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ORIGINAL PAPER

Drug-induced apoptosis of *Echinococcus granulosus* protoscoleces

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Abstract Parasitic infection by *Echinococcus granulosus* in humans induces hydatidosis (echinococcosis), which is a zoonotic disease that seriously endangers public health. This study was to determine the status of cell apoptosis in the protoscoleces of *E. granulosus*, which were isolated from hydatid cysts in livers or lungs of sheep. Those protoscoleces were incubated with drugs (at the concentration of 1 mmol L^{-1} H_2O_2 and 5 mmol L^{-1}dexamethasone) for 8 h, the apoptosis were examined by transmission electron microscopy and TUNEL assay, the expression of caspase-1 and caspase-3 were detected by immunohistochemistry and caspase-3 activeity was detected by colorimetric assay. Our results have clearly demonstrated the presence of cell apoptosis in protoscoleces in the absence or presence of drug (H_2O_2, dexamethasone) treatment, but drug-induced apoptosis rate, caspase-1 and caspase-3 expression levels were higher than no-drug induce and caspase-3 activity were significantly increasing. We found H_2O_2 and dexamethasone can induce the cell apoptosis of protoscoleces. Our results implied the existence of a CED-3 like apoptosis gene in protoscolces and provide a rationale for further exploring the induction of apoptosis as non-surgical treatment method in treating this parasitic disease.

This research was supported by chinese natinal scientific fund, the project of the fund is the study on Hydatid induced apoptosis and its molecular mechanism. This Parasitic infection of *Echinococcus* in humans induces echinococcosis (hydatid disease), which is a zoonotic disease that seriously endangers public health. our aims of this study is to determine the status of cell apoptosis in *Echinococcus granulosus* by working with isolated protoscoleces. Results demonstrated clearly the presence of cell apoptosis in protoscoleces in the absence or presence of drug treatment and provide a rationale for further exploring the induction of apoptosis as non-surgical treatment method in treating this parasitic disease. The result of the research provided us a basement for futhur study on exploring the induction of apoptosis as a non-surgical treatment method in treating this parasitic disease.

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Introduction

Cystic echinococcusis caused by the larval stage of the cestode *Echinococcus granolosus* is a life-threatening disease of serious public health and economic concern of global proportion (Eckert and Deplazes 2004) Surgery is the first choice for single hydatid cyst treatment nowadays, but it is not useful for patients with several lesions in different organs or for patients without appropriate physical conditions for surgery. On the other hand, the surgery results were not always successful and surgery sometimes had been associated with local recurrence or secondary dissemination (Anadol et al. 2001; EL-On 2002).Finding effective non-surgical way to treat the disease becomes the goal of this study. Apoptosis is a form of cell death which is triggered by external factors and ultimately leads to the cell's self destruction. It is usually not associated with inflammation (Casciola et al. 1996). Therefore, apoptosis provides an ideal way to kill the parasitic *E. granulosus*. Protoscoleces are important components of the parasite; both share many identical or similar cell structures (Morsth

1967 and Wang et al. 1994). This study has investigated whether the apoptosis mechanism is present in *Echinococcus* protoscoleces and the possibility of applying drug-induced apoptosis in these protoscoleces.

Materials and methods

Materials

Protoscoleces were collected from hydatid cysts of *E. granulosus*, which were freshly isolated from livers or lungs of slaughtered sheep in a slaughterhouse in Urumqi City, Xinjiang province, West China. Briefly, the surface of the infected organs were disinfected and cut to expose the hydatid cysts; hydatid cyst fluid and free protoscoleces were harvested as much as possible by aspiration with a sterile syringe. Hydatid cyst walls were surgically removed, and rinsed with aspirated cyst fluid to harvest the residual protoscoleces and brood capsule, which were stored for further use.

RPMI 1640 medium was purchased from Sigma (Sigma–Aldrich, USA); dexamethasone sodium phosphate injection (Lot 20070102) was provided by Qianjiang Pharmaceutical Co., Ltd. (Hubei, China); ATP injection (Lot 200708085) was from Jinxin Double Crane Pharmaceutical Co., Ltd. (Shanxi, China); rabbit anti-caspase-1 (BS-0169R), rabbit anti-caspase-3 (BS-0081R), HRP-labeled SP (streptavidin) kit (ZYMED), and DAB (diaminobenzidine) kit were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) in situ apoptosis detection kit was provided by Nanjing KeyGen Biotech. Co., Ltd. (Jiangsu, China); Caspase-3 Activity Assay Kit and Apoptosis Kit were purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

Methods

Grouping: (1) Harvested hydatid cysts used directly in the assay and designated as “natural group”. (2) For the experiment, collected cyst fluid and hydatids were forceably pipetted to completely release and disperse the protoscoleces from brood capsules. The protoscoleces were rinsed with sterile saline until the microscopic morphology of the protoscoleces and the calcareous corpuscles were clean and bright and intact. More than 95% of the protoscoleces were negative in eosin exclusion test. This group of protoscoleces was cultivated with RPMI 1640 medium at 37°C, in a 5% CO_2 incubator: these were designated as “control group”. (3) The protoscoleces that were prepared the same way as control group but treated with drugs. These were designated as “induced group”.

