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TREATMENT AND PROPHYLAXIS - ORIGINAL PAPER

Ultrastructural changes in hydatid cyst walls obtained from human cases, exposed to different therapeutic approaches

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Abstract

Recurrence of cystic echinococcosis as a result of treatment failure is frequently reported to cause a major problem in management of such serious parasitic infection. The deeply seated innermost germinal layer of hydatid cysts is a relatively delicate layer, yet responsible for viability maintenance of this parasitic stage. In this study, a trial was done to explore the ultrastructural changes in germinal and laminated layer of the hydatid cyst for the first time in human cases exposed to different therapeutic approaches which were done earlier to the final open surgical intervention. Four groups were included: group 1 did not receive any earlier form of treatment; group 2 was previously treated with only medical therapy; group 3 was treated with a single course of medical treatment, plus a single PAIR technique; group 4 was treated with multiple courses of medical treatment plus multiple PAIR techniques. Complete alteration of ultrastructural features of germinal and laminated layers were observed only with samples from group 4, indicating a kind of failure of the therapeutic approaches used in group, 1, 2, and 3, unless repeated in group 4 to achieve a real change regarding the fitness of the parasitic cystic lesions. Searching for more effective, safe, therapeutic method is highly recommended which may end the suffering of the affected patients.

Keywords Hydatid cysts · Germinal layer · Laminated layer · PAIR technique · Albendazole · Transmission electron microscopy

Introduction

Cystic echinococcosis is a chronic serious zoonotic disease, frequently reported in Mediterranean region including Egypt. The infection is caused by ingestion of mature eggs of *Echinococcus granulosus* by humans. The eggs are shed in the feces of dogs that harbor the adult worms in their small intestines. The first priority regarding location of these slowly growing cystic lesions is the liver, which is the site of infection of about 70% of human cases. There are variable therapeutic strategies to deal with symptomatic cases of hepatic hydatid cysts which include the use of chemotherapeutic agents, conservative surgical intervention as PAIR technique (percutaneous aspiration, injection and re-aspiration), and open surgery, intended for radical cure, either by performing partial resection by deroofing technique or by performing complete resection of the cystic lesions (Brunetti et al. 2010; Manouras et al. 2007). Recurrence is frequently reported to cause a major problem in management of such serious infection (Atmatzidis et al. 2005). Unfortunately, dealing with recurrence is mainly based on specialists' estimation with deficient quality of evidence, putting more risk on the suffering cases (Velasco-Tirado et al. 2017). The deeply seated inner most germinal layer of hydatid cyst is a relatively delicate layer, yet responsible for capability maintenance of this parasitic stage. Appropriate damage of this layer by any therapeutic means will certainly end the torment journey of the affected

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patients. In this study, a trial was done, for the first time, to explore the ultrastructural changes, specifically in germinal layers obtained from human cases exposed to different therapeutic approaches.

Subjects and methods

Specimens enrolled in the current study were taken from Egyptian patients suffering from cystic hydatidosis attending Surgery Department, National Hepatology and Tropical Medicine Research Institute, Cairo, Egypt, in the period from January 2017 to December 2018. The cases were subjected to comprehensive history and clinical examination, ultrasonography, and serological testing to detect anti-*Echinococcus* antibodies, in addition to the routine preoperative investigations. The enrolled cases were diagnosed to have hepatic hydatid cystic lesions and legible for open surgical intervention. Based on ultrasound examination and according to the World Health Organization (WHO) classification of hydatid cysts, large liver cysts (> 5 cm) with multiple daughter cysts, single liver cysts (> 5 cm), and cysts that were located superficially and posed the risk to rupture spontaneously or as a result of trauma were included in this study. In addition, viable cysts with signs of active infection, cysts communicating with the biliary tree, cysts that cause local pressure to adjacent organs, and complicated cysts were also involved (Brunetti et al. 2010). Pregnant women, patients with liver cysts < 30 mm in diameter, patients with cysts that are difficult to access, and patients with an active malignant disease were excluded from the study as recommended by the institutional ethical committee.

Study groups

Twenty six cases were enrolled in the present study and divided according to treatment they have received into the following groups:

- Group 1: 2 cases did not receive any form of treatment.
- Group 2: 8 cases previously treated with only medical therapy (albendazole, 400 mg twice daily for 4 weeks repeated up to 12 cycles separated by 2 weeks rest).
- Group 3: 8 cases previously treated with single course of medical treatment plus single PAIR technique.
- Group 4: 8 cases previously treated with multiple courses of medical treatment plus multiple PAIR techniques.

