Original Article

Nitric oxide synthase: A potential factor in loss of hydatid protoscoleces viability *via* its role in cholesterol immunomodulation

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ABSTRACT

Background: Presence of cholesterol in hydatid cyst fluid is associated with cyst degeneration through its immunomodulatory effect as a non-immune inducer of inducible nitric oxide synthase (iNOS) expression.
Objective: To investigate the association of cholesterol crystals (CCs) in hydatid fluid, viability of protoscoleces and iNOS expression in hydatid cyst wall.
Patients and Methods: Cases were divided into two groups; group (1) included six patients with CCs in their hydatid cyst fluid and group (2) included 12 patients without CCs in their hydatid cyst fluid. Protoscoleces' viability was assessed and correlated with iNOS expression in the hydatid cyst walls in both groups by immunohistochemistry.
Results: Protoscoleces' viability was significantly decreased in G1 containing CCs. High expression levels of iNOS were recorded in sections from the hydatid cyst walls of G1, as compared to minimal iNOS expression observed in sections from G2. A significant negative correlation was recorded between the degree of iNOS tissue expression and viability percentage of hydatid protoscoleces.
Conclusion: The decreased viability of protoscoleces suggests a role for cholesterol in immunomodulation, possibly through the involvement of the nitric oxide pathway.

Keywords: cholesterol crystals; hydatid cyst; iNOS; protoscoleces; viability.

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INTRODUCTION

Cystic echinococcosis (CE) represents a chronic re-emerging serious zoonotic disease[1]. It is found on every continent except Antarctica and is frequently reported in the Mediterranean region including Egypt[2]. Transmission of these parasites needs a predator-prey relationship that involves domestic and wildlife animals according to the species[3].

Nitric oxide (NO) is considered as a pro-inflammatory mediator, synthesized by nitric oxide synthase (NOS). It plays an important role in the process of immunoinflammation[4]. Three NOS isoforms were identified: neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and iNOS[5]. Neuronal NOS is expressed mainly in neurons, while eNOS is expressed mainly in epithelial cells. Notably, iNOS is a calcium-independent enzyme induced by various immune cells, such as T cells, macrophages, and mature dendritic cells. The enzyme plays a role in the regulation and differentiation of these cells via the nitration of important molecules involved in signaling or transcriptional pathways[6]. The effect of iNOS on immune cells mediates the antimicrobial action of NO[7]. In addition, iNOS is expressed by non-immune cells such as fibroblasts, endothelial cells, and keratinocytes. Besides its role in pathogen killing, iNOS also exerts immune-modulatory effects, such as the inhibition of T cell activity[8]. The expression of iNOS can be induced by cytokines in addition to other stimuli[4]. High fat diet and cholesterol are among the factors that increase the mRNA expression of iNOS leading to a consequent increase in NO levels[9]. As an important component of the eukaryotic cell membrane cholesterol mediates various functions including the pathogen entry into the host cells[10]. Cholesterol is also suggested to have an immune modulatory effect by promoting the proliferation and function of immune cells[11]. The presence of cholesterol crystals in hydatid cysts was recognized as a rare finding in several case reports. They were described as having a glittering appearance, and hyperechoic heterogenicity (that having greater echogenecity than that of subcutaneous fat or it is equal to that of fibroglandular parenchyma), i.e. mimicking cyst infection[12,13]. The presence of cholesterol was found to be associated with the degeneration of hydatid cysts[14,15].

This study was designated to investigate the possible association between the expression of the iNOS inflammatory marker in the hydatid cyst walls and the viability of protoscolices in relation to the presence of cholesterol crystals in the hydatid cyst fluid.

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PATIENTS AND METHODS

This case-control study was conducted in Parasitology and Pathology departments of the National Hepatology and Tropical Medicine Research Institute, Cairo, Egypt during the period from March 2018 to June 2020.

