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Short Communication

Limited fertility of the subcutaneous cysts of *Echinococcus multilocularis*

Dang, Z.[1], Zhang, X.[2], Luo, X.[2], Gao, Z.[2], Jia, W.[2], Xiao, N.[1], Huang, F.[3], Zhao, Y.[4], Xu, S.[1,4], Hu, W.[1] and Zheng, Y.[2,5*]

[1]Key Laboratory on Biology of Parasite and Vector, Ministry of Health, Shanghai, China; National Center for International Research on Tropical Diseases, Shanghai, China; WHO Collaborating Center for Tropical Diseases, Shanghai, China; National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, Shanghai 200025, China

[2]State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, CAAS, Lanzhou, Gansu 730046, China

[3]Parasitology Laboratory, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan

[4]Department of Biomedical Engineering, College of Biotechnology, Guilin Medical University, Guilin, Guangxi 541004, China

[5]Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou 225009, China

[*]Corresponding author e-mail: zhengyadong@caas.cn

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Abstract. *Echinococcus multilocularis* is a tiny devastating worm that causes alveolar echinococcosis in humans. This disease mainly occurs in the liver but rarely in other organs. We report the subcutaneous encystment of *E. multilocularis* metacestodes in experimentally infected mice. Subcutaneous cysts had remarkably fewer protoscoleces (2.05 ± 1.47, n = 20) and small irregular-shape vesicles (ISVs) in the lumen as compared to liver cysts (69.6 ± 55.65, n = 10). Moreover, abnormal development of a protoscolex was also observed in a subcutaneous cyst. The results suggest that subcutaneous encystment may have potential adverse effects on the reproductivity and development of protoscoleces, providing potential explanations for high tissue preference of metacestode encystment.

Echinococcosis is one of the 17 neglected tropical diseases as defined by WHO, and two of them, cystic echinococcosis (CE) and alveolar echinococcosis (AE), are major public health concerns, caused by *Echinococcus granulosus* and *Echinococcus multilocularis*, respectively (World Health Organization, 2010). These two diseases are globally distributed with approximately 2-3 million human cases of CE and 0.3-0.5 million human cases of AE (Craig *et al.*, 2007) and they are considered as emerging or re-emerging diseases (Atanasov *et al.*, 2013; Moro *et al.*, 2009). Encystment of *E. multilocularis* larva is highly tissue-preferred and nearly 99% of cases occur in the liver (McManus *et al.*, 2003). AE infections are also clinically observed in the lung but rarely in other organs.

Here we report the subcutaneous encystment of larval *E. multilocularis* in BALB/c mice subsequent to intraperitoneal injection of 100 µl protoscolex-containing medium (approximately 2,000 protoscoleces). The proscoleces were prepared by scissoring vesicles dissected from the mouse liver into pieces, and filtration followed by incubation in 10% FBS DMEM

(HyClone) at 37°C overnight. After 3 months, 5 inoculated mice were euthanized and 2 were found to have both 1 subcutaneous cyst and 1-2 liver cysts. These cysts were located in the peritoneum of mice. As the importance of formation of acellular laminated layer (LL) in metacestode development (Spiliotis *et al.*, 2008; Zheng 2013), we conducted glycan staining using periodic acid Schiff kit (Sigma) (Spiliotis *et al.*, 2008) and found that the LL of both liver cysts and subcutaneous cysts was well formed (Fig. 1 A1 and B1). However, the subcutaneous cysts were remarkably different from those in the livers. First, there were only a few protoscoleces in the daughter cysts with the whole LL in the subcutaneous cysts (the number of protoscoleces was 3, 3, 2, 1, 6, 1, 1, 1, 2, 1, 1, 1, 4, 1, 1, 1, 2, 1, 4 and 4 (2.05 ± 1.47, n = 20), respectively). In the liver cysts there were more protoscoleces (the number of protoscoleces was 52, 8, 124, 154, 70, 52, 3, 41, 38 and 154 (69.6 ± 55.65, n = 10), respectively) (Fig. 1 A2 and B2), demonstrating the decreased or limited reproductivity of subcutaneous cysts. In addition, a number of large and irregular-shape vesicles (ISVs), which have not described before, were present in the liver cysts (Fig. 1 A1). The subcutaneous cysts only harbored a few small ISVs (arrowed, Fig. 1 B1). For these ISVs, some appeared to be directly extended from the LL towards the cyst lumen but others not. All ISVs were wrapped by a PAS-positive LL (Fig. 1 A1 and B1). Moreover, the fill-in of liver and subcutaneous cysts was also lightly colored by periodic acid Schiff staining, suggesting the presence of glycan. All vesicles in the liver cysts were coated by a layer of cells or protoscolex (Fig. 1 A1). Although the exact actions of ISVs are unclear, this suggests that these vesicles have a potential role in the production of protoscoleces or daughter cysts, for instance, serving as an energy source.

