Full Length Research Paper

Viability loss and ultrastructural changes on protoscolices of human hydatid cysts induced by retinoic acid

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Received 6 November, 2014; Accepted 22 December, 2014

Surgical removal of intact hydatid cyst is the most effective treatment for hydatid disease. Recurrence of hydatid cyst is mainly due to dissemination of protoscolices (PSCs) rich fluid during the surgical operation. Therefore, preoperative instillation of a scolicidal agent into the cyst is a common practice with adverse side effects of the used drugs. All-trans retinoic acid (atRA) is the physiological mediator of most of the functions of vitamin A, particularly as a cellular differentiation and apoptosis regulating factor. We hypothesized that instillation of atRA could provide an alternative safe scolicidal approach. We tested the scolicidal effects and ultrastructural changes imposed by atRA on human hydatid cyst PSCs in vitro. Freshly isolated hydatid cyst PSCs were subjected to atRA (at 16.7, 1.67, 0.167 µM and 16.7 nM/L). Changes in protoscolices viability (0.1% eosin exclusion) and morphology (scanning and transmission electron microscopy; SEM and TEM) were investigated. Dose-dependent PSCs death within few minutes to 7 days of exposure to atRA was observed. SEM demonstrated ultrastructural damages including rosteller disorganization, loss of hooks and distortion of hooks morphology. TEM revealed loss of the integrity of the internal tissues of PSCs, an increased vacuolization, formation of large lipid droplets in the distal cytoplasm and aberrant, rounded abnormally large sized mitochondria. atRA is a promising alternative to the available synthetic and chemical scolicidal agents. However, in vivo scolicidal activities of atRA and the possible side effects necessitate further studies.

Key words: Hydatid cyst, protoscolices, retinoic acid, ultrastructure, scolicidal activity.

INTRODUCTION

Cystic echinococcosis (CE) is a chronic zoonosis caused by the larval stage of the cosmopolitan parasitic cestode Echinococcus granulosus (Lv et al., 2013). It is a serious disease to human and domestic livestock having as
significantly high fatality rate (Himsworth et al., 2010; Grosso et al., 2012). In all North African countries, including Egypt, CE is highly endemic and is re-emerging as a major public health issue (Sadjjadi, 2006; Elshazly et al., 2009; Dakkak, 2010; Cardona and Carmena, 2013).

Although surgery is still the preferred method for symptomatic cases of CE, there is an increasing risk of intra-operative spillage of protoscolices rich fluid leading to local reoccurrence (10% in post operative cases) or secondary echinococcosis (Kilicoglu et al., 2006; Moro and Schantz, 2009). Intracystic instillation of scolicidal agents is a common practice to prevent such spillage along with percutaneous aspiration (Brunetti et al., 2010). However, no ideal (both effective and safe) scolicidal agent has been deviced yet. The unacceptable side-effects of the scolicidal agents cause limitation to their use (Naguleswaran et al., 2006).

Surgery (radical or conservative) is usually complemented by pre and/or post-surgical chemotherapy. In massive infestation and in inoperable cases, chemotherapy is the sole intervention. Several drugs, notably mebendazole, albendazole, praziquantel, mitomycinic, isoprinosine and other antihelminthic drugs showed promising reduction and induction of ultrastructural damage of the larval cystic mass (Satoh et al., 2005; Taran et al., 2009). However, their adverse side effects included abnormalities in liver function, leucopenia and alopecia, along with teratogenesis (albendazole). It should therefore be avoided during pregnancy and lactation (Kern, 2003; Nunnari et al., 2012; Verma et al., 2013). Moreover, drug resistance and high recurrence rates after interruption of therapy added further complications (Reuter et al., 2004; Sadjjadi et al., 2008). This mandates the development of new therapeuetic agents for such disease (Moazeni et al., 2012).

Retinoids are a class of lipophilic chemicals that include active natural metabolites of vitamin A (retinol), as well as a diverse array of synthetic derivatives. The major physiological retinoid, all-trans retinoic acid (atRA) modulates a wide variety of cellular processes including; cell proliferation, differentiation and apoptosis, cell-matrix interaction, angiogenesis, homeostasis, polyamine metabolism and immune performance through modulation of hormonal/growth factor signal transduction and expression hundreds of genes. It is a promising chemopreventive agent for bladder cancer (Al Tanoury et al., 2013), and the first line therapeutic agent for acute promyelocytic leukemia and is a potent inducer of liver and other tissue regeneration (Degos and Wang, 2001; Gudas, 2012; Hx et al., 2014). Anti-inflammatory (Thielitz et al., 2006; Andrea and Zaenglein, 2008), antifungal and antimicrobial effects of RA has also been observed against several fungi and bacteria (Mikhlin et al., 1983). RA antiparasitic activity was rarely investigated (Hurst and Else, 2012). For instance, RA was correlated with successful clearing of Trichinella. spiralis (Carman et al., 1992) and vitamin A supplementation reduced re-infection of Ascaris lumbricoides in children (Long et al., 2006; Payne et al., 2007). Hall et al. (2011) demonstrate that vitamin A insufficient animals experienced lack of Th1 responses to Toxoplasma gondii infection leading to increased parasite burdens.