Study population and collected samples: The study enrolled eighteen patients with cystic echinococcosis attending the hospital for surgery. Inclusion criteria included patients with large cysts (>5 cm), with multiple daughter cysts, superficially located cysts at risk of rupture, cysts with biliary communication, cysts with local pressure effect on adjacent organs and complicated cysts[16]. Exclusion criteria included small cysts <3 cm, pregnant women, patients with active malignant disease, and cysts that are difficult to access[17]. The patients were divided according to the presence of cholesterol crystals in their hydatid cyst fluid (HCF) into two groups; six patients with cholesterol crystals in their hydatid cyst fluid (G1), and twelve patients without cholesterol crystals (G2). From each patient, samples collected from surgically removed cysts included HCF and parts of the hydatid cyst wall.

Examination of HCF[18]: The HCF was microscopically examined as wet mount to detect protoscoleces after simple sedimentation and also after centrifugation at 4000 rpm for 5 min. The supernatant fluid was transferred to a sterile tube and its physical characters, regarding color and aspect were recorded. In addition, the presence of cholesterol crystals was identified microscopically from the hydatid sand content. Each slide was examined under x100 and x400 magnification.

Eosin stain for assessment of viability: Eosin solution is a vital stain that can differentiate living from dead protoscoleces. Live protoscoleces do not accept the stain, while dead ones stain red due to changes in their osmo-regulatory mechanisms[16]. One ml of hydatid sand was mixed with 0.1 ml of 0.1% eosin solution. After 1-2 min, a smear was made, air dried and examined under the compound microscope[17]. The percentage of viable protoscoleces (viability%) was estimated by calculating the ratio between the number of viable protoscoleces and the total number of protoscoleces, i.e., Viability percent = number of viable protoscoleces/total number of protoscoleces.

Immunohistochemical assay: Fresh samples of the hydatid cyst walls were fixed in 10% formalin and embedded in paraffin blocks for immunohistochemical examination. All chemicals were obtained from Biological, P.O Box 261, Swampscott, Massachusetts, USA. According to Prophet *et al*.[19], tissue sections of 4-5 µm thickness were placed on adhesive-coated glass slides. After deparaffinization and rehydration of the sections' epitope retrieval was performed by adding the Tris-EDTA buffer to unmask calcium ions. Covered tissue was incubated for 10 min in the buffer and then washed twice. To block nonspecific background staining, ultra-vision protein block was applied to the tissue and incubated for 5 min. Primary antibodies of iNOS were applied for 10 min. Cyst sections were washed using phosphate buffered saline four times. After each step, 1-2 drops of 3,3' Diaminobenzidine (DAB) chromagen were added to 1 ml of DAB substrate, mixed by swirling then applied to the tissues and incubated for 10 min.

Data analysis by real time quantitative morphocytometry[20]: The pathological and morphometric analysis were evaluated using the Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd, Cambridge, England). Observation of each slide was with a light microscope at x100 and x200 magnification. Ten fields were randomly selected, for color index measurment. Brownish color indicated the positive expression of iNOS marker. The area of reaction to be measured was automatically covered by a blue mask (binary image). The area of this binary image was automatically calculated by the software.

Statistical analysis: Results were statistically analyzed by the SPSS software, Version 20 (SPSS Inc., Chicago, IL, USA). Numerical data were expressed as means and standard deviations and were analyzed by students *t*-test. Mann Whitney non-parametric t-test was used for non-normally distributed data which were expressed as median and inter quartile range. Correlation between tissue iNOS and viability of protoscoleces were tested by the Pearson correlation test. Statistical significance was considered at $P<0.05$.

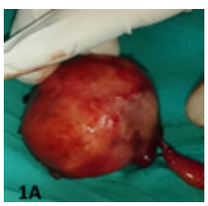
Ethical considerations: This study was conducted according to the professional and institutional guidelines for the management and follow up of patients for post-operative care, written informed consent was provided by each patient. Ethical approval was obtained from the ethical committee of the Faculty of Medicine for Girls, Al-Azhar University.

