Suppressive effect of purified 1-hydroxyphenazine pigment on phagocytosis against experimental hydatidosis

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Abstract

The effect of 1-hydroxyphenazine pigment which was isolated and purified from *Pseudomonas aeruginosa* on phagocytosis process of peritoneal macrophages inside the body of white BALB/C mice against experimental secondary hydatidosis and the infectivity of protoscoleces was studied.

In comparison with control mice groups which injection with Phosphate Buffered Saline (P.B.S.) the results showed that the higher purified concentrations (75,100) µmole/ml of this pigment had suppressive effect on this non-specific immune response cells and this effect was highly significant($P<0.001$) on (6) weeks post challenge dose with protoscoleces infection against this pigment, and this effect reflects the protoscoleces infectivity which increased due to suppression of non-specific phagocytic activity of macrophage, while the mitogen phytohemagglutin (PHA) showed a significant stimulation of this non-specific cellular response which leads to decrement in protoscoleces infectivity in comparison with higher pigment concentrations .

KEYWORDS. 1-hydroxyPhenazine, phagocytosis, Protoscolex, Candida.

ألتأثير ألكابت لصبغة 1- هيدروكسي فينازين النقيه في عملية ألبلعمه ضد الخمج التجريبي بالاكياس العداريه

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الخلاصة

تمت دراسة تأثير الصباغ 1-هيدروكسي فينازين ألمعزوله والنقيـه مـن *Pseudomonas aeruginosa* في عملية ألبلعمه لخلايا ألبلعم ألكبير الصفاقيه داخل جسم الفئران البيض BALB/C ضد الخمج التجريبـي بالاكياس العدارية ومدى تأثيرها على خمجية الروؤيسات الاولية (infectivity of protoscoleces) .

اظهرت النتائج مقارنة مع مجموعة السيطرة بان التراكيز العاليه لهذه الصباغ (100,70) مايكروموول/مل لها تأثيرا كابتا على هذه ألعمليه وان هذا التأثير قد ازداد بصوره معنوية عاليه ($P<0.001$) بعد مـرور سـتة اسابيع من ألخمج التجريبي بجرعة التحدي بالروؤيسات البدائية

وهذا التأثير يعكس مدى خمجية هذه الروؤيسات والتي ازدادت لكبت خلايا ألبلعم ألكبيرMacrophages ، فيما أظهر ألمشطر أللانوعي (PHA) تحفيزآ في ألأستجابه ألمناعيه ألخلويه غير ألمتخصصه والتي أدت الى نقصان في خمجية ألروؤيسات ألبدائيه مقارنة مع تراكيز الصباغ كافه.

كلمات مفتاحيه-1.هيدروكسي فينازين ، ألبلعمه ، روؤيس بدائي ، كاندیدا.

Introduction:

Cystic Echinococosis(CE) is a widespread chronic endemic helminthic disease caused by larval stage hydatid cyst of the tapeworm *Echinococcus granulosus* which affects human and a wide range of livestock species (McManus *et a*l.,.2003;Zhang and McManus,2006).

The young cyst may die and calcify, but it often continues to grow inexorably, eventually seriously compromising the function of the effecting organ in which it situated (Greenwood , 2002).

This parasite secretes some antigens that are thought to be responsible for immunomodulatory activities promoting its survival within a mammalian host (Mamuti *et al*., 2006) and they have extraordinary abilities to control host immune rejection mechanisms (Heath,1995).

Recent studies were directed to modulate this relationship between the parasites and the immune system arms (specific and non specific) by using chemical or natural substances that may modulates the immune response to control the infection (AL-Taei, 1996).

1-1- *Pseudomonas aeruginosa.*

It is a prevalent opportunistic pathogen in human, causing many adverse effects, like chronic lung infection associated with cystic fibrosis patients (Lyczak et al.,2002), burns victims(Singh *et al.*,2000;Singh *et al*,2002), or bronchiectasis (Wilson *et al.*,1996).

Many virulence factors produced by this pathogen effect the immune system during infection causing both acute and chronic diseases, these factors are either enzymes like proteases which may cleave antibody (Wilson *et al.*,1996), and maybe endotoxins like lipo- polysaccharide (Usher *et al*.,2002), or sometimes poison pigments like a redox cycling phenazine pyocyanine(Gallagher *et al.*,2002) and its derivative 1-hydroxyphenazine(Lauredo *et al.*,1998).

Many of these factors have biological effects on host cells that may contribute to pathogenesis of this pathogen or some of them effects the specific immune response cells T or B lymphocytes (Ulmer *et al.*,1990), while others may effects non-specific immune response like complement (Kindt *et al.*,2007) and macrophages(Buret and Cripps,1993).

1-2-Phagocytosis.

It is non-specific process by which the macrophages ingest particulated materials, destroyed it, and eliminated it through plasma membrane (Hyde,2000) because the macrophages are considered as one of the first line of non-specific immune defenses which play an important role in the elimination of protozoa and worms (Stewart,2002).