In both liver and subcutaneous cysts, protoscoleces were at multiple developmental phases: some did not have hooks, but some did with an invaginated or evaginated scolex (Fig. 1 A3, A4 and B3). A protoscolex with an unusual scolex was observed in one of the subcutaneous cysts, which was never seen in the liver cysts (Fig. 1 B4). It was in a one-protoscolex-containing daughter cyst (data not shown). Compared to the protoscoleces with an evaginated hook-harboring scolex commonly found in the liver and subcutaneous cysts, this parasite had a longer scolex part (Fig. 1 A4, B3 and B4). In addition to the abnormally-developed scolex, there was a scolex-like bulge that was never seen in the rest of protoscoleces in both liver and subcutaneous cysts either (arrowed, Fig. 1 B4).

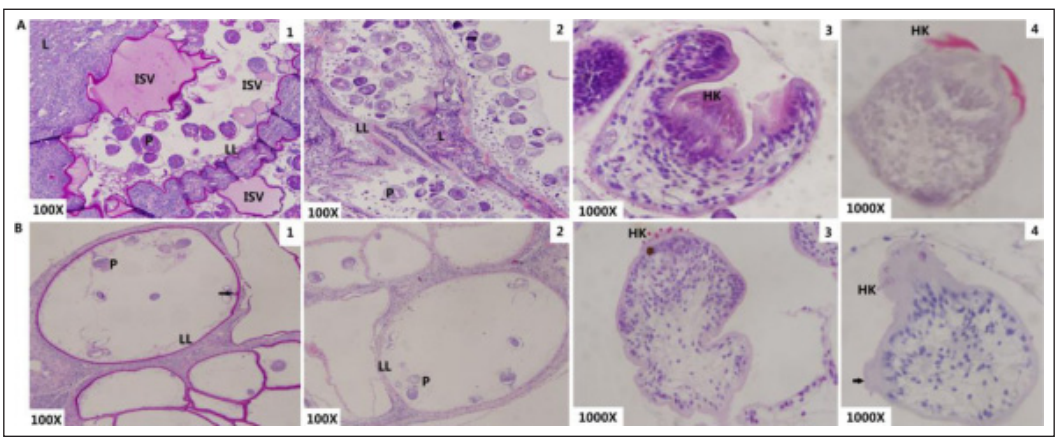

Fig. 1. Comparison of liver and subcutaneous cysts of *E. multilocularis* larvae. Paraffin-embedded sections of liver (A panel) and subcutaneous (B panel) cysts were stained using periodic acid Schiff kit (1) or hematoxylin and eosin (2, 3 and 4). A small vesicle (B1) and a scolex-like bulge (B4) in the subcutaneous cyst are indicated by arrowed. L: liver; LL: laminated layer; P: protoscolex; ISV: irregular-shape vesicles; HK: hooks.

In human cases, protoscolex formation is rarely described, but the mechanisms involved remain unclear (Moro *et al.*, 2009). In this study, results show that, although *E. multilocularis* larvae developed in a subcutaneous environment, subcutaneous encystment has adverse effects on protoscolex production and development. The decreased reproductivity and increased risk of abnormal development of protoscoleces provide potential explanations for high tissue encystment preference of *E. multilocularis* metacestodes. Due to the possibilities of deficiency in protoscolex production, it should be taken a precaution to use the subcutaneous encystment as a model for studies of echinococcosis, which has recently been applied for assessments of radiotherapy and secondary infection (Kuster *et al.*, 2013; Zhang *et al.*, 2011).

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Conflict of interest
None.

Ethical standards
The experimental protocol was approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China.

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