Evaluation of experimentally induced early hepatic alveolar echinococcosis in rats with ultrasonography

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Abstract

Objectives: The aim of this study was to explore the value of ultrasonography in screening the early stage of hepatic alveolar echinococcosis (HAE) in animal model, and to demonstrate characteristic imaging of early HAE in ultrasonography.

Materials and methods: The early stage of experimentally induced secondary HAE in 157 rats was studied by ultrasonography. The features of all lesions were recorded, and compared with pathological results.

Results: The sensitivity and specificity of ultrasonography was 93.5% (95% confidence interval: 76%—98%) and 83.3% (95% confidence interval: 65%—93%), respectively. The typical features of early HAE in ultrasonography presented as solid hyperechoic masses and mixed lesions. Blood flow was detected in only three lesions by Color Doppler Flow Imaging. Calcifications were detected in 17 lesions which were larger than 8 mm. Lesions presented as single or multiple cystic structure, surrounded with the proliferation of small blood vessels, epithelioid cells, macrophages, fibroblasts, lymphocytes and fibrous tissue in the liver.

Conclusions: Our data suggested that ultrasonography could be used as a screening method for early Echinococcus multilocularis lesions in the animal model. Calcification could be occurred in those lesions with fast growth of the parasite in short cycle. The typical features of early HAE presented as hyper-echoic with small size in ultrasonography.

Keywords: Experimental alveolar echinococcosis; Echinococcus multilocularis; Ultrasonography

1. Introduction

Hepatic alveolar echinococcosis (HAE) is caused by the development of the larval stage of the canine tapeworm Echinococcus multilocularis (E. multilocularis). The severity of HAE is related to a slow infiltrative growth in the liver of the larva of E. multilocularis in a tumor-like way [1]. Moreover, currently there is no fully effective parasiticidal drug to kill metacestode of the parasite, resulting in that surgical removal remains the best choice for treatment of HAE [2]. Due to that the development of HAE may result in high mortality if remaining untreated, accurate detection of the early stage is urgently required.

As a space occupying lesion, diagnosis of human HAE should be achieved, first and foremost, using imaging techniques, especially ultrasonography (US). In endemic communities, active mass screening programs using US as the primary screening tool, have facilitated to detect the asymptomatic or undiagnosed human AE cases [3—6]. However, the early HAE is easy to be confused with other diseases in the liver due to its atypical features [4]. Thus, it is very meaningful for...
radiologists to know more features of early HAE to diagnose HAE in early stage at the preliminary screening.

In view of ethic requirements, it is very difficult to get the details of pathology timely in the progression of human HAE to compare with imaging. Therefore, the preparation of a satisfactory AE animal model would be a better choice for researches, which can be used for imaging, immunology and pharmacokinetics research. Currently, the preparation of animal model has been successfully utilized in the cerebral AE [7], pulmonary AE [8], intraabdominal AE [9], and HAE [10]. And it has been verified that there are similar pathological progresses of animal models to these of human AE. During experiment, it is crucial to identify the development status of the larva of *E. multilocularis* in the liver, so as to determine the time point for research. Accordingly, it is meaningful to establish a method for the exact and early diagnosis of HAE in laboratory animal model and to investigate its usefulness.

Our previous data [10] have shown that early HAE presented typical enhancement manifestations according to the different size of HAE lesions. In this study, we further verified the value of US in screening early HAE in animal model. Most importantly, we also demonstrated the ultrasonographic features of early HAE in rats to give a comparison between results of ultrasonography and pathological changes in the liver.

2. Materials and methods

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals, and the experimental protocol was approved by the Animal Experiment Ethics Committee of the First Teaching Hospital of Xinjiang Medical University (Permission No. IACUC2015423-06), which had been certificated by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.1. Experimental animals and reagents

157 pathogen-free female SD rats (200 ± 20 g) were purchased from the animal breeding center of Xinjiang Medical University. Injectable Ketamine hydrochloride (Batch number: 091102) was purchased from Jiangsu Hengrui Medicine Co., Ltd., China, and Injectable Diazepam (Batch number: 0907291) was purchased from Tianjin Jin Yao Amino Acid Co., Ltd., China.