The aim of this study is to see the effect of this pathogen pigment (1-hydroxyphenazine) on one of the non-specific cell-mediated reaction against

experimental hyadatidosis *in vivo* and that may affect hydatid cyst protoscoleces infectivity.

Materials and Methods

Six groups of white male BABL/C mice (each group with 12-13 mice; age 5-6 weeks; weight 22-27gm) were used for experimental infection.

All hydatid cysts were collected from resident's patients in some of Baghdad hospitals
In Iraq.

Protoscoleces were isolated from cysts in very sterile conditions according to (Smyth,1985) method, and their numbers were adjusted to 2000 protoscoleces / 1ml of sterile Phosphate Buffer Saline (P.B.S) with (pH = 7.2) and their viabilities were tested according to AL_Qaoud and Abdel-Hafes(2008) method using eosin stain(**Viability must be more than 98%).**

2-1- **Design of experiments:**

The inbred male BALB/C mice groups were prepared to be injected as follow:

1- Four groups were inoculated intraperitoneally (I.P) with four purified(Risan,1998) concentrations of 1-hydroxyphenazine(25, 50 , 75 , 100) μmole/1ml, and after seven days they were given the same concentrations as a booster dose, and after same period they were infected (I.P) with 2000 protoscoleces/1mL (P.B.S) as a challenge dose .
2- The fifth group was inoculated (I.P) with 1mL of sterile (P.B.S) and used as a control group.
3- The sixth group was inoculated (I.P) with (10 mg/ml) non-specific mitogen phytohemaagluttin (PHA) and challenge dose with same number of protoscoleces.
4- After (2, 4, and 6) weeks peritoneal macrophages cells were isolated according to Owaki *et al.* (2000).
5- These macrophages were cultured with heat killing *Candida albicans* according to AL_Kaisy *et al*.(2008) method, and two hundreds macrophage cells were counted and the numbers of active yeast phagocytic cells(**MORE THAN 5 YEAST CELLS / ONE MACROPHAGE)** was calculated using haemocytometer,and phagocytic index was measured according to the following equation(AL_Jorany *et al.*,1992).

$$\text{Phagocytic Index (P.I) \%} = \frac{\text{NO of yeast phagocytic cells}}{\text{Total phagocytic cells}} \times 100$$

6-After 25 weeks, infectivity of protoscoleces was investigated by killing mice and recording cysts number and their diameters using Vernier micrometer.

Statistical Analysis

The usual statistical methods were done to analyze data and assess the results using SPSS. Ver. 15. Under windows XP as follow:

1. Mean
2. Standard Deviation (S.D.) and Standard Error (S.E).
3. Analysis Of Variance (ANOVA).
4. Least Significance Difference (LSD). The results were considered high significant if $P<0.001$, significant if $P<0.05$, and non- significant if $P>0.05$.

Results and discussion

Despite inducing a strong cellular and humoral immune response, *Echinococcus granulosus* is a highly successful parasite that develops, progresses and ultimately causes chronic disease(Rigano *et al* .,2007).

After two weeks of mice groups exposure to protoscoleces as a challenge dose, 1-hydroxyphenazine caused decrement in the phagocytosis of peritoneal macrophages(Table-1), this decrement was highly significant($P<0.001$) specially among mice groups which exposed to high concentrations of pigment which were (51.75±2.065 ,37.5±4.655) for both concentrations(75,100)µmole/ml respectively, and this decrement in macrophages phagocytosis is continue highly significant $P<0.001$ after 4 weeks from mice groups exposure to challenge dose of protoscoleces which were (47.25±3.096 , 39.5 ±6.807) for both higher concentrations (75,100)µmole/ml respectively in comparison with control group (84±3.367) and PHA (87.5±1.291) (Table-2) .

Pigment concentrations (75,100)µmole/ml showed high significant decrement $P<0.001$ in macrophages phagocytosis after 6 weeks of the challenge dose which were (40.5±1.732 , 27.75± 1,893) respectively in comparison with control group(83.75±2.63) and the PHA (86±3.162)(Table-3).

This decrement affected the infectivity of protoscoleces which showed significant increment in cyst growth and development (numbers and diameters) in comparison with PHA which decreased significantly the infectivity of protoscoleces (Table - 4).

From all above results it can be conclude that the higher concentrations of 1-hydroxyphenazine have toxic and suppressive effects on the phagocytic index of macrophages which is considered very important non-specific (innate) cells against protoscoleces infection, while PHA causes enhancement of macrophages phagocytic index which causes decrement of the protoscoleces infectivity.

On contrary of these results, pigment concentrations below 25µmole **(private data under laboratory research and confirmative tests? not shown**) may not effect macrophages and may be cause modulation of these cells.