Hypothesizing that atRA could prove scolicidal, for the first time, the present work planned to investigate changes in viability and ultrastructure of hydatid cyst PSCs treated with different concentrations of atRA in vitro as an initial screening of the anti-parasitic potential of retinoids against hydatid cysts.

MATERIALS AND METHODS

Parasitological procedures

For this in vitro study, intact hydatid cysts were removed aseptically from seven in-patients with hepatic hydatidosis underwent pericystectomy following laparotomy in the General Surgery Department, Faculty of Medicine, Assiut University Hospitals during May, 2012 to April, 2013. The intact cysts were immediately placed in an ice box and transported within 10 min to the Parasitology Laboratory, Medical Parasitology Department, Faculty of Medicine, Assiut University, Assiut, Egypt.

Collection of hydatid fluid

Cysts were washed three times in sterile phosphate-buffered saline (PBS; pH 7.2) and cyst fertility was determined by the presence of free PSCs in hydatid fluid by wet mount drop. Sterile cysts were discarded.

PSCs preparation

Fertile cyst surfaces were disinfected with 70% ethyl alcohol to guard against surface contaminations and hydatid fluid was evacuated completely and transferred into 15 ml falcon tubes by aseptic techniques. The fluid was left to precipitate at room temperature for 15 min to obtain hydatid sand without centrifugation. PSCs with enough volume were obtained and the supernatant was removed into another 15 ml tubes. The viability of PSCs was confirmed prior to the experiments by 0.1% eosin staining exclusion test and flame cells motility according to Esfandiari et al. (2010). Only PSCs with an initial viability of at least 95% or more were used.

Protoscolices preservation fluid

Preservative solution consisted of hydatid cyst fluid and normal physiological saline (0.85% NaCl) 4:1 mixture, v/v, without antibiotic or antifungal drugs.

All-trans retinoic acid solution

atRA was dissolved in endotoxin-free dimethysulfoxide (DMSO) for biological uses (Sigma-Aldrich Chemical Co., Saint Louis, MA,
USA) at 100, 10, 1 µM, and 100 nM/L stock concentrations under dim dark yellow light (the condition under which all experiments were conducted) and maintained in dark brown vials.

**Treatment**

The effect of different concentrations of atRA on the viability of PSCs was compared to albendazole sulfoxide (ABZSO) 800 µg/ml as a positive standard anti-hydatidosis control drug (Polat et al., 2009). Treatment of PSCs was initiated within 15 min of isolation. Five ml of the preservative solution was added to 1 ml of viable PSCs (approximately 2,000 PSCs) in separate tubes and 1.0 ml of each stock concentrations of atRA (100, 10, 1.0 µM and 100 nM/L) was added into three tubes for each dose to give rise to final atRA concentrations of 16.7, 1.67, 0.167 µM and 16.7 nM/L in the experiment mix. All tubes were mixed gently and incubated at 30°C without changes of preservative solution. Percentage of viable PSCs was determined in triplicate samples withdrawn from each tube after 5, 10, 15, 30, 60 and 120 min and every 6 h thereafter, until all of the PSCs were dead. ABZSO tubes were processed the same. One ml volume of each of the sterile preservative solution and the DMSO vehicle were used as negative controls in their respective tubes.

**Viability assay**

The viability of PSCs was assessed by microscopic observation of characteristic motility (Figure 1C). The viability of PSCs was determined using eosin staining exclusion method; where dead PSCs absorbed eosin and stained red and ordered hooks. By time, most of these PSCs attained evaginated form (scolices) with clearly visible suckers. In preservative solution and dimethyl sulfoxide (DMSO) vehicle control, the viability of PSCs decreased from more than 95% at the beginning to total death in an average of 13 days incubation at 30°C (Figure 1D). Microscopically, viable PSCs exhibited distinct movements, membrane integrity and ordered hooks. By time, most of these PSCs attained evaginated form (scolices) with clearly visible suckers. In preservative solution and DMSO vehicle controls non-significantly different.

**Results**

Intact hepatic hydatid cysts were dissected by pericystectomy following laparotomy (Figure 1A for example). Cyst fertility was examined by wet mount drop detection of free PSCs in cystic fluid (Figure 1B). PSCs viability was determined using eosin staining exclusion test; where dead PSCs absorbed eosin and stained red and alive PSCs remained colorless and showed characteristic motility (Figure 1C). The viability of PSCs was also confirmed by flame cell activity and muscular movements.