2.2. Preparation of animal model

SD Rats were infected by *E. multilocularis* as described in previous study [10]. Briefly, Gerbils infected with *E. multilocularis* for 18 weeks (Breeding Center of Xinjiang Medical University) were euthanized rapidly by cervical dislocation and used as an *E. multilocularis* source. Under sterilized conditions, the metacestode was removed and cut into pieces and the vesicle membrane was removed. It was then washed with 0.9% sterile saline, centrifuged, and re-suspended. The process repeated from three to six times. Hematoxylin and eosin (H&E) staining was used to count the number of microvesicles and protoscolices, and a supernatant with 2000 section head at 0.1 mL was made. After adding 0.1 g penicillin and 0.1 g streptomycin, 0.1 mL of supernatant was injected into the liver of each rat. All rats were housed in cages with a 12-h light/dark cycle and provided with conventional rodent chow and filtered ion-exchanged water.

2.3. Ultrasound screening

Since the sixth weeks after inoculation, 20 rats were randomly detected by US weekly to optimize the time point to screen all of the rats until at least half of the selected rats were detected with focal AE lesion by US. In the ninth week, 157 surviving rats were screened by using a Sequoia 512 scanner (8–14MHz, Siemens Medical Solutions, Mountain View, CA) and a LOGIQ7 (7–12 MHz, GE, USA). The scanning parameters, including the gain, field of view and time gain control, were optimized for each region independently. Each examination lasted for 2 min. Details of the location, shape, boundary, maximum diameter, echogenicity and features of HAE lesions were recorded, and the examinations were recorded in Workstation (Landwind Ltd., Shenzhen, China).

2.4. Image interpretation and analysis

All images were analyzed by two independent radiologists who had 8 and 11 years' experience respectively in the diagnosis of human AE, and the location, shape, boundary, maximum diameter, echogenicity and features of HAE lesions were assessed. Imaging characteristics of US were categorized into the following four types according to the echogenicity and the size of lesions [10]: (1) single hyperechogenic lesion less than 3 mm in diameter (hyper-echoic spot, type 1); (2) clustering of multiple fine hyper-echoic spots (granular hyper-echoic spots, type 2); (3) single hyper-echoic lesion larger than 3 mm in diameter (hyper-echoic lesion, type 3); and (4) mixed pattern with hyper- and hypo-echoic area in lesion (mixed pattern, type 4). Additionally, lesions were divided into two patterns according to whether lesions contained vesicle structure or not. One is solid mass, which presented as homogeneous hyperechoic lesions in the liver. The other is a mixed type, which presented as hyperechoic lesions accompanied with hypechoic or anechoic.

2.5. Histopathological examination

After US examination, 10 rats were selected randomly from different patterns, euthanized by cervical dislocation. After opening the abdomen and removing the whole liver tissue, details of the number, shape, location and diameter of HAE lesions were recorded. All specimens were then fixed in 10% formalin, and embedded in paraffin. Five-micrometer sections were prepared for H&E staining for pathological changes and Masson staining for fibrous tissue.

2.6. Statistics

Statistical analysis was performed with commercially available software (SPSS 13.0 Version, Chicago, IL, USA). An
independent sample \( t \)-test was used to compare the diameters of lesions between solid mass type and mixed type. Two tailed \( P \) value of less than 0.05 was considered statistically significant. The sensitivity and specificity of US was evaluated using RevMan 5.0 (The Cochrane Collaboration).

3. Results

3.1. Results of screening by US

At least one lesion was observed in 133 of 157 rats detected. 142 lesions were detected in 133 rats. 24 rats were not detected any lesion in the liver. After US screening, these 24 rats were further identified by laparotomy. It was shown that 7 rats had AE lesions less than 1 mm in the liver and 2 \textit{E. multilocularis} lesions (the diameter was 3 mm and 3.5 mm respectively) were discerned in the liver of rats. No \textit{E. multilocularis} lesion was visible in the liver of 15 rats by laparotomy examination. Through the experiment, 3 rats did not show any lesions in the liver that identified by US in the ninth weeks after infected.

Of all 142 lesions visualized by US, the largest diameter measured was 16 mm, and the smallest one was 1 mm. The sensitivity, misdiagnosis rate, specificity, misdiagnosis rate, positive predictive value, negative predictive value, positive likelihood ratio, negative likelihood ratio, diagnostic accuracy, was 93.5%, 6.48%, 83.33%, 16.67%, 97.74%, 62.50%, 5.59, 0.078, 92.36%, respectively.

3.2. Ultrasonographic features of different classifications

According to different ultrasonographic features, all of lesions could be divided into 4 types. Twenty-five lesions (17.60%, 25/142) were identified as type 1, 55 (38.73%, 55/142) as type 2, 34 (23.94%, 34/142) as type 3, and 28 (19.71%, 28/142) as type 4. The features of US in type 1, type 2 and type 3 were corresponded to the solid mass type, and that of type 4 were corresponded to a mixed type.