RESULTS

Gross and microscopic features of hydatid cysts contents: Regarding the color of HCF in G1, three cases had hydatid cysts with whitish and viscous content (Fig. 1B), and the other 3 cases contained turbid HCF. While in G2, the color of the hydatid cyst fluid varied being translucent in six cases, reddish in one case, greenish in two cases and yellowish in three cases.

Structural characteristics of the protoscoleces: Viable protoscoleces had well-constructed contour, intact tegument, invaginated head, well arranged hooks and were full of refractile calcareous corpuscles. Hooks appeared as complete or half circles according

to the position of the protoscoleces during observation (Fig. 2A-C). Non-viable protoscoleces accepted the Eosin stain and showed different features, included tegumental destruction or fragmentation and distortion of hooks (Fig. 2D). Cholesterol crystals were detected during the examination of hydatid fluid and appeared as typical rectangular shaped crystals with notched corners (Fig. 3A-C). These crystals were seen associated with taenoid hooks and non-viable protoscoleces (Fig. 3B).



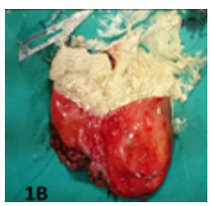
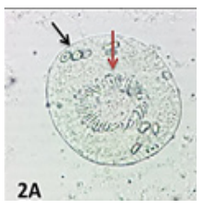
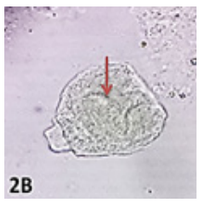
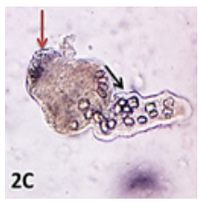


Fig. 1. Photographic picture showing surgically extracted hepatic hydatid cyst. **1A:** Intact hydatid cyst. **1B:** Opened hydatid cyst revealing whitish and viscous appearance of its contents.







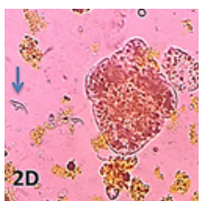
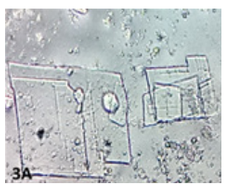
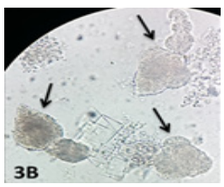


Fig. 2. Light microscopic picture of hydatid cyst fluid showing various morphological features of protoscoleces. **(A)** Protoscolex containing a well-arranged crown of hooks (red arrow) with refractile calcareous corpuscles (black arrow). **(B)** Protoscolex with intact tegument and invaginated rostellum. **(C)** Relaxed (evaginated) protoscolex (red arrow) with refractile calcareous corpuscles (black arrow). **(D)** Non-viable protoscolex accepting Eosin stain and with distorted hooks (blue arrow) (X 400).





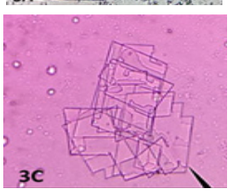


Fig. 3. Light microscopic picture of hydatid cyst fluid showing: **(A)** Cholesterol crystals appearing as typical rectangular-shaped crystals with notched corners. **(B)** Cholesterol crystals associated with non-viable protoscoleces (black arrows). **(C)** Cholesterol crystals stained with Eosin (X 400).

Viability of protoscoleces in the HCF: The mean percentages of viability were 4.5±3.9 in G1, while in G2 it was 35.4±20 with a significant statistical difference (*P*=0.002) (Fig. 4).

Expression levels of iNOS detected by immunohistochemistry: The means of iNOS expression were 2.9±3.3 and 0.13±0.1 for G1 and G2, respectively (Fig. 5). Significantly high iNOS expression levels (*P*<0.001) were visually demonstrated by the densely stained brown areas in tissue sections in cases of G1 as compared to sections from G2 (Figs. 6 and 7). A negative correlation was detected between iNOS expression and viability of hydatid protoscoleces in G1, while no significant correlation between both parameters was found in G2 (Table 1).