Transmission electron microscopy

Immediately after surgery, specimens from hydatid cyst wall were trimmed into small pieces approximately 0.5 × 0.5 mm^3, then fixed in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3 at − 4 °C for 24 h. Specimens were then washed with 3 changes of 0.1 M sodium cacodylate buffer. Post-fixation was carried out in 1% osmium tetroxide in cacodylate buffer for 2 h at − 4 °C. Dehydration of the specimens was done after washing using 3 changes of propylene oxide over a period of 90 min. Tissue was impregnated with equal parts of epoxy resin, epon 812 mixtures, and propylene oxide for 8 h, then mixture of 3/1 epoxy resin and propylene oxide for 8 h, then in 2 changes of pure resin 8 h in each change. Finally, the specimens were embedded in pure resin and were left for 24 h in an oven at 40 °C to harden then for another 24 h at 60 °C for resin polymerization. The sections were cut on an LKB ultramicrotome. Following semi-thin sections' examination (0.5 μm semi-thin), ultra-thin sections were cut (80 nm) and stained with uranyl acetate for 30 min (Johannessen 1978), followed by lead citrate for 15 min (Reynolds 1963; Robinson et al. 1987). The ultra-thin sections were examined and photographed by JEOL 100S transmission electron microscope (Jeol; Tokyo, Japan) at 60-kV accelerating voltage. Quantitation of different cellular structures in the germinal syncytium (e.g., undifferentiated cells) was done in 5 fields of magnification power × 4000, and then mean ± SD was calculated individually then calculated in each group.

Results

Electron microscopic examination of hydatid cyst wall from patients in group 1 showed regular arrangement of the entire hydatid cyst layers with intact structural features. Concerning germinal layer, it appeared intact, densely packed with its free border possessing microvilli-like extensions (microtriches) of papilliform type that protrude into the matrix of the laminated layer (Fig. 1a). Abundant glycogen storage vesicles were also seen in the germinal layer (Fig. 1a). The cellular components (syncytium) of the germinal layer included a large number of undifferentiated cells with vesicular (euchromatin) and condensed (heterochromatin). Heterochromatin appears relatively small, darkly stained with irregular particles scattered throughout the nucleus or accumulated adjacent to the nuclear envelope. Euchromatin was dispersed as seen in Fig. 1b. The mean number of undifferentiated cells was 10.7 ± 2.5/field (× 4000) (Table 1). The internal cellular structures and their organelles were intact with clear mitochondria, rough endoplasmic reticulum, Golgi apparatus, and variable vacuoles noticed in the germinal layer's cellular components. Cellular activity was also observed during mitotic division as obvious in Fig. 1c.