This reduction in phagoctic index is increased due to higher concentrations of 1-hydroxyphenazine pigment which may causes suppressive effects on respiratory chain in mitochondria and electron transport system within cells (Stewart-Tull and Armstrong,1972), or may have directly effect on cell membranes and suppressive the metabolites transport (Eagon *et al.*,1979), Muller and Sorrell (1995) said that this pigment may causes inhibition of cyclooxygenase response from human platelets and inhibit leukotriene production by human neutrophils .

Our results confirmed the results which conducted by Buret and Cripps(1993) ; Risan (1998)who they said that the exoproducts of this pathogen may alter the immune effectors cells function and reduced macrophages activity.

However, the toxic effect of 1-hydroxyphenazine on the macrophages phagocytosis specially at higher concentration lead to increasing the infectivity of protoscoleces which grows and develops inside the body of the host because the macrophages are considered as one of the first line of non-specific immune defenses which play an important role in the elimination of protozoa and worms (Stewart,2002).

Table 1-Effect of purified 1- hydroxyphenazine pigment on phagocytosis *in vivo* after (2 weeks) from protoscoleces infection.

Pigment concentrations μmole/ml	Phagocytic index (P.I)			
	Mean	±	S.D	#
*P.B.S (control)	87.25	±	3.202	ae
25	84.25	±	3.096	ab
50	80.25	±	1.708	b
75	51.75	±	2.065	c
100	37.5	±	4.655	d
**PHA	88.5	±	1.291	e

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*One way ANOVA of P.B.S f-test = 87.25 p< 0.001 High significant.
**One way ANOVA of PHA f-test= 455.65 P<0.001 High significant.
#Different letters are indicated significant differences between each two groups

Table 2-Effect of purified 1- hydroxyphenazin on phagocytosis in *vivo*_after (4 weeks) from protoscoleces infection.

Pigment concentrations μmole/ml	Phagocytic index (P.I)			
	Mean	±	S.D	#
*P.B.S (control)	84.00	±	3.367	a
25	85.25	±	0.957	a
50	83.00	±	2.449	a
75	47.25	±	3.096	b
100	39.5	±	6.807	bc
**PHA	87.5	±	1.291	e

*One way ANOVA of P.B.S f-test = 138.67 p< 0.001 High significant.
**One way ANOVA of PHA f-test = 163.58 P<0.001 High significant.
#Different letters are indicated significant differences between each two group.

Table 3-Effect of purified 1- hydroxyphenazine on phagocytosis *in vivo* after (6 weeks) from protoscoleces infection.

Pigment concentrations μmole/ml	Phagocytic index (P.I)			
	Mean	±	S.D	#
* P.B.S (control)	83.75	±	2.63	a
25	81.25	±	2.63	ab
50	79.25	±	2.449	b
75	40.50	±	1.732	c
100	27.75	±	1.893	d
**PHA	86.00	±	3.162	ae

*One way ANOVA of P.B.S f-test= 454.33 p<0.001 High Significant.
**One way ANOVA of PHA f-test= 455.8 p<0.001 High Significant.
Different letters indicated significant difference between each two groups.

Table 4- Effect of purified 1- hydroxyphenazine pigment on cysts numbers and diameters after 25 weeks from protoscoleces infection.

Pigment concentrations μmole/ml	Cysts numbers			Cysts diameters(mm)		
	mean	±	S.D	mean	±	S.D
25	a4.00	±	0.814	a3.297	±	0.631
50	a4.25	±	1.5	a3.550	±	0.214
75	b10.5	±	1.291	b7.260	±	1.107
100	c12.25	±	1.258	c9.102	±	0.602
*PHA	d1.75	±	0.957	d1.564	±	0.562

*One way ANOVA (PHA)
Cysts numbers f=50.05 P<0.001 High significant.
Cysts diameters (mm) f test=72.988 p<0.001 High significant.
Different letters indicated significant difference between each two groups.

No studies were done previously as we sure about the effect of this pigment on non-specific phagocytosis process against secondary experimental hydatidosis, but to some extent these results goes with (Miller *et al*.,1987) who confirm the fact that phenazine pigment at higher concentrations causes inhibition of host phagocytes function and result in ineffective bacterial killing, while some factors secreted by this pathogen causes tissue damage and many patho physiological effects (Leidl *et al*.,2001), in addition to that , our results not going so far from results conducted by Look *et al.*(2005), who they said that a number of virulence factors express by this bacterium *Pseudomonas aeruginosa* have a direct damage on host tissues, or contribute to host tissue damage by impacting the host immune response .

In our conclusion, 1-hydroxyphenazine is toxic pigment (**dose dependent**) causing decrement of non-specific macrophage phagocytosis which allowing protoscoleces to develop and grow.

Lastly, till now numerous questions regarding mechanism of the phenazine action remain unanswered (Look *et al.*,2005)

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Footnotes

1-Address correspondence and any modifications should be done with the author Dr. **Zuhair Ghalib O. Al-shaheen'''**

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2005.

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