Microscopic observation The protoscoleces were put onto slides, covered with coverslips, and observed microscopically under various magnifications.

TUNEL assay This was done according to instructions from the manufacturer. Briefly, protoscoleces were fixed with 4% neutral formaldehyde for 24–48 h; sections were cut according to previous descriptions (Chen et al. 2008). After color development with DAB and hematoxylin staining, slides were observed under the microscope. Nuclei of apoptotic cells were stained brown with TUNEL reagents; normal nuclei had no brown staining but showed a blue color with hematoxylin.

Observation with transmission electron microscopy (TEM): Protoscoleces were rinsed once with saline, fixed in 4% glutaraldehyde (24 h) and 1% osmium tetroxide sequentially, dehydrated with acetone gradient, and embedded in Epon 812 epoxy resin. The 60-nm sections were cut with an ultrathin section machine, stained with uranium and lead electron stains, and observed under the TEM. (JEOL1230)

Immunohistochemistry Protoscoleces were prepared according to a previous description Chen et al. (2008) Caspase-1 and caspase-3 antibodies were diluted 1:300; all sections were subjected to high-pressure, high-temperature antigen retrieval and subjected to staining procedures according to the kit's instructions. The slides finally went through color development with DAB, counterstained with hematoxylin, and observed under light microscopy. Yellow-brown staining in the cytoplasm (may include the nuclei) was considered as caspase-1 or caspase-3 positive; the slides stained with PBS replacing primary antibodies were used as controls to exclude false positive.

Caspase-3 activity assay Detections were performed according to the caspase-3 activity colorimetric assay kit

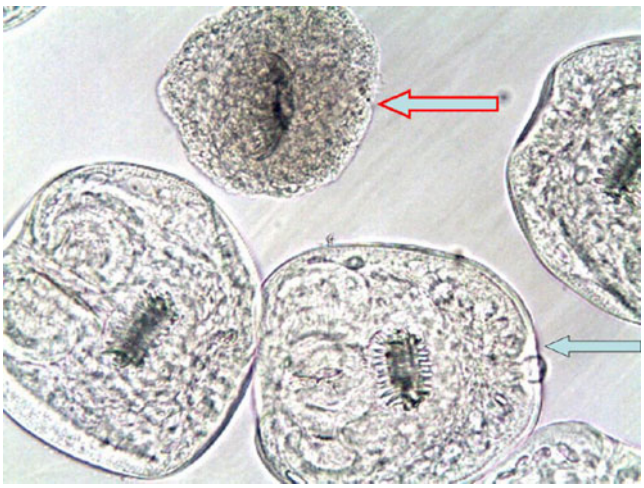

Fig. 1 Mophology of protoscolex under light microcopy (×400)

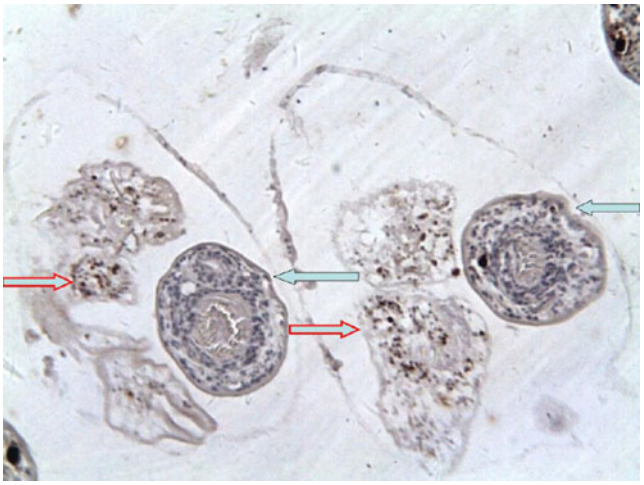

Fig. 2 Result detected by TUNEL assay in natural group

instruction (Beyotime Institute of Biotechnology, Jiangsu, China. Briefly, 3-mg samples were added with 100-μl lysis buffer, ground, kept on ice for 15–20 min and centrifuged at 4°C, 17,000×*g* for 15 min. The supernatants were harvested and added into the reaction system on an assay plate with a control group according to the kit's instruction; plates were incubated at 37°C for 15 h and detected with a microplate reader for the absorbance at 405 nm (A_{405}). The activated caspase-3 in samples catalyzed colorless substrate Ac-DEVD-*p*NA into yellow *p*NA: which concentrations could then be calculated according to *p*NA standard curve and sample A_{405}; the activity of caspase-3 in samples was finally deduced based on the *p*NA concentration.