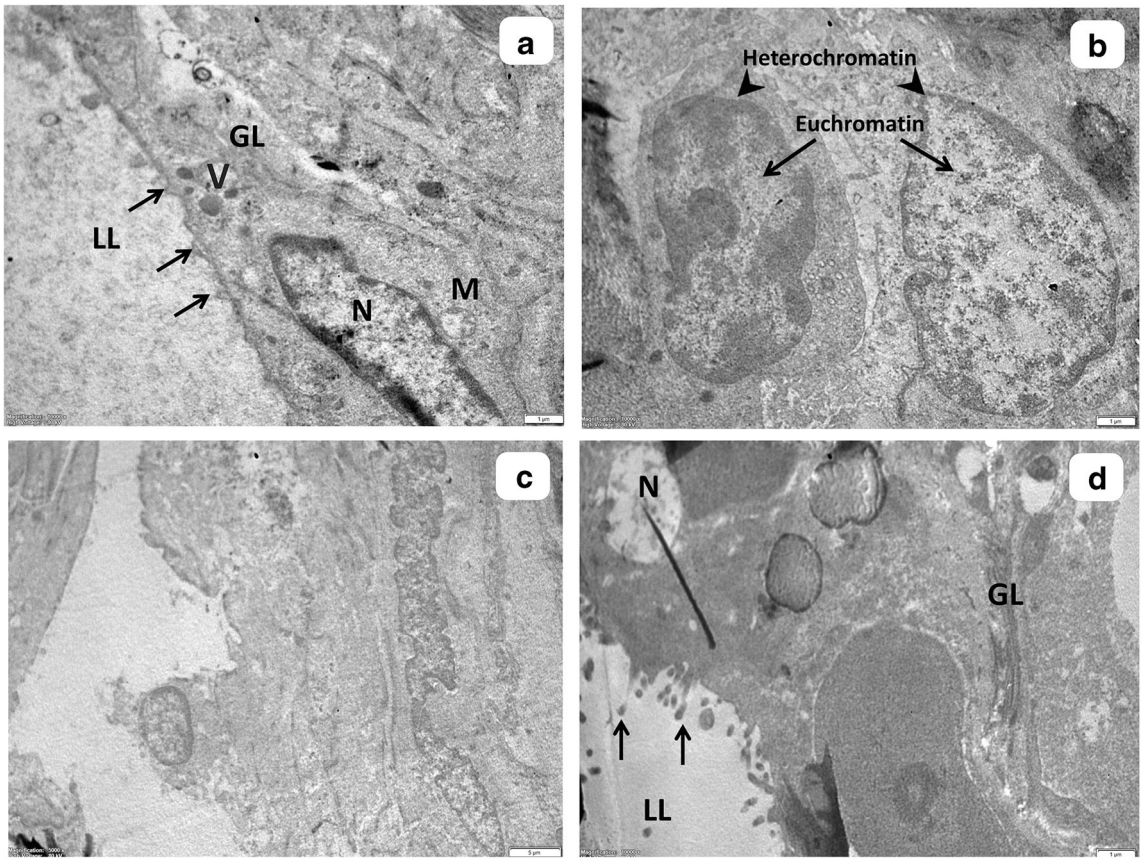

Fig. 1 Electron micrographs of human hydatid cyst ultrastructural features in group 1 (**a**–**c**) and group 2 (**c**) showing **a** germinal layer (GL), nucleus (N), mitochondria (M), laminated layer (LL), short papilliform microtiches (arrows), and storage vesicles (V). **b** Nuclei with condensed (heterochromatin) and vesicular (euchromatin) with prominent nucleolus within the left nucleus. **c** Cell with an elongated and lobulated nucleus in the stage of mitosis. **d** Germinal layer with interrupted spiniform microtriches (arrows). Scale bar = 1 μm × 10000

Microtriches of the germinal layer related to group 2, who received medical treatment only, were still found. They were either spiniform or papilliform (Fig. 1d). The mean number of undifferentiated cells was 9.2 ± 3.9/field (× 4000). Mitosis was also observed in this group. Apoptotic changes were seen in very few numbers of cells.

In patients who received single medical treatment and single PAIR technique (group 3), germinal layer appeared disturbed in the vast majority of the studied areas, with atrophied microtriches (Fig. 1c). The mean number of undifferentiated cells was 3.8 ± 1.4. The difference between the numbers of undifferentiated cells in different groups was statistically significant (Fig. 2).

Table 1 Different ultrastructural parameters investigated in different studied groups

		Group 1	Group 2	Group 3	Group4	ANOVA	
Germinal layer	Microtriches	Papilliform	Papilliform or spiniform	Atrophied	Damaged	*f*	*p* value
	Undifferentiated cells (counts ± SD/field)	10.7 ± 2.5	9.2 ± 3.9	3.8 ± 1.4	0 ± 0	42.73	< 0.001**
	Cellular organelles	Intact	Intact	Disturbed	Disturbed		
	Mitotic division	Seen	Seen	Seen	Not seen		
	Apoptotic changes	Not seen	Seen	Seen	Seen		
	Glycogen storage vesicles	Seen	Seen	Seen	Not seen		
	Inflammatory cells	Not seen	Seen	Seen	Seen		
Laminated layer	Arrangement	Compact	Compact	Interrupted	Severely interrupted		
	Granular bodies	Seen	Seen	Seen	Not seen		
	Lamellar bodies	Not seen	Seen	Seen	Not seen		
	Collagen fibers with lipids vesicles	Not seen	Seen	Seen	Not seen		
	Inflammatory cells	Not seen	Seen	Seen	Seen		