**Changes in the viability of hydatid cyst PSCs induced by atRA**

In preservative solution and dimethyl sulfoxide (DMSO) vehicle (negative and vehicle controls, respectively), PSCs viability decreased from more than 95% at the beginning to total death in an average of 13 days incubation at 30°C (Figure 1D). Microscopically, viable PSCs exhibited distinct movements, membrane integrity and ordered hooks. By time, most of these PSCs attained evaginated form (scolices) with clearly visible suckers. In preservative solution and DMSO vehicle controls, PSCs viability in comparison with ABZSO (800 µg/mL) caused a significant reduction in the viability of PSCs at day 6 incubation-time point (p < 0.05). Although it was significantly scolicidal, atRA at 16.7 µM/L was clearly less efficient than the higher concentrations. It increased the percentage of dead PSCs with time where a small fraction of PSCs could be seen viable at day 5 and total death occurred at 7 days time-point of incubation with 0.167 µM/L atRA (p < 0.05). The viability of PSCs treated with 16.7 nM/L atRA did not show any changes till the 13th day time-point similar to the performance of the preservative and vehicle controls without significant difference amongst them. ABZSO (800 µg/mL) caused a significant reduction in the viability of PSCs at day 6 incubation-time point, where 70% of the incubated PSCs died. All of the remaining PSCs died by the end of 10th day post treatment (p < 0.05). The effect of different concentration of atRA on PSCs of hydatid cyst viability in comparison with preservative solution, DMSO vehicle and ABZSO is illustrated in Figure 2.

**Statistical analysis**

Statistical calculations were carried out by one way analysis of variance (ANOVA), using the software statistical package for social sciences (SPSS 16) for windows. P-values of < 0.05 were considered significant.
Ultrastructural changes in hydatid cyst PSCs induced by atRA

Morphological changes were observed in tegument and parenchyma of PSCs treated with 1.67 and 0.167 μM/L atRA at the end of the experiments. These changes were more evident in evaginated than invaginated PSCs. Similar changes were observed in PSCs treated with ABZSO. No similar tegumental or parenchymal ultrastructural alterations were observed in control PSCs. SEM showed that the primary site of damage was the rostellum of the scolices. Compared to the normal arrangement of hooks on the rostellum (Figure 3A), damages varied from disorganization of rostellar hooks (Figure 3B) to total loss of rostellar hooks (Figure 3C). Distortion of hooks morphology was also observed especially in the guard and handle regions of the hook (Figure 4A). The tegumental scolex (especially the sucker regions) appeared to be altered or contracted (Figure 4B). Several blebs were detectable in the surface of PSCs (Figure 4C). There was also aggregation and adherence of the PSCs together (Figure 4D).

Transition electron microscope (TEM) showed that the internal tissues of PSCs were severely affected in response to treatment with atRA compared to the normal tissues (Figure 5A). Loss of its integrity, an increased vacuolization and significant reductions in the relative numbers and lengths of the microtriches of the distal portion of the tegument were observed (Figure 5B). There were formation of large lipid droplets in the distal cytoplasm and many undifferentiated cells, the cytoplasm of which contained large vacuoles filled with electron-dense bodies, granular particles, and small vesicles (Figure 5C). These vacuoles occupied a large portion of the cytoplasm of these cells. The treatment with atRA also resulted in the formation of aberrant, rounded mitochondria which had abnormally increased in size, and the cellular disintegration process had advanced, rendering the tissue largely necrotic (Figure 5D).

DISCUSSION

Chemotherapy, surgery and puncture with aspiration are the three traditional treatments for hepatic hydatid cysts. Treatment option is still subject of controversy (Frider and
Figure 2. Loss of viability of human hydatid protoscolices during *in vitro* atRA treatment. Viability was determined through eosin staining exclusion test. Note the dose-dependent effect of atRA in comparison with preservative solution, DMSO vehicle and ABZSO.

Figure 3. SEM of scolices in presence or absence of retinoic acid (A) the normal arrangement of hooks on the rostellum (arrow), (B) disorganization of rostellar hooks (x), (C) total loss of hooks from the rostellum. Arrow-heads pointed to the hooks and arrows to the rostellum.
Larrieu, 2010). For inoperable patients and those refusing surgery, chemotherapy and puncture are recommended as alternative medication (Lv et al., 2013). Inactivation of the scolex with a protoscolicidal agent before surgery is intensely recommended because the spillage of the cyst contents is a major cause of return (Zhang et al., 2003). A number of drugs are being used and various degrees of success have been claimed. However, the metabolites of certain drugs including benzimidazole mebendazole, albendazole and albendazole sulfoxide are potentially toxic in some subjects (Polat et al., 2005; Mandal and Mandal, 2012).