Results

Observations with a light microscope: Under a light microscope, the morphology of isolated protoscoleces was

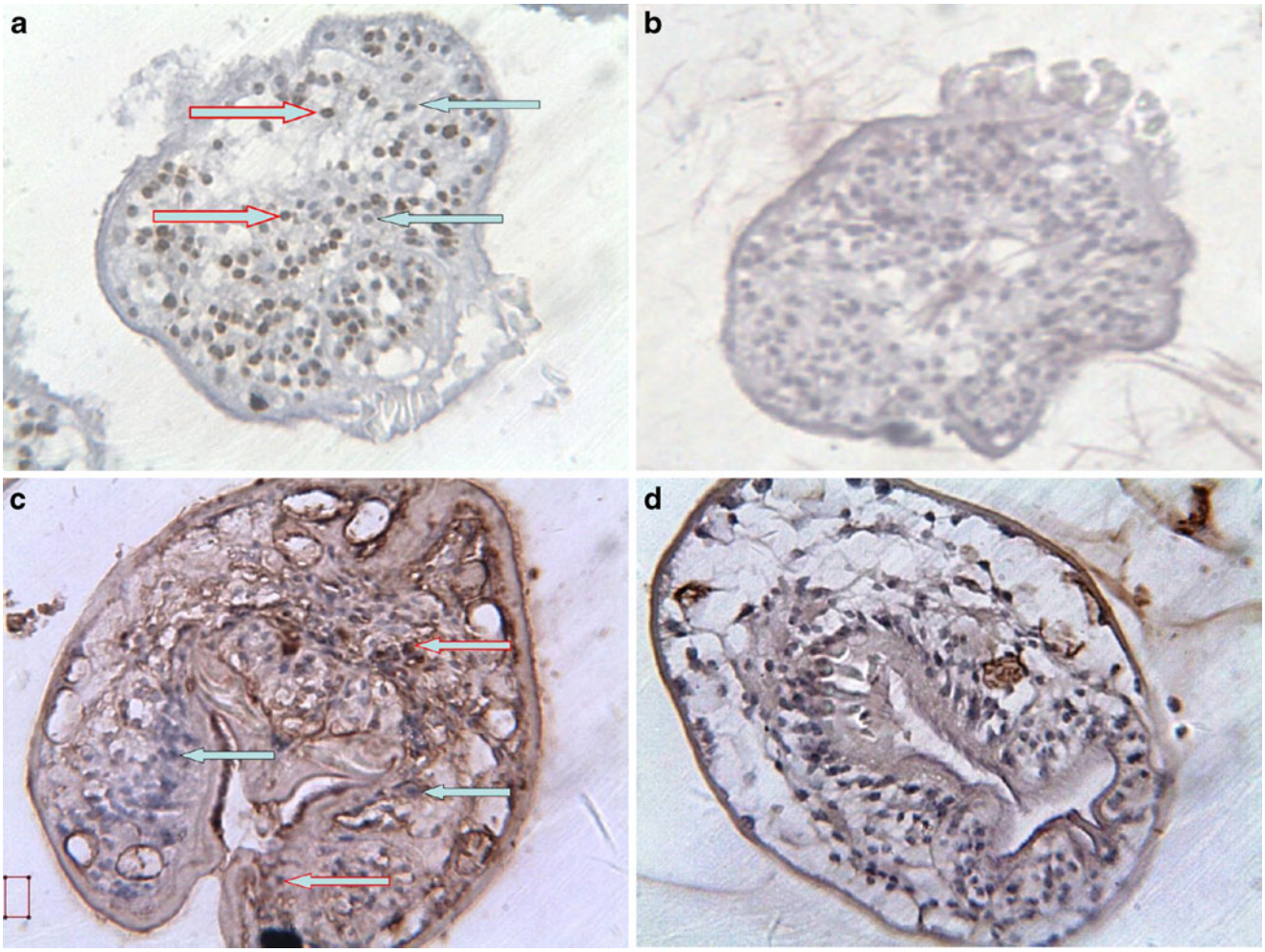

Fig. 3 Result detected by TUNEL assay in induced group, **a** the treatment 1mmol L-1 for 8 h, **b** the control group, **c** the treatment with 5 mmol L-1 dexamethasone for 8 h, **d** the control group

clear and intact with good light transmittance and the calcareous corpuscles were clear and bright (Fig. 1 indicated with the blue arrow). However, some protoscoleces from the natural group were shrunken with weakened light transmittance and shrunken or unclear calcareous corpuscles, showed the sign of wilt (Fig. 1, note red border arrow).