**Highly significant

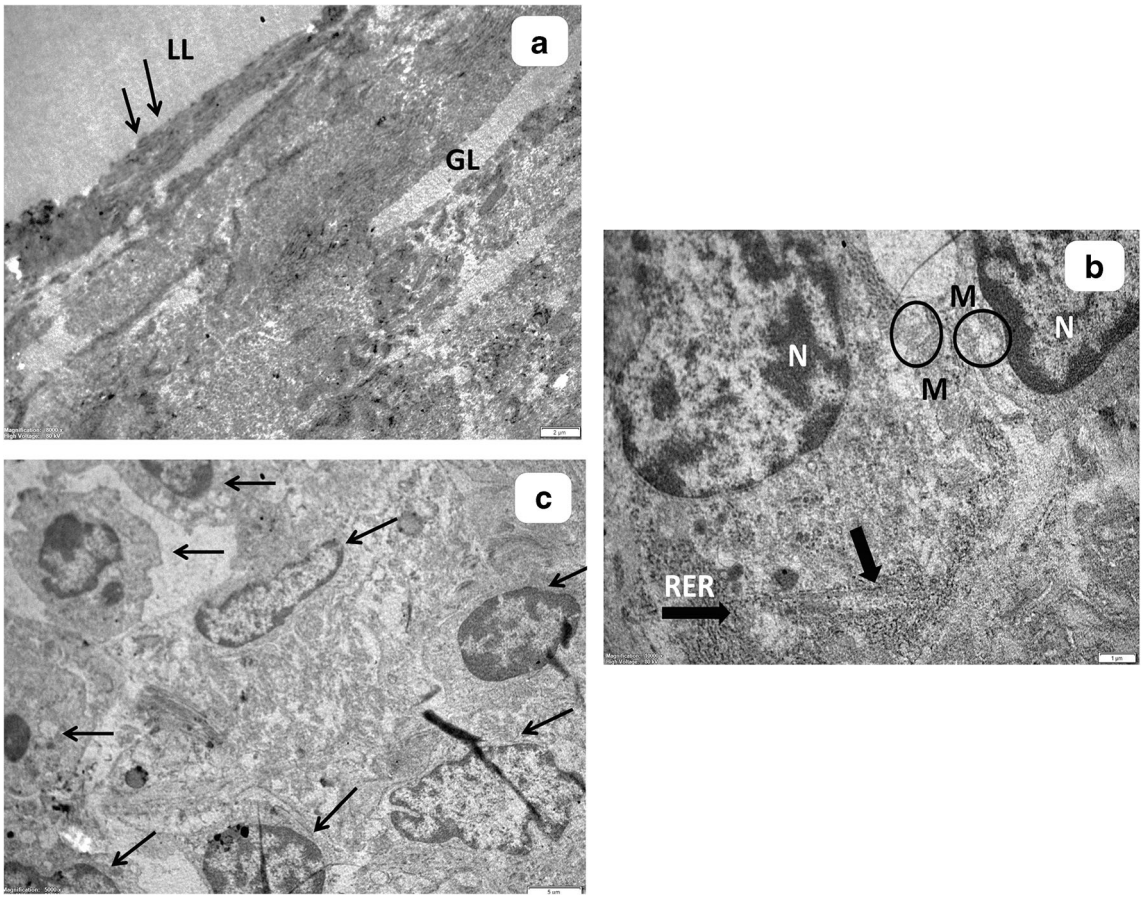

Fig. 2 Electron micrographs of some ultrastructural features in the germinal layer from human hydatid cysts in group 3 (**a**) and group 1 (**b**, **c**). **a** Injured germinal layer with wrecked microtriches. **b** Intact internal cellular structures and organelles in germinal layer of group 1 with clear mitochondria and rough endoplasmic reticulum (RER) in addition to some vesicles beside RER. **c** Numerous undifferentiated cells containing heterochromatin and euchromatin. Scale bar = 1 μm × 10000

Apoptosis was also seen in a relatively large number of cells in group 3. The apoptotic characters were in the form of nucleus with condensed chromatin, irregular nuclear membrane, plasma membrane blebbing, and the cell shrinkage from the surrounding tissue (Fig. 3b). In addition, cup-shaped chromatin condensation was also seen in some apoptotic cells (Fig. 3a).

In patients who received multiple courses of medical treatment and multiple PAIR techniques (group 4), undifferentiated cells were not seen; instead, pyknosis of the germinal layer's cells was noticed (Fig. 3 c and d) with condensed fragmented chromatin. Necrosis was noticed in the germinal cells, together with broken nuclei and disappearance of nucleoli; relatively swollen mitochondria was noticed in the germinal cells (Fig. 3d), combined with increase of lysosome and formation of myeloid body. Vacuolization and lipid formation was also increased in the germinal layer. Complete cellular necrosis with vacillation of the cytoplasm and destruction of the organelles and dilatation of smooth endoplasmic reticulum were also seen (Fig. 3 d and e).

Concerning laminated layer, it appeared in group 1 as well-defined compact layer comprised of an acellular, amorphous, and heavily glycosylated layer. Ultrastructurally, in this group, this layer is composed of a microfibrillated matrix containing some aggregates of electron-dense material (Fig. 4a). In group 2, few lamellar bodies were seen within the laminated layer in cases related to this group (Fig. 4b), yet the laminated layer appeared relatively compact with electron-dense bodies. In group 3, disturbed collagen fibers with large cytoplasmic vacuoles filled with moderately electron-dense material, query lipid vacuoles, were seen within the laminated layer (Fig. 4c), in addition to numerous lamellar bodies. While in group 4, the laminated layer appeared completely disrupted with electron-dense elongated structure (Fig. 4d).

On the other hand, there was inflammatory cellular infiltration as eosinophil, plasma cell, and phagocytic cell reaching the germinal layer (Fig. 4a), observed in group 3 within some cells, either undifferentiated or apoptotic. The inflammatory cells were also seen in group 4 within the huge number of the necrotic and apoptotic cells. This indicated successful invasion of inflammatory cells through the disrupted laminated layer, reaching the germinal layer. Other inflammatory cells were also seen as plasma cell that demonstrated the characteristic cartwheel appearance of the nucleus due to the presence of large, peripheral clumps of heterochromatin. Eosinophils