All of lesions presented two types in US, solid mass type and mixed type. 114 lesions (114/142, 80.28%) were identified as solid lesions (the diameter is from 1 mm to 7 mm), which presented as hyperechoic spot, granular hyper-echoic spot or hyperechoic lesion in the liver with clear boundaries, and no significant anechoic or hypo-echoic in lesions, no color blood signal was detected in the center of those lesions (Fig. 1a). 28 lesions (28/142, 19.72%) presented as mixed-echoic type (the diameter was from 5 mm to 16 mm) as hyperechoic mixed with hypoechoic or anechoic. The color blood signals were only found in three lesions (Fig. 1b).

3.3. Comparison with US and pathology according to classification

Lesions of solid mass (Fig. 1a) and mixed type (Fig. 2a), which presented as single (Fig. 1b) or multiple milky white cyst-like nodules in the liver (Fig. 2b), were observed as well.
by pathological examination. It was a typical feature that single cyst-like nodules were corresponded to the thick fiber layer and fluid contents. Multiple cyst-like nodules, which presented as milky white multi cyst-like nodules in the liver, were filled with a large amount of milky white material and a small amount of hydatid fluid. The larger lesions pathologically presented thinner layer and more hydatid fluid in lesions.

The layer of the vesicle was stained in red by using HE staining (Figs. 1c and 2c), and the fibrous tissues surrounding the lesion were stained in blue by using Masson staining (Figs. 1d and 2d). Additionally, the proliferation of small blood vessels, epithelioid cells, macrophages, fibroblasts and lymphocytes were shown in the area surrounding *E. multilocularis* lesion. The small protoscoleces were demonstrated in pathology.

Only three lesions in 142 presented single or multiple granuloma structure surrounded with inflammatory reaction belt. Those three lesions presented as multiple hyper-echoic dots in US, which corresponded to type 2.

3.4. Correlation of diameter of lesions with ultrasonographic classification

The different diameters of lesions presented as different features in US. The smaller lesions were easy to be as solid mass. It was shown in Table 1 that all lesions that diameters were smaller than 4 mm presented as solid mass type (Table 1). The vesicle was easy to be detected in larger lesions, which presented as hypoechoic or anechoic in lesions. We also found that all of lesions lager than 7.1 mm presented as mixed type in US, in which the vesicle structure could be detected. Lesions (diameter ranged from 4.1 mm to 7 mm) that presented as solid mass or mixed type was determined by the size of vesicles. If the single vesicle was larger than 2.5 mm, the lesion presented as mixed type, on the contrary, presented as solid mass type. From type 1 to type 4, the diameters of lesions were gradually increasing (Fig. 2).

3.5. Correlation of calcification of lesions with ultrasonographic classification

No internal calcification was detected by US in 125 lesions of type 1, type 2 and type 3. Scattered tiny calcification was detected in 17 lesions of type 4 (the diameter of lesions ranged from 8 mm to 16 mm), and the maximum diameter of calcification was 0.7 mm. The calcification was scattered in the internal lesions, the boundary of lesions, and the internal wall of the vesicle. No calcification was detected in lesions smaller than 8 mm.

4. Discussion

Ultrasonography has been proven to be a reliable, portable, cheap and noninvasive method for screening of symptomatic and asymptomatic echinococcosis cases in mass surveys [5,6].
And because of its low cost, it helped particularly in developing countries to diagnose parasitic diseases in clinical settings. The preliminary reports have suggested that US is more accurate than serology in mass survey [5], which has been the most widely used diagnostic tool in AE screenings. But, up to now, no correlative report of US value in screening early HAE in animal model and the typical ultrasonographic features of early HAE has been published. In this study we showed the high sensitivity and specificity by using US in screening in rats infected with *E. multilocularis* in the liver, and the typical ultrasonographic features of early HAE.

In this study, we found that there were 114 lesions of 142 presented as hyper-echoic in the liver of *E. multilocularis* infected rats. In those lesions, 111 lesions of 114 were identified as single vesicle structure or multiple vesicle structure with surrounding inflammatory reaction belt and proliferation of small vessels around lesions, and only three lesions presented as central granular structure surrounded with inflammatory reaction belt, which was also shown in the cerebral AE of animal model [7] and HAE model in mice [11]. The similar ultrasonographic features in human AE have been reported by Suzuki et al. [12], which demonstrated lesions presented as hyper-echoic were in the early stage of infection. Thus, the early HAE in rat has the similar ultrasonographic features to human HAE.

