may help exclude many influencing factors. When the cercariae transform into schistosomula and then adult

worm to lay eggs, alternative and complicated immunological reactions may be induced during these stages, which are too complicated to be studied. On the contrary, this method can simplify the infection progress, because the brain-blood barrier has set a unique immune environment different from the host systemic immune environment. After injection of egg suspension, the histological observations showed clearly 3 stages: a necrotic-exudative stage, a productive stage, and a healing-by-fibrosis stage^[11,14].

However, there are still some questions for consideration in further studies. Firstly, The antigenic property of eggs varies due to the difference in the development of embryo or miracidia in the eggs. This is perhaps the main cause for the variations of the time to reach the peak of granulomas. Some articles agree that more eggs may cause more intense inflammation, and this inflammation also has connections with the host immune system, especially in the acute stage. In this study, it took approximately 1-3 weeks to induce egg granulomas by 1 mL dead egg suspension (1 mg/mL). Secondly, the time of sacrificing rabbits needs to be carefully determined. Mostly, it is based on the rabbits' neurological symptoms and the lifespan of eggs' granulomas, according to the 3 stages. Necrotic-exudative granulomas are characteristics of early stages of infection^[11-14]. Freshly-laid eggs are immature and the leukotactic responses to immature eggs are initially slight. As the miracidia become mature and die, florid granuloma occurs. The granulomas reach a peak size 4-8 d after embedding the eggs in the nervous tissue^[11-14]. Here we use the suspension of dead schistosome antigenic eggs, and microscopy observation revealed the presence of egg granuloma reaction as shown in Figs.1-4. Thirdly, the clinical manifestations are important. At the early stage especially the first week, most rabbits exhibit various symptoms, such as loss of appetite, seizures, paralysis, and unconsciousness^[12]. In the present study, partial and generalized tonic-clonic epileptic seizures occurred frequently. Afterwards, the rabbits acted as normal as the rabbits in the sham and the normal groups. On day 30, 2 rabbits in the operation group suddenly experienced paralysis and partial seizures. Finally, hematoxylin-eosin pathological findings (Figs.1-4)

showed activities of specific cells within granulomas at different stages, such as T cells, B cells, macrophages, fibroblasts, and eosinophils (the most predominant cells). Many necropsy studies have indicated that unlike the eggs injected into the cortex or other brain tissues, eggs injected into the cistern of the brain can not induce the formation of typical granulomas. The granulomas may be more clear and more typical if there are enough antigenic egg stimuli in the brain cortex.

Apart from the issues mentioned above, individual variation has also raised some attention. NS can occur in many mammalian species, such as pigs, humans, mice, rabbits, monkeys, *etc.* The experimental results may also vary due to different experiment observers, even in the same procedure. Some data and information need to be verified in human NS^[7,9]. Investigating the granulomas in the host must take into consideration the timing, symptoms, as well as quantity and quality of the egg antigen involved. With the development in medical techniques such as magnetic resonance imaging (MRI), polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), it is entirely possible that the pathogenesis of NS can be clearly revealed, and NS can be effectively prevented and treated.

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家兔脑型血吸虫病动物实验模型的建立及影响因素

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摘要: 目的 本研究旨在建立家兔脑型血吸虫病模型, 为观察脑型血吸虫病的形态学和临床症状、研究其免疫反应以及探讨其发病机理提供重要手段。**方法** 新西兰大耳兔60只随机分成手术组($n=30$)、假手术组($n=20$)和对照组($n=10$)。在手术组中, 直接将血吸虫虫卵悬浊液注入家兔大脑, 形成血吸虫虫卵肉芽肿。假手术组采用相同的手术方式, 但是注入等剂量的生理盐水。对照组不采用任何手术。术后对动物神经系统临床症状进行观察, 并于第3、5、7、10、20和30天对家兔脑组织切片, 并进行苏木精-伊红染色观察。**结果** 手术组家兔术后出现各种神经系统症状, 包括厌食、局部抽搐、全身抽搐以及瘫痪。病理切片证实在术后第3天, 家兔神经系统中形成血吸虫虫卵肉芽肿, 并伴有轻微的炎症。在第7–10天, 红细胞临近区域形成一些虫卵肉芽肿, 伴随有数量较多的中性粒细胞和嗜酸性粒细胞聚集在其周围。在第14–20天, 虫卵肉芽肿主要处在增生期。在第30天, 血吸虫虫卵肉芽肿处于纤维化治愈阶段, 肉芽肿区域逐渐被纤维性神经胶质细胞取代。此外, 假手术组和对照组家兔术后表现正常, 病理切片未见明显异常。**结论** 本方法可以成功建立脑型血吸虫病动物模型。在实验中, 血吸虫虫卵的抗原性、动物的临床表现以及实验动物的处死时间等因素十分重要。

关键词: 动物模型; 血吸虫病; 脑型血吸虫病; 肉芽肿