Attenuation of human hydatid cyst protoscolices viability by 1-Hydroxyphenazin (1-HP) pigment: In vitro and in vivo study

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Summary:

Background: Hydatidosis is a widespread chronic zoonotic disease caused by helminthic larval stage of tapeworm Echinococcus granulosus. It has a serious medical and public health problem. Recently many substances are used to activate and modulate the immune system of the host in order to control the cyst growth and development.

Materials and Methods: Protoscolices were isolated from human hydatid cysts and treated with four purified concentrations (25, 50, 75, 100μm/ml) of 1-HP plus hydatid cyst fluid as a positive control group for 4, 24, 48 and 72 hrs period of exposure. Then After 72 hours, the protoscolices which were treated with different concentration of 1-HP were inoculated intraperitonially of male white balb / c mice to see the infectivity of the protoscolices in vivo.

Results: The results showed that the higher purified concentration 75,100 μmole/ml of this pigment had a highly significant toxic effect on the viability of protoscolies, especially after 72 hrs.This toxicity of the pigment decreases the number of the protoscolices to develop and grow in vivo.

Conclusion: It has been found that 1-hydroxyphenazine is a dose dependant toxic pigment causing attenuation of the protoscolices viability in vitro which decreases the number of protoscolices to develop and grow in vivo.

Keywords: 1-HP. Protoscolex.Viability. Attenuation. Human. Cyst.Hydatid.

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Introduction:

Hydatidosis (or cystic echinococcosis) is a parasitic zoonotic disease caused by metacestode tapeworm Echinicoccus granulosus**.** (1).The definitive host is the dog, in which adult tapeworms attach to the intestinal epithelium and undergo sexual reproduction, leading to the development of eggs. These eggs are shed into the environment with the faeces. The eggs contain an oncosphere, which upon ingestion by a suitable intermediate host (human, cattle...etc) and subsequent passage through stomach and intestine become activated, penetrate the mucosa, enter blood and lymphatic vessels and are disseminated in the body (2). This parasite usually settles and forms cysts in liver and lung with a frequency of 60% and 20-30% respectively. The involvement of other organs may occur in case of dissemination of scolices by the blood (3).After an undefined incubation period, E. granulosus metacestodes are formed. These are single, fluid-filled cysts, which are surrounded by a thick, host-derived layer of connective tissue (adventitia). The parasite tissue is delineated by the laminated layer, an acellular, carbohydrate-rich outer surface structure of considerable thickness, which separates the living parasite tissue at the inside from the outer host environment. Most human patients harbour a solitary cyst within a single organ. In 70% of patients, infection occurs in the liver, the lung being affected in 20% of patients. Other sites of infection include kidney, spleen, muscle, skin, abdominal and pelvic cavity, mediastinum, heart, brain, spinal cord and others. (4). Within fertile metacestodes, brood capsules are formed out of distinct cells originating from the germinal layer, which themselves contain protoscolices. Infertile metacestodes only contain the germinal layer. Often, the metacestode contains one or more daughter cysts, which give rise to the septate appearance in diagnostic imaging (5).The cysts slowly expand over several years, often without being noticed. Spread of infection into other sites, such as the peritoneal cavity, has been described, and this can be caused by cyst rupture and spillages of protoscolices.These have the potential to develop into metacestodes in the intermediate host and into adult parasites when ingested by the final host (2). Pseudomonas aeruginoa is an opportunistic Gram-negative rod pathogen that has a long and infamous association with many diseases like burn wounds infections (6), cystic fibrosis (7), Urinary tract infection (8).This pathogen secretes many virulence factors like elastase, protease and exotoxins(6). Among these exotoxins is the low molecular –weight phenazine pigment pyocyanin and its derivative 1-hydroxyphenazine (9). These phenazines pigments have been suggested to contribute deleterious effects on eukaryotic cells including epithelial cells, endothelial cells, and leukocytes (10).

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Materials and methods:

Source of protoscolices:

E. granulosus intact hydatid cysts containing protoscolices were removed under aseptic conditions from infected patients attending many hospitals in Baghdad city –IRAQ. Briefly, in the laboratory, the

hydatid cysts were cut open and vesicle fluid (containing protoscolices) was separated from the metacestode tissue according to method adopted by (11). The Viability of protoscolices was assessed by flame cells and 0.1% eosin staining exclusion test respectively according to (12).

Design of experiment.

Part 1:

In vitro study

Four pyocyanine concentrations 25, 50, 75 and 100µmole/ml plus hydatid cyst fluid, which sterilized by 0.2 µM Millipore filter, as control group are use for protoscolices treatment during the experiment with exposure time (4, 24, 48, 72) hrs .The number was adjusted to 2000 protoscolices /1ml of each concentration and for each period as follow:

A- (4, 24, 48, 72) hrs after treated with hydatid cyst fluid.

B- (4, 24, 48 72) hrs after treated with 25 µmole/ml of 1-HP.

C- (4, 24, 48, 72) hrs after treated with 50 µmole/ml of 1-HP.

D- (4, 24, 48 72) hrs after treated with 75 µmole/ml of 1-HP.

E- (4, 24, 48, 72) hrs after treated with 100 µmole/ml of 1-HP.

All these groups are incubated in 37 °c. After those periods the viability was assessed by taking 0.1 ml of the suspension mixed well with 1% eosin. (Viable one; green, while nonviable appear with red color). One hundred random protoscolices viabilities under compound microscope were examined.

Part 2:

In vivo study

Five groups of white male balb/c mice, each group containing 10-12mouse, aged 12-13 weeks and weighted 20-24 gm ,were infected intraperitonially(I.P.) with four 1-HP concentrations treated protoscolices and the fifth groups was infected with hydatide cyst fluid containing protoscolices after 72 hrs exposure. Finally, 22 weeks later, all mice groups were etherized and dissected under a dissecting microscope. The infectivity of the protoscolices was investigated by recording cyst numbers and their diameters were determined by vernier micrometer.