Kodama et al. [13] showed that the cysts, granulation, fibrous tissues, necrosis and calcified tissues were recognized as the main pathological constituents of HAE in human. Moreover, they confirmed that multiple small cysts without or with a solid structure in HAE lesion presented as the early stage HAE. Therefore, Kodama et al. [13] considered that the multiple vesicle lesions marked as very early stage, and multiple vesicles with a solid structure marked as later stage of HAE. However, those were only identified in MRI with no pathological data verified. In the present study, as basic pathological feature, vesicles were only observed in 28 lesions in US, which were larger than 7.1 mm. Those lesions that presented as solid structure without cyst or with cyst were identified as single vesicle structure or multiple vesicle structure with surrounding inflammatory reaction belt with proliferation of small vessels around lesions on pathology. Thus, the data suggested that the imaging could only demonstrate the enough large vesicles. And even the imaging both without cyst in lesions and with cysts in lesions could mark as the early stage HAE.

Due to the natural or experimental rodent infections, the metacestode tissues grew rapidly and usually it became highly fertile (produce protoscoleces) within 2–4 months. Therefore, in this study, the typical ultrasonographic features of HAE were identified after 9 weeks in rats. All solid hyper-echoic lesions on US were verified as single or multiple vesicle structure in pathology except 3 lesions, and vesicles were usually smaller than 2.5 mm. But for type 4 which was larger in size, the typical vesicle could be detected by US. Therefore, the typical vesicle structure could be detected only when the size of lesions was large enough to display the vesicle structure on US. The specific features of vesicles in US have been reported by Suzuki et al. [12] by describing the small cystic components of HAE lesions on US presenting as hypoechoic structure.

The size and echoic aspect of lesions were classified as types 1, 2, 3 and 4, and the average size was relatively smaller in type 1 but larger in the type 4, which was similar to the previous report [10]. Moreover, we further demonstrated that the larger size of HAE lesions was easy to display vesicle structure in US. Pathologically, the features of all lesions were identified as single vesicle or multiple vesicles except 3 lesions. Those 3 lesions were shown as granulation-like in pathology, which were not detected in the previous study [10]. The occurrence of the atypical pathological features in those three lesions could be accused by the allergic reaction in focal liver tissue by the *E. multilocularis* suspension after injection. Further study needs to be undertaken to explore why lesions in this experiment displayed different pathological features. However, the data obtained in this study did suggested that sometimes *E. multilocularis* in the liver in rats could not grow into AE lesions due to the host-immune reaction to *E. multilocularis*.

Calcification in lesions has been considered the hallmark as diagnostic criteria in HAE. And the occurrence of calcification in lesions was always considered as abortive form of the disease [4]. However, In this study, calcification was detected in 17 lesions of type 4 with size larger than 7 mm, but no calcification was detected in lesions with smaller size. The data suggested that the calcification could be detected in those lesions growing rapidly in short cycle in early HAE. It was similar to the report by Wang et al. [14] that they considered the mild calcification was easy to be detected in those lesions growing rapidly in short cycle.

3 rats were identified hyper-echoic dot in US, but didn't show any lesion in the liver of rat during the experiment. We retrospectively analyzed the ultrasonographic features, and all of these lesions presented as hyper-echoic dot in US. The diameters of lesions were 1 mm, 1.5 mm and 1.6 mm, respectively. The possible reasons might be: 1) the HAE lesions were gradually decayed and thus difficult to be identified

### Table 1

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Type 1</th>
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<th>Type 3</th>
<th>Type 4</th>
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<td>0</td>
<td>3</td>
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<td>0</td>
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<td>3</td>
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<td>5</td>
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<tr>
<td>~16</td>
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<td>1</td>
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by gross pathology, or 2) the focal fibrosis in the liver, which was easy to be confused with the hyper-echoic in US.

Previous report showed that HAE lesions smaller than 7 mm in the liver were unable to delineate by US [15] (Reuter S et al., 2001). In this study, we utilized high frequency transducers (7–12 MHz and 8–12 MHz) to screen E. multilocularis infected rats, which allowed for a higher spatial and temporal resolution than abdominal transducers. Therefore, it could provide the higher ability to distinguish lesions as small as 1 mm in size. However, the frequency of abdominal transducers used for abdominal scans in humans was lower (usually 3–7 MHz), and thus the resolution (3 mm) was lower. Therefore, the clinical practical questions required further study.

In conclusion, US showed a high sensitivity in screening HAE animal model. As a result, US could be used as an effective screening method for early E. multilocularis lesions in the animal model. And the typical features of early HAE were in smaller size and presented as hyper-echoic or mixed-type lesions, while calcification might be detected in early HAE.

Financial disclosure

The authors have no financial interests related to the present manuscript.

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