TUNEL assay The apoptosis of the protoscoleces was detected on histological sections using TUNEL method. On the same section or even in the same brood capsule of the natural group, there were some protoscoleces with little or nearly no yellow-stained apoptotic cells (Fig. 2, note the blue arrow), while other protoscoleces were with a large number of yellow-stained nuclei (Fig. 2, indicated by the red border arrow). In the induced group, after the treatment with 1 mmol/L (working concentration) H_2O_2 or 5 mmol/L (working concentration) dexamethasone sodium phosphate, there were mixed yellow or blue-stained nuclei, showing scattered distribution of apoptotic cells in the protoscoleces; on the contrary, there were few or no apoptotic cells in the control group, and nearly all the nuclei in the control group were stained blue (Fig. 3).

TEM observations Aligned microtriches, covered by a thick layer of periodic acid-Schiff (PAS)-positive substance, was observed on the tegument syncytium of normal protoscoleces in the natural group. Internal parenchymal cells of the protoscoleces were round or oval with clear and large nuclei and centrally localized nucleoli; the chromatin was fine and smooth with less amount of heterochromatin (Fig. 4a). Typical apoptotic cells in protoscoleces were seen in both the natural and the induced groups. In the natural group, there were protoscoleces, which showed typical apoptosis-induced morphological changes, which were