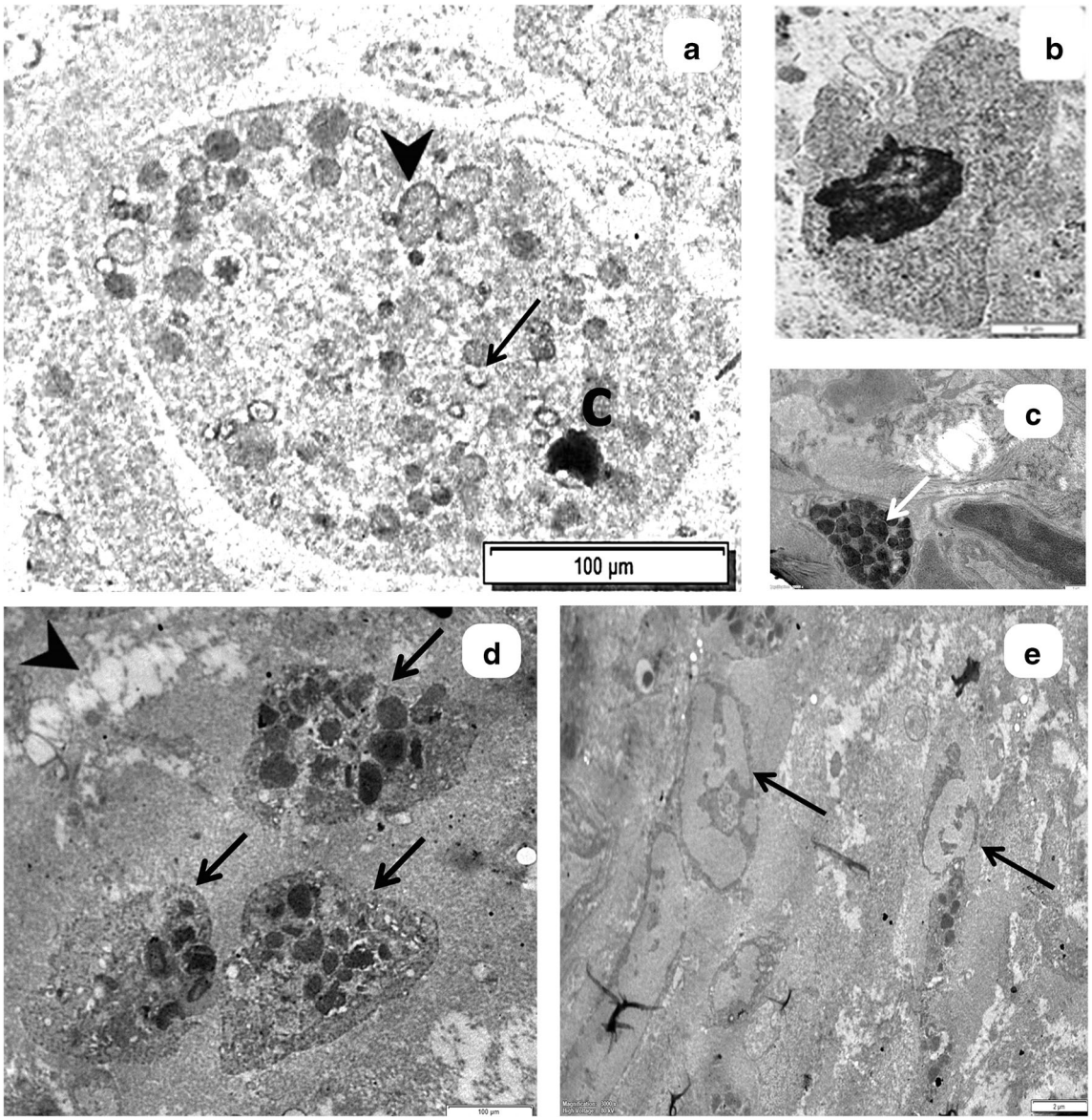

Fig. 3 **a** Electron micrograph showing apoptotic cell characterized by swollen mitochondria (arrow head) and cup-shaped chromatin condensation, notice the vacuolization of the cell (arrow). **b** Electron micrograph showing apoptotic cell characterized by a nucleus with condensed chromatin and irregular nuclear membrane and shrinkage of the cell from the surrounding tissue. **c**, **d** Electron micrograph showing pyknosis of the cells (degradation and condensation of the nucleus), cytoplasmic apoptotic bodies (arrows), vacuolization of the cytoplasm, and destruction of the organelles. Dilatation of smooth endoplasmic reticulum is seen in **d** (arrow head). **e** Necrotic cells with disruption of the nuclear membrane (arrows) and destruction of the organelles. Scale bar = 100 μm × 6000 (in **a**, **d**), 1 μm × 10000 (in **b**, **c**), and 2 μm × 3000 (in **e**)

were also seen that were characterized by its major population of specific granules either intra- or extracellular, with a unique morphology with an internal often electron-dense crystalline core and an outer electron-lucent matrix with the typical bi-lobed nucleus (Fig. 4c, d). Table 1 summarizes the results of ultrastructural changes in different groups.

Discussion

The present study aimed to study the ultra-structures of some surgically removed portions of hydatid cyst layers which were earlier exposed to different therapeutic trails. This was done to determine whether treatment was successful in eliminating the ability of the parasite to spread or not (Fig. 5).