Manterola et al. (2006) reported that the prevalence of fertile human hydatid cyst was low and fertility is associated with the type of cyst and the presence of biliary communications. The viability assessment in CE is important for in vitro and in vivo studies and also drug experiments. Several vital stains like neutral red, methylene blue, eosin, giemsa, Ziehl-Neelsen and trypan blue have been used to assess viability of protoscolices in fertile cysts (Yildirim et al., 2007). In the current study, cyst fertility was determined by the presence of free PSCs in cystic fluid by wet mount drop. This was important to exclude sterile hydatid cyst which is common in human. In the present study, 0.1% aqueous eosin stain exclusion was used for initial PSCs viability assessment and its time- and treatment-induced changes in vitro.

Hydatid PSCs remain viable for long periods in different media at different temperatures. In the absence of nutritive factors, the PSCs of Echinococcus granulosus do not survive in vitro for more than 4 day at 37°C. The survival time depends on temperature, pH and ionic concentration (Al-Sudani, 2007). In the present study hydatid PSCs remain viable for 13 days after being incubated in the preservative solution (HCF and NS; 4:1 v/v) that provided PSCs with the minimal requirement to stay alive for such period of time at 30 °C. Moreover, evagination of some PSCs may indicate further growth in such conditions. Similar PSCs morphological changes in culture media were discussed to indicate morphological
Figure 5. TEM of scolices in presence or absence of retinoic acid (A) Normal internal tissues of PSCs; G pointed to germinal layer and L to laminated layer (×7200), (B) Significant reductions in the relative numbers and lengths of the microtriches of the distal portion of the tegument and increased vacuolization (v) (×14000), (C) Formation of large lipid droplets (l) in the distal cytoplasm, many undifferentiated cells (arrows) (×19000), (D) Formation of aberrant, rounded, abnormally increased in size mitochondria (arrows) and advanced cellular disintegration (×29000). Bars in A = 3.5 mm, B = 2.9 mm, C = 5.8 mm, D = 1.6 mm.

evolution, whereas, presence of free hooks in culture medium indicated their detachment and destruction of the tegument (Al-Sudani, 2007).

The present study demonstrated the dose- and time-dependent protoscolicidal effect of atRA. The biologically apoptotic high atRA concentration (16.7 µM/L) caused such effects within standardized very short time of 6 min, whereas, the retinoid receptor saturating concentration of atRA (1.67 µM/L) induced such protoscolicidal effect within five days. The lowest concentrations atRA used (0.167 and 16.7 nM/L) induced no effect on PSCs compared to negative and vehicle controls. This was confirmed by morphological and ultrastructural deformities induced by the two effective atRA doses at different incubation periods. Such atRA-induced morphological and ultrastructural changes that included contraction in soma region, formation of blebs on the tegument, rosteller disorganization, loss of hooks, and destruction of microtriches were consistent with that observed with several established protoscolicidal compounds including praziquantel (Urrea-Paris et al., 1999, 2000), benzimidazole (Pe´rez-Serrano et al., 2001), albendazole (Ceballos et al., 2011) and ivermectin (Casado et al., 1989; Elissondo et al., 2009).

Rosteller disorganization and destruction of microtriches might be major reason for loss of viability of PSCs as they are the means for nutrient absorption, physiological homeostasis and defense (Satoh et al., 2005; Afifi and Harba, 2012). Similarly, the formation of numerous blebs on the tegument which became detached, leaving debris was described for the effects of pure albendazole and its sulphoxide combination therapy on E. granulosus. These blebs could had enlarged with time and burst out leading to the disturbance in osmoregulatory system of PSCs (Lv et al., 2013).

Our TEM findings of increasing vacuolization, small
lamellated bodies, and presence of lipid droplets in atRA treated PSCs reflect general tissue stress for subsequent apoptosis/necrosis. These vacuolations might result in major leakage of the cytoplasm contents (Kim et al., 2011). The presence of lipid droplets in the cytoplasm of tegumentary cells of the germinal layers indicates metabolic disruption of the cyst (Verma et al., 2013). Very similar tissue degeneration was observed, when treated with netobimin (García-Llamazares et al., 2002) and isoprinosine (Sarciron et al., 1993). Disruption of the muscle and tegumental layers, which is the fundamental interface of the helminth body with its environment, and responsible for selective absorption of nutrients, secretory activities and sensory perception renders it specifically susceptible to anthelmintic agents (Roy et al., 2007; Shalaby et al., 2012).


Conclusion

The in vitro scolicidal activity of all-trans retinoic acid was described for the first time. Retinoic acid that is a major physiological player with low drug toxicity in a proof-of-the-concept study showed promising scolicidal activity - indicated by massive reduction in PSCs viability and unfavorable ultrastructural deformations. Further in vitro studies are required to dissect the molecular machinery of such atRA actions along with in vivo experimentation using Echinococcus infection models.

Conflict of interest

The authors declare that there is no conflict of interests.

REFERENCES