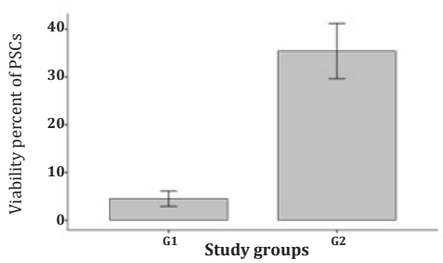


Fig. 4. Viability percent (Mean±SD) of hydatid protoscoleces in both study groups. **G1:** Cysts containing cholesterol crystals; **G2:** Cysts devoid of cholesterol crystals.

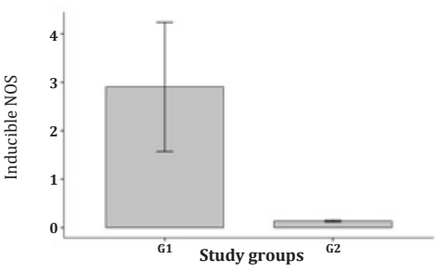


Fig. 5. Inducible NOS (Mean±SD) in the hydatid cyst walls of both study groups. **G1:** Cysts containing cholesterol crystals; **G2:** Cysts devoid of cholesterol crystals.

Table 1. Correlation between iNOS expression in hydatid cyst wall and viability of protoscoleces in both study groups.

Study group	Correlation between iNOS expression and viability of protoscoleces	
	Pearson correlation test	*P* value
G1 (HCF containing cholesterol crystals)	-0.93*	0.00729
G2 (HCF without cholesterol crystals)	0.56	0.0609

*Statistical significance at P<0.05.

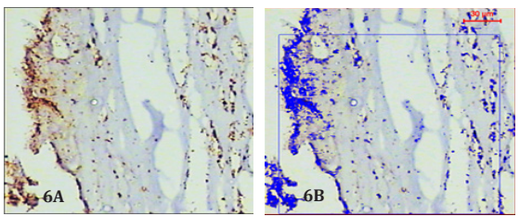


Fig. 6. Light microscopic picture of sections from hydatid cyst walls isolated from G1 patients showing positive immunoexpression of iNOS. **6A** represents the immunohistochemically stained tissue before analysis and **6B** represents the real time analysis, where bluish discoloration reflects a positive enzyme expression.

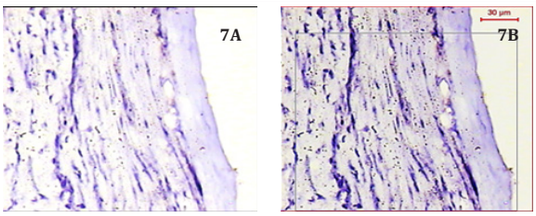


Fig. 7. Light microscopic picture of sections from hydatid cyst walls isolated from G2 patients showing minimal immune-expression of iNOS. **7A** represents the immunohistochemically stained tissue before analysis and **7B** represents the real time analysis where the near absence of bluish discoloration can be noticed.

DISCUSSION

The immune response against hydatidosis is mainly Th2 mediated but has a recognizable Th1 component[21]. Nitric oxide is an important effector of the Th1 response and was found to be associated with diminished vitality of protoscoleces[22]. Patients with a dominant Th1 response, showed better response to chemotherapy than patients with a dominant Th1 response[23].

In the current study, viable protoscoleces had a well-defined smooth tegument, invaginated head with well-arranged hooks, and contained refractile calcareous corpuscles. Hooks appeared as complete circles or half circles according to the position of the protoscoleces during examination. These observations were similar to those made by Casado *et al.*[24]. Calcareous corpuscles in the protoscoleces are unique structures[25]. It was suggested that these corpuscles form major reserves for inorganic and organic compounds. This enables the parasite to establish the optimal chemical intra- and extracellular environment in the hydatid cyst fluid[26].