Ultrastructural examination of the germinal layer related to the 2 cases in group 1 revealed intact microtriches that were of papilliform-type as described by Hoberg et al. (1995). These microtriches increase the resorbing surface of the parasite and fix the germinal layer well into the richly covering laminated hyaline layer (Mehlhorn et al. 1983; Stettler et al. 2003). Galindo et al. (2008) stated that the destruction of the microtriches might be the major reason for loss of viability of hydatid protoscolices (PSs). The authors added that these

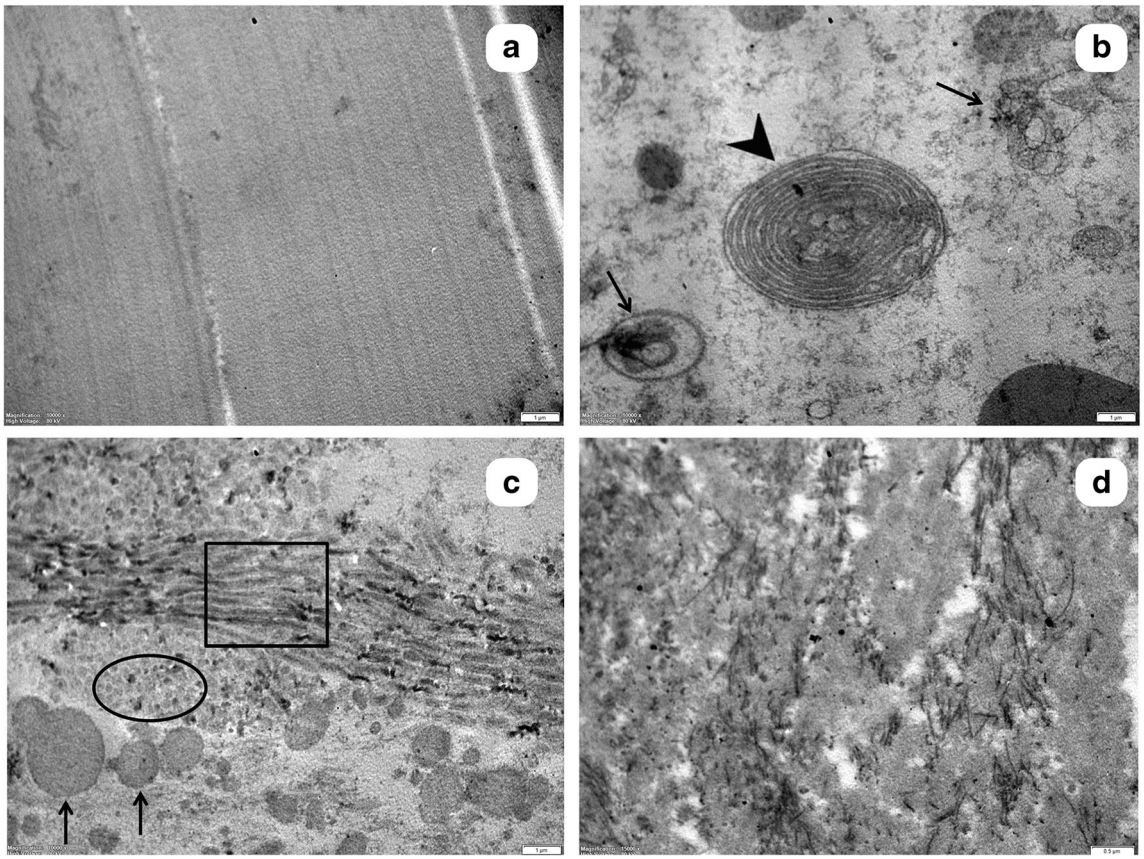

Fig. 4 **a** Electron micrograph showing compact regular acellular laminated layer from group 1. It appears as an amorphous layer composed of a microfibrillar matrix containing some aggregates of electron-dense material. **b** Lamellar body seen within the laminated layer (arrow head) with starting formation of new lamellar bodies (arrows) in groups 2 and 3. **c** Longitudinal collagen fiber (square contour), transverse fibers (circular contour), and cytoplasmic vacuoles filled with moderately electron-dense material and query lipid vacuoles (arrows) in groups 2 and 3. **d** Completely disrupted laminated layer in group 4. Scale bar = 1 μm × 10000 (in **a**, **b**, **c**) and 0.5 μm × 15000 in **d**

vital structures are the means for nutrient absorption, physiological homeostasis, and defense for the protoscolices. Thus, the presence of these microtriches in all the studied groups, except the fourth one indicates failure of the used therapeutic approaches to radically cure the enrolled cases in groups 2 and 3. Medical treatment alone and single PAIR technique seemed non-effective to radically cure the infected human cases.

The nuclei of the undifferentiated cells in the germinal syncytium with both their euchromatin and heterochromatin components were also helpful to add more illumination on such vital issue. Euchromatin is predominant in active cells in the transcription process, while heterochromatin is most abundant in cells that are less active or inactive (Allis and Jenuwein 2016). This may reflect variable degree of activity of germinal layer in different groups. Significantly higher numbers of these active cells were found in group1 then group2, while the minimum number was counted in group 3. Complete loss of such cells was reported in group 4. This strengthened again what was mentioned before about failure of medical treatment and single PAIR technique to totally treat this aggressive parasitic infection which possibly needs multiple medical and several invasive surgical interventions to reach final destination of cure, thus exposing the suffering patients to more risk with a possible morbidity or/and mortality. Therefore, this study may open the way to find more effective therapeutic agent to minimize the risk of multiple surgical procedure.

