Effect of Purified 1-Hydroxyphenazine Pigment on Lymphocytes Viability Against Experimental Secondary Hydatidosis

Zaman A. A. Ibrahim, PhD*
Farhan A. Risan, PhD**
Zahid J. Mohammed, PhD***

الخلاصـــــة

الهـدف: يهدف البحث لمعرفة تأثير صبغة (1-هيدروكسيفينازين) في عيوشية الخلايا اللمفاوية التائية وتأثير ذلك على خمجية الروزيسات البدائية داخل الجسم الحي.