Statistical Analysis: The data were analyzed by one-way ANOVA. Using Pentium-4 computer through the SPSS program (version-10) and Excel application (13). The results are represented as the means ± standard deviation (S.D.). Results were considered significant at P<0.05, highly significant at P<0.01, non significant at P>0.05·

Results:

The main result of this study is that 1-HP pigment, generated by Pseudomonas aeruginosa, was able to attenuate protoscolices viability especially at higher concentration 70, 100 µmoles in vitro. It can be seen from Table 1 that the protoscolices viability affected by different concentrations of the pigments, but this effect was not significant (P > 0.05) at lower concentrations during first 4 hours after exposure to the pigment (89.33±4.521, 87.33±3.512) for both concentrations 25, 50 µmoles respectively in comparison with hydatid cyst fluid positive control (90.33± 5.242) .Table (1). Nearly, lower pigment concentrations (25, 50 µmole/ml) have no significant effect (P>0.05) on the protoscolices viability after 24 hours exposure which were (88.66±2.221, 80.33±6.412) respectively .This effect was highly significant P<0.01 at higher concentrations 75,100 µmole/ml which were (44.33±6.412, 33.66±2.571) after 48 hrs and (46.00 ±2.042, 30.66 ±2.432) after 72 hours respectively in comparison with hydatid cyst fluid treatment (87, 66±2.321, 82.00±5.512). Table (1). The attenuated protoscolices by this toxic phenazine pigment especially at higher concentration may showed as evidence of the highly significant decrease in numbers and sizes (P <0.01) of cysts recovered from mice injected with 75 and 100 µmoles after 72 in comparison with cysts recovered from hydatid cyst fluid positive control group which allow protoscolices significantly to develop and grow. Table (2).

Table 1- Effect of purified 1-HP pigment on protoscolices viability in vitro.

Pigment concentrations µmole/ml	Viability :Mean ±S.D.			
	After 4 hrs	After 24 hrs	After 48 hrs	After 72 hrs
Hydatid cyst fluid	90.33± 5.242	89.33± 3.666	87,66± 2.321	82.00± 5.512
25	^89.33 ±4.521	88.66± 2.221^	^79.00 ±1.321	79.33± 3.211^
50	^87.33 ±3.512	80.33± 6.412^	77.33± 2.211^	#68.00 ±7.331
75	^86.33 ±2.231	#74.00 ±2.121	44.33± 6.412*	*46.00 ±2.042
100	*70.66 ±1.222	*70.66 ±3.123	1*33.6 6±2.57	30.66± 2.432*

Data are shown in mean ±standard deviation
*** HS= P<0.01 # S = P< 0.05 ^ NS = P>0.05**

Table 2- Effect of different purified concentrations of 1-HP pigment on protoscolices infectivity in vivo following 72 hrs treatment.

Pigment concentrations µmole/ml	Cysts numbers Mean ± S.D.	Cysts diameters(mm) Mean ± S.D.
Hydatid cyst fluid(+control)	9.66 ± 0.614	2.046 ± 0.921
25	^9.33 ± 1.666	^1.135 ± 0.214
50	^9.00 ± 1.33	#1.222 ±0.432
75	*2.33 ± 0.443	*0.624 ±1.432
100	*0.66 ± 0.129	* 0.423 ± 1.321

Data are shown in mean ±standard deviation
*** HS= P<0.01 # S = P< 0.05 ^ NS = P>0.05**

Discussion:

In this investigation, we studied the efficiency of different 1-hydroxyphenazine pigment concentrations against protoscolices viability of Echinococcus granulosus for different period's exposures. There were no studies have been found to investigate the effect of pseudomonas aeruginosa exotoxin 1-hydroxyphenazine pigment on the viability of protoscolices neither in vitro nor in vivo.

Most Pseudomonas aeruginosa strains secret pyocyanine and its derivate 1-hydroxyphenazine (1-HP), which are both among the cytotoxic secretory factors secreted by this bacterium that felt to contribute to organism virulence and pathogensis. This pathogenicity due to direct damage to host tissue or contribute to host tissue damage by impacting the host immune response (7), but this mechanism remains uncertain (14, 15, and 16). Numerous questions regarding the mechanism of phenazine action remain unanswered (9). It has been found that phenazine products of Pseudomonas aeruginosa can induce death in Caenorhabditis elegan and fungi (17, 18). Analysis of C. elegans mutants with altered responses to oxidative stress suggests that phenazines exert their toxic effects on C. elegans through the generation of reactive oxygen species (ROS) (17). It has been found that the phenazine pigment administrated to airway epithelial cells in vitro, it will move to sub-cellular site in which mitochondrial reside and causes decrease ATP levels, both mitochondrial and cytoplasmic aconitase activity and mitochondrial membrane potential. The ability of the phenazine pigments to generate reactive oxygen species (ROS) linked to its cytotoxicity for both eukaryotic and prokaryotic cell (19). For that reason, the attenuated protoscolices stimulate and activate the macrophage and the complement system which both play very important role in control the protoscolices by phagocytic activity (20, 21). We found that the HCF was the better preservative solution than other concentrations of the pigment because this fluid has nutritional factors and a numbers of minerals, compounds such as triglycerides, proteins, fatty acids, carbohydrates and others (22 and 23),this preservation allows the injected protoscolices to well develop. Finally, it was well known that phenazine pigment is a toxic dose dependant especially at higher concentrations; it has both inhibitory and stimulatory effects on human specific and non-specific cells (24).

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