Ultrastructurally, the laminated layer is composed of a microfibrillated matrix containing some aggregates of electron-dense substances. Consequently, we believe that the laminations seen in light microscopy studies are the result of different degrees of compaction of these components. The granular element was described in detail by Richards et al. (1983) to involve naturally electron-dense bodies of defined size that are found irregularly dispersed, individually or in aggregates, among the laminae. Individual granules were also observed within vesicles in germinal layer cells and seemed to be exocytosed towards the LL. The compact microfibrillar matrix is a common feature of the LL of all *Echinococcus* species, yet the granules have been described only in *E. granulosus* (Ingold et al. 2000, 2001). Richards et al. (1983), Rogan and Richards (1989), and Irigoín et al. (2004)

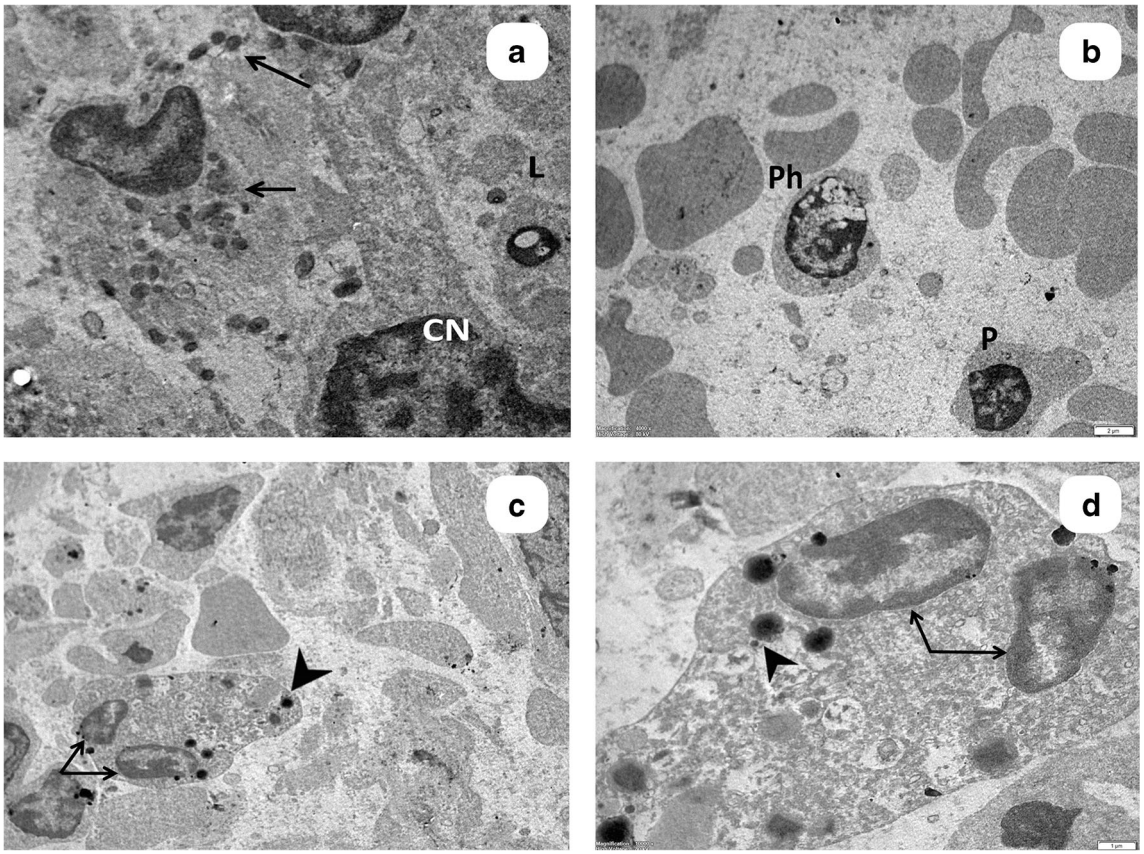

Fig. 5 **a** Electron micrograph showing eosinophil with its characteristic intra- and extracellular granules (arrows) in groups 2 and 3. Compact nucleus of an undifferentiated cell is seen in the field (CN). Lysosome (L). **b** Electron micrograph showing phagocytic cell (Ph) and plasma cell with the characteristic cartwheel nucleus (P) and RBCs. **c** and **d**: Electron micrograph showing eosinophils with the characteristic granules (arrow head) and the typical bi-lobed nucleus (arrows). Scale bar = 5 μm × 5000, 2 μm × 4000, 2 μm × 3000, and 1 μm × 10000, respectively

reported that the LL is formed by two ultrastructural elements, a predominant microfibrillar matrix and granular bodies. The earlier matrix is usually stained positive for carbohydrates and is thus thought to be made up from the glycoconjugates that account for the bulk of the LL chemically. More recently, Corfield (2015) reported that the LL is based on mucins with a particular type of glycosylation (mucin O-type glycosylation), which form the loose mucus-rich glycogen barriers. This may explain the presence of some glycogen vesicles in the germinal layer of active hydatid cysts as observed in groups 1, 2, and 3 (Table 1). While absence of these glycogen storage vesicles in group 4 may be due to impact of multiple treatments which forcibly reduce glucose uptake and storage in human hydatid cysts as explained by Lacey (1990) which studied the effect of medical treatment on cellular microtubular structures of the metacestode.

Regarding the other element of the LL of *E. granulosus*, Irigoín et al. (2004) and Casaravilla et al. (2006) stated that a laminated layer contains abundant nano-deposits of a calcium salt of inositol hexakisphosphate (InsP6). This InsP6 is considered an intracellular molecule in all biological systems, so may perhaps indicate some sort of viability (Irvine and Schell 2001). These reports may explain the presence of these particles which appeared as electron-dense materials in groups 1, 2, and 3, while disappeared from group 4, indicating useless laminated layer following exposure of repeated surgical manipulations of hydatid cyst wall in such group.

Generally, the LL is widely thought to be a crucial element of the host parasite relationship in echinococcosis. Its roles include shielding the parasite from direct attack by host immune cells, and probably downregulating local inflammatory reaction (Díaz et al. 2011a, b). This shelter is successfully devastated, only in group 4, allowing the inflammatory cells to functionally reach the germinal layer to achieve real changes. The infiltration of the cyst wall up to germinal layer by eosinophils and the change in there granules in this study can be explained by Burkitt et al. (1993), who mentioned that all eosinophils have receptors for IgE which may be important in the immune response against the parasites. For phagocytosis of large object (e.g. parasite), the eosinophil appear to release its granular contents into the external environment in a trial to attack the parasite. The release of such eosinophilic granules is evident in this study in groups 3 and 4, in which the laminated layer was interrupted in the earlier group and completely destructed in the latter group.

Detection of increasing cellular vacuolization, swollen mitochondria, presences of lipid droplets within the germinal layer and lamellar bodies within the laminated layer that may reflect a general tissue stress due to struggle against the parasite with subsequent apoptosis or/and necrosis. This vacuolization may cause a major leakage of cytoplasmic contents (Kim et al. 2011). The presence of lipid droplets within the syncytium of the germinal layer may indicate metabolic disruption of the cyst as a result of therapeutic manipulation (Verma et al. 2013). Cellular vacuolization, fat droplets, and abnormal mitochondria due to progressive changes within the germinal layer were also reported by Walker et al. (2004) after investigating the effect of in vitro medical treatment on hydatid cyst. Such changes were noticed in group 3, which were exposed to medical treatment plus single PAIR technique. In general, medical treatment for human hydatid disease is not successful in vast majority of cases with recurrence rate reaching 80% (Safioleas et al. 1994; Dziri et al. 2004).

Concerning apoptotic changes which started within the cellular component of groups 3 and 4, it is a type of programmed cell death that can be identified by morphological and biochemical events such as chromatin condensation resulting in nuclear pyknosis, DNA fragmentation, formation of apoptotic bodies, and cysteine aspartate-specific protease activation (Gupta 2001; Zimmermann et al. 2001). Apoptosis may be a normal program of tissue reorganization during development or it could be a response to damaging external stimuli as the surgical therapeutic technique done for our patients in groups 3 and 4. Moreover, parasitic stage may elicit programmed cell death in host immune cells, mainly for the regulation of host parasite interactions. Otherwise, may be related to effective modulation of the host response to the parasite as a response to different treatment (Flores-Pérez et al. 2003; Tato et al. 2004). On the other hand, cellular organelles were intact in groups 1 and 2, while some mitochondria were seen swollen in group 3. Group 4 showed destruction of the organelles and dilatation of smooth endoplasmic reticulum as a result of intensive surgical manipulation.

Unfortunately, there is no satisfactory treatment for hydatid cysts. More than 40 years have passed since the introduction of benzimidazoles (Bekhti et al. 1977; Saimot et al. 1983) and more than 30 years since the initiation of the PAIR technique (Mueller et al. 1985); however, this ambition is still distant. In spite of the enormous information about the variable therapeutic strategies of human hydatidosis, the currently used methods are based on low-to-mild quality of evidence and instruction strength. Therefore, the current therapeutic strategies are possibly inadequately assessed concerning their efficiencies, effectiveness, and safeties (Brunetti et al. 2010; Tamarozzi et al. 2014).

The results of the current study confirmed such disappointment about the current therapeutic approach. Germinal layers of the cystic lesions are only affected after repeated invasive surgical manipulations in addition to repeated medical therapy. Thus, more effort should be made by the clinicians in collaboration with the basic medical scientists to find a successful, better, non-invasive, effective, and safe therapeutic protocol to radically cure such serious parasitic infection to stop the affected patients from suffering.

Compliance with ethical standards This work was performed according to the national institutional ethical and professional guidelines for the management, follow-up, and post-operative care of hydatidosis cases. Informed written consent was supplied by individual patients.

Conflict of interest The authors declare that they have no conflict of interest.

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