Caseated or milky hydatid fluids were observed in our G1 cases that contained cholesterol crystals. Sheriff *et al.*[27] reported that thick or caseated hydatid fluids were associated with higher concentrations of cholesterol in cysts originally obtained from camels and cattle. This could be attributed to the cysts undergoing degeneration.

Higher cholesterol levels were observed in HCF collected from human, camels, cattle, and sheep[28]. On the other hand, Hasona *et al.*[14] conducted a biochemical analysis of the hydatid cyst content of hepatic hydatid cysts of sheep in relation to the fertility of the cysts. They noticed that fertile cysts had a low cholesterol, lipid, protein, and glucose content, yet they contained a high mineral content. Fallah *et al.*[15] also reported that infertile hydatid cysts of human and animal origin had lower cholesterol contents than fertile ones. Al-Zubaidy[29] described higher cholesterol content in the germinal layer. Meerkhan[30] confirmed that cholesterol concentrations were higher in germinal than laminated layers of cysts obtained from infected livers and lungs of several animal hosts; the highest concentration was in the germinal layer of hydatid cysts obtained from infected cattle liver. Regarding the source of cholesterol in the hydatid cysts, Irshadullah and Rani[31] stated that *Echinococcus* is incapable of de novo lipid synthesis and thus derives the necessary lipids needed for survival and cyst wall formation from their host. Silva-Alvarez *et al.*[32] reported that AgB secreted by *E. granulosus* larvae carries host lipids necessary for the parasite metabolism, in addition to exerting immunomodulatory functions on the host's immune response. The fact that cholesterol is absorbed into the hydatid cyst was also confirmed by Romano *et al.*[33].

Cholesterol crystals are believed to induce inflammation by the activation of the NLRP3 inflammasome[34]. This activation of the inflammosome pathway can explain the effect of cholesterol on protoscoleces viability which was observed in our study. The mean viability percent was 4.5±3.9 in G1, while in G2 it was 35.4±20 with significant statistical difference. This finding agreed with Thompson and Lymbery[35] and Eslami[36] who reported that there was an inverse relationship between protoscoleces viability and concentration of cholesterol in the HCF. Shahideh *et al.*[37] suggested that while echinococcosis is a rare cause of cholesterol effusion, the occurrence of such effusions is an indication of the chronicity of infection.

Cholesterol and high fat diet were also found to induce the iNOS mRNA expression and increase NO level, which suggests an anti-inflammatory and immune modulatory role for cholesterol[9]. In our study, the local expression of iNOS in the isolated hydatid cyst walls was significantly higher in G1 as compared to G2. A negative correlation between the viability of the hydatid protoscoleces and the expression of iNOS was also observed in G1, but not in G2.

Several previous studies explained the different mechanisms of antimicrobial activity of iNOS[38,39]. In an *in vitro* study conducted by Zeghir-Bouteldja *et al.*[40], reactive nitrogen species were found to have a protoscolecidal effect, in addition to inducing

degeneration of the germinal and laminated layers. The higher expression of iNOS in G1 and the marked affection of viability could indicate that NO has antimicrobial activity against protoscoleces.

In conclusion, the presence of cholesterol crystals in hydatid cyst fluid has not yet been sufficiently explained. The decreased viability of protoscoleces suggests a role for cholesterol in immunomodulation, possibly through the involvement of the NO pathway. Further studies are required to clarify the source of cholesterol in hydatid cysts and the metabolic interactions between parasite and host and their effect on infection outcome. Further studies are recommended to understand the specific mechanism of action involved in the development of inflammatory response (increase expression of iNOS) associated with presence of cholesterol crystal in hydatid cyst.

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Author contribution: Shaheen HA formulated the study design, performed data collection and analysis, conducted methodology, manuscript writing, formulation and submission. Hamed AM contributed to manuscript formulation, editing and revision. Abdeltawab MSA performed data analysis, manuscript writing, formulation, editing and revision.

Conflicts of interest: Authors declare that they have no conflict of interest.

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