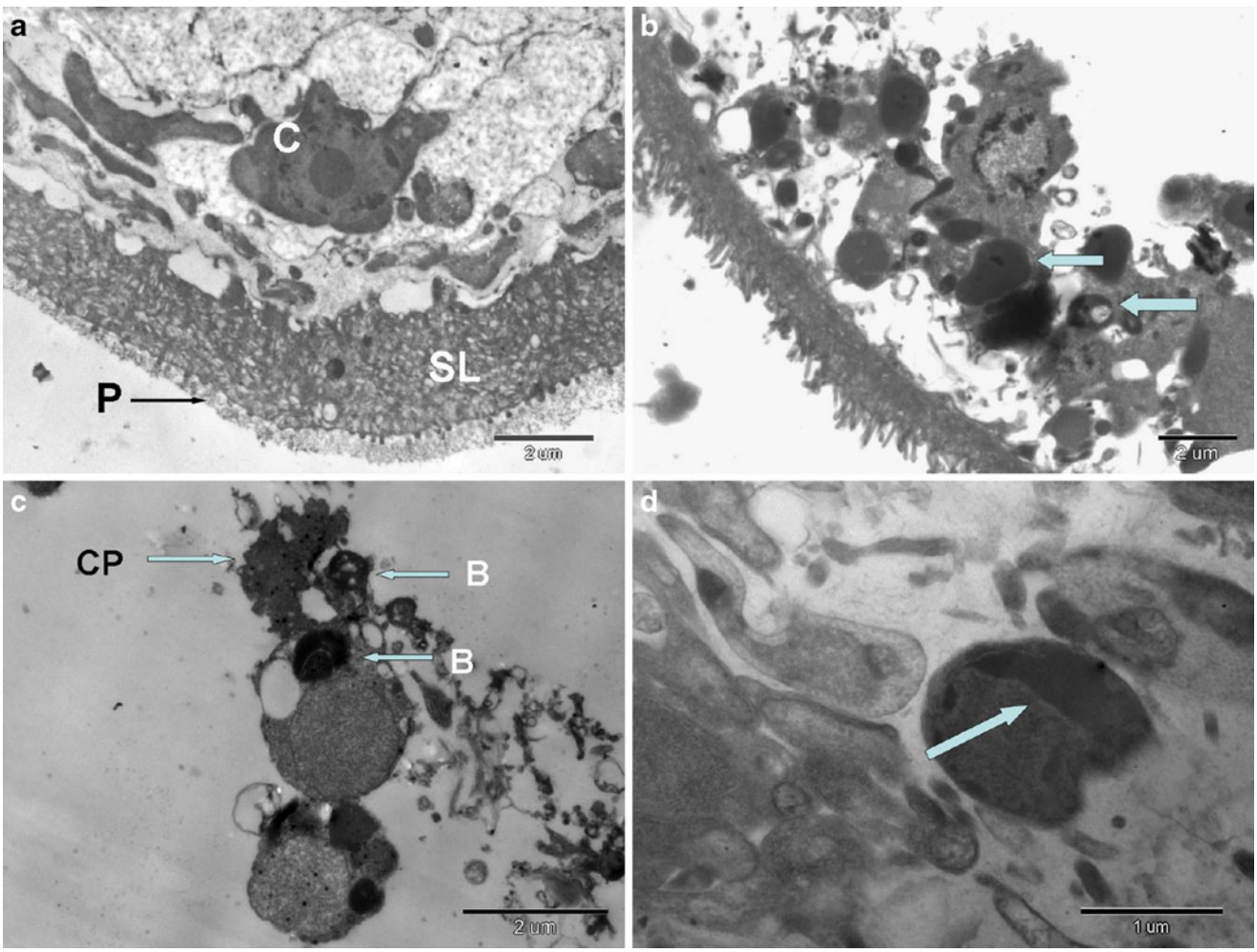

Fig. 4 Observation on protoscalex by TEM, **a** Normal protoscolex show syncytial layer of tegument and parenchymae cell (×10, 000) (*SL* syncytial layer, *C* parenchyma cell, *P* PAS positive material), **b** apoptotic protoscolec show the PAS positive material has disappeared, the cytoplasm of parenchyma cell concentrated and apoptotic bodies (*arrow*) formed (×8,000), **c** Show the concentrated cytoplasm of parenchyma cell and apoptotic bodies (×15, 000), **d** Show crescent-shaped heterochromatin mass on the nuclear membrane (*arrow*) of parenchyma cell (×30,000)

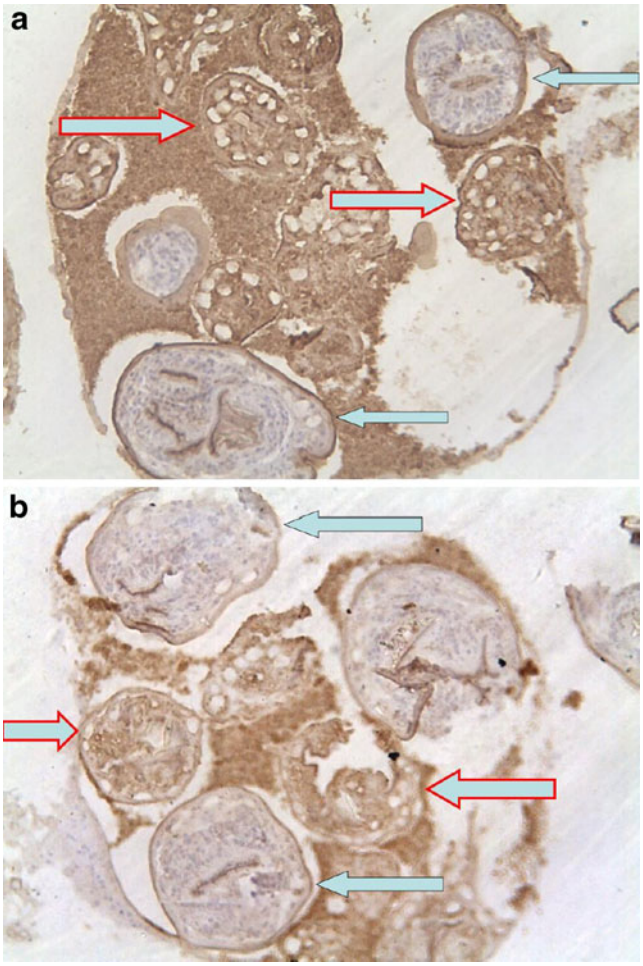

Fig. 5 Expression of caspase-1, caspase-3 in nature protoscolex of hydatid cyst, **a** Antigen-I is rabbit anti-caspase-1, **b** antigen-I is rabbit anti-caspase-3 (*Red border arrow* indicating a positive protoscolex, *blue arrow* indicating negative protoscolex)

characterized by: disappearance of the PAS-positive material outside the tegument microtriches; condensed cytoplasm in the internal parenchymal cells; and the formation of apoptotic bodies (Fig. 4b, c). In the induced group that was treated with 5 mmol/L (working concentration) of dexamethasone sodium phosphate and 1.6 mmol/L (working concentration) ATP for 8 h, some protoscolex cells were shrunken with reduced processes on the cell surface, condensed cellular matrix, and crescent-shaped heterochromatin that was marginalized to the nuclear membrane (Fig. 4d), showing the characteristics of apoptotic cells.

Immunohistochemistry In the natural group, some protoscoleces showed few or no expression of caspase-1 and caspase-3; however, in the same section or even the same brood capsule, there were some protoscoleces with strong expression of caspase-1 and caspase-3 (Fig. 5). These results are consistent with the results from TUNEL assay. The expression of caspase-1 and caspase-3 in induced group after 8 h of treatment with 1 mmol/L^{1} (working concentration) H_2O_2 is significantly higher than that in control group (Figs. 6, 7), while the expression of caspase-3 in induced group after 8 h of treatment with 5 mmol/L^{-1} (working concentration) dexamethasone sodium phosphate was significantly higher than that in control group (Fig. 8). When using PBS, which was used to replace the primary antibodies with other procedures unchanged in staining the above slides, all the slides showed no yellow staining, which excludes false positive.

Caspase-3 activity assay Caspase-3 activity assay was done in the induced (and control) groups after 8 h treatment with 5 mmol/L (working concentration) dexamethasone sodium phosphate. The calculated results showed that *p*NA production in the induced group was about eight times higher than that in the related control group, indicating that much higher caspase-3 activity in the induced group than that in control.

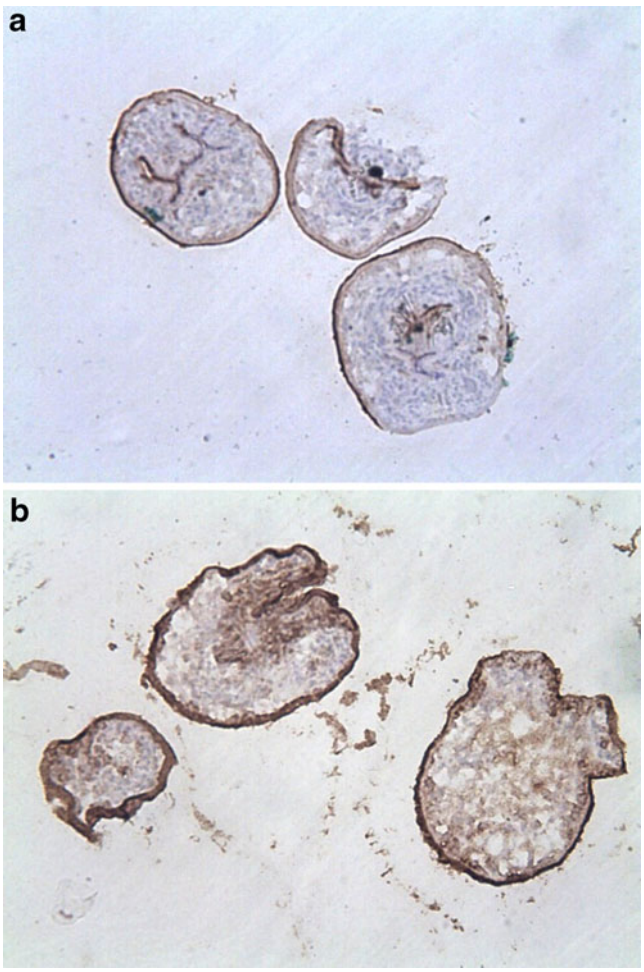

Fig. 6 Expression of the caspase-1 in the experiment group protoscolex of hydatid cyst, **a** the control group, **b** the induced group treated with 1 mmol L-1 H_2O_2 for 8 h

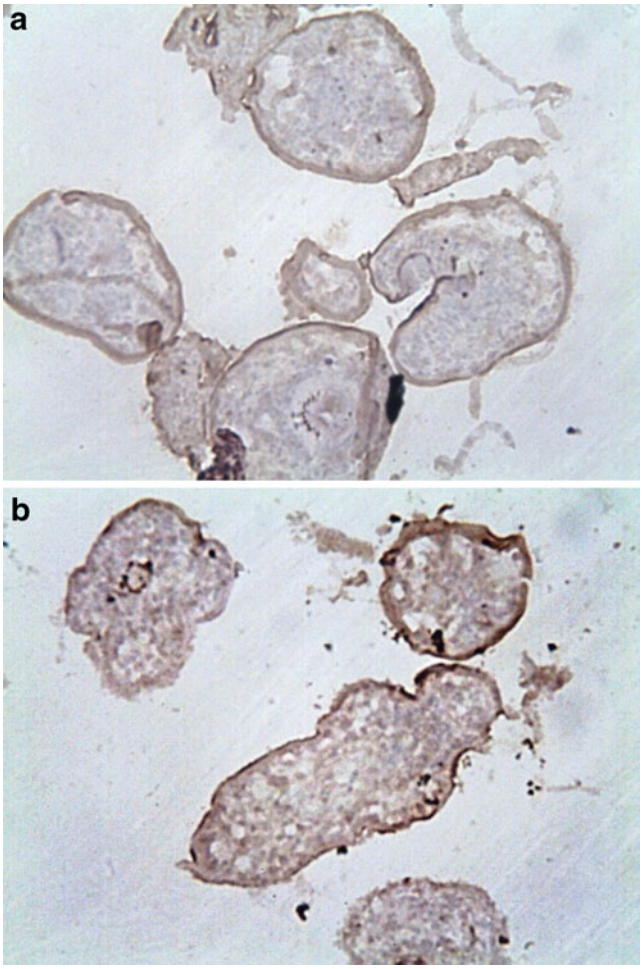

Fig. 7 Expression of the caspase-3 in the experiment group protoscolex of hydatid cyst, **a** the control group, **b** the induced group treated with 1 mmol L-1 H_2O_2 for 8 h

Discussion

Programmed cell death (PCD) or apoptosis is the most common form of eukaryotic cell death (Kerr et al. 1972). Due to the activation of endogenous endonuclease in apoptotic cells, nuclear DNAs are cut into many double-stranded high-molecular-weight DNAs, with single-strand break points (nicks) that expose a large number of 3′ hydroxyl ends. These can be end-labeled with deoxyuridine triphosphate (-dUTP) by desoxyribonucleic nucleotide transferase (TdT) to reveal the apoptotic cells in situ. This process has been named in-situ terminal deoxynucleotidyl transferase dUTP nick end-labeling, for which one can use the TUNEL assay (Negoescu et al. 1996). TUNEL assay is a research method that combines molecular biology and morphological techniques to detect the intact single apoptotic nucleus or apoptotic bodies in situ accurately, thus reflecting the typical biochemical and morphological characteristics of apoptotic cells, for identification of apoptosis not only in cultured or freshly isolated cells, but also in paraffin-embedded or frozen tissue sections. TUNEL method can detect a small number of apoptotic cells and is, therefore, widely used in apoptosis research (Negoescu et al. 1998). This study has used TUNEL method to detect apoptosis in protoscoleces under natural condition and found that there are various degrees of spontaneous apoptosis in some of the protoscoleces.

Dexamethasone and H_2O_2 are common apoptosis inducers, which have been reported by Wang et al. to induce apoptosis of adult cells and eggs in *Schistosoma japonicum* (Wang and Li 2000). A protoscolex is composed by multiple cells; although smaller, it is an intact worm. To observe the significant effect in a short time, this study used higher dosages of dexamethasone and H_2O_2. All the drug-induced protoscoleces that were detected with TUNEL method showed clear yellow staining of nuclei, and the corresponding control group showed few or no nuclei with yellow staining, indicating that dexamethasone and H_2O_2 can induce cell apoptosis in protoscoleces.

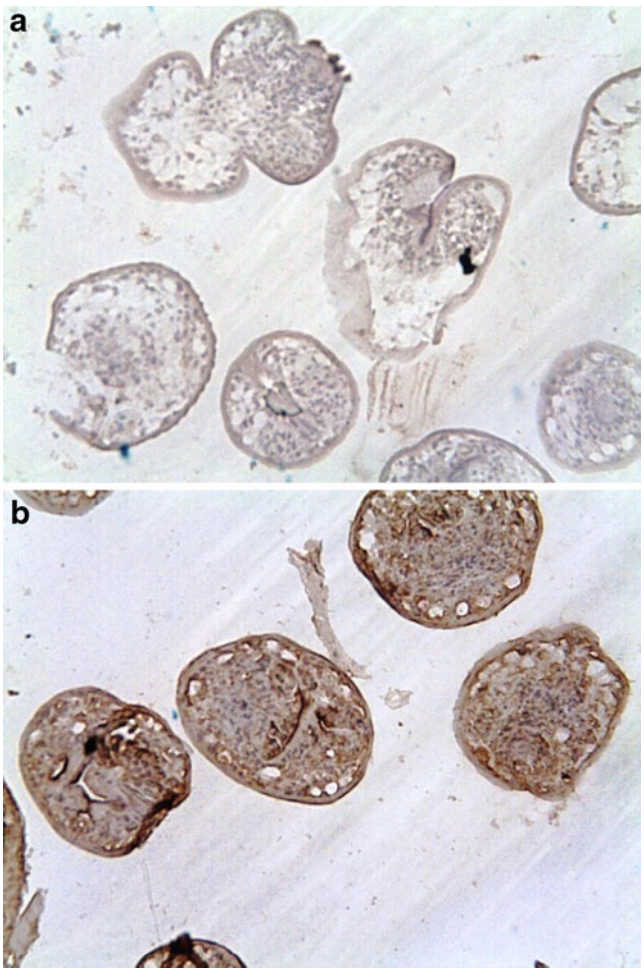

Fig. 8 The caspase-3 expression of 5 mmol L-1 dexamethasone sodium phosphate induced by 8 h, **a** the control group, **b** the induce group

To date, TEM is still the most classical and reliable method for determination of cell apoptosis and considered as the gold standard; TEM can clearly reveal characteristic ultrastructural changes in apoptotic cells, such as reduced volume, condensed cytoplasm, disappearance of surface microtriches, condensation and marginalization of crescent-shaped chromatin, cytoplasm compaction with concentrated organelles, and membrane blebbing. At the late stage of apoptosis, the nuclei are broken into pieces with the formation of apoptotic bodies, which are often seen phagocytosed by the neighboring macrophages (Gao 1998).We observed the characteristic changes of apoptosis including cytoplasm condensation and formation of apoptotic bodies in both natural and induced groups. These ultra-stracture changes were similar to (Celina Elissondo et al. 2009) study. They have been demonstrated that *Echinicoccus* protoscoleces have PCD patterns and may be induced to apoptosis by drugs.

Caspase-1 and caspase-3 are the two cysteine-aspartic acid proteases that play central roles in the execution-phase of apoptosis. Caspase-1, the IL-I-converting enzyme (ICE), shows 28% homology in primary structure with the nematode suicide gene CED-3 and plays similar roles. Caspase-3, the 32 KD cysteine protease (CPP32), is the major terminal proteolytic enzyme in the process of apoptosis. Inactivated caspase-3 (pro-caspase-3) shows the highest homology with CED-3 in the caspase family (with 35% homology). Caspase-3 is similar with CED-3 in both structural homology and substrate specificity and commonly recognized as mammalian homologue of CED-3. In particular, there are reports showing that the detection of caspase-3 can be used to distinguish cell death pathways between apoptosis and necrosis (Gao 1998). While it plays central roles in apoptosis, caspase-3 is negative in necrotic cells. However, the specific mechanism of apoptosis in *Echinococcus* cells remains unclear (Van-Cruchten and Van-Den-Broeck 2002).

This study has applied immunohistochemistry techniques by selectively applying caspase-1 and -3 polyclonal antibodies that can cross-react to a variety of mammalian proteins in detecting caspase-1 and -3 proteins in *Echinococcus* cells, with PBS replacing primary antibody as negative control to rule out the possibility of a false positive. Enhanced expression of caspase-1 and -3 was detected in TUNEL positive protoscoleces, and the activity of caspase-3 in induced group was significantly higher than that in the controls. These results not only further confirm the presence of apoptosis, but also imply the existence of a CED-3 like apoptotic gene in protoscoleces. Further deciphering the mechanisms of cell apoptosis in protoscoleces will provide a theoretical basis for the development of novel treatment of hydatid disease in future.

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References

Anadol D, Ozcelik U, Kiper N, Gogmen A (2001) Treatment of hydatid disease. Paediatr Drugs 3:123–135

Casciola RL, Rosen A, Petri M, Schlissel M (1996) Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythematosus. Proc Natl Acad Sci USA 93:1624–1629

Celina Elissondo M, Ceballo L, Alvarez L, Sánchez Bruni S, Lanusse C, Denegri G (2009) Flubendazole and ivermectin in vitro combination therapy produces a marked effect on *Echinococcus granulosus* protoscoleces and metacestodes. Parasitol Res 105:835–842

Chen R, Kong JF, Baishanbieke A (2008) Agarose-embedded section of hydatid of the protoscolex of the first experience of paraffin sections. J Xinjiang Med Univ 31:1541

Eckert J, Deplazes P (2004) Biological epidemiological and clinical of Echinococcosis, a zoonosis of increasing concern. Clin Microbiol Rev 17:107–135

EL-On J (2002) Benzimidazole treatment of cystic Enchinococcosis. Acta Trop 85:243–252

Gao YZH (1998) Caspase. Foreign Med Sci (Mol Biol) 20:145–149

Kerr JFR, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with ranging implications in tissue kinetics. Br J Cancer 26:238–257

Morsth DJ (1967) Fine structure of the hydatid cyst and protoscolex of *Echinococcus granulosus*. Parasitol 53:312–325

Negoescu A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillermet C, Brambilla C, Brambilla E (1996) In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. J Histochem Cytochem 44:959–968

Negoescu A, Guillermet C, Lorimier P, Brambilla E, Labat-Moleur F (1998) Importance of DNA fragmentation in apoptosis with regard to TUNEL specificity. Biomed Pharmacother 52:252–258

Van-Cruchten S, Van-Den-Broeck W (2002) Morphological and biochemical aspects of apoptosis oncosis and necrosis. Anat Histol Embryol 31:214–223

Wang WS, Li YL (2000) [Studies on apoptosis and its induction in *Schistosoma japonicum*]. Chin J Parasitol Parasit Dis 18:269–271

Wang H, Yan HY, Wu HY (1994) Observations on the ultrastructure of protoscoleces from different hosts by transmission electron microscope in Qinghai plateau. Bulletin of Endemic Diseases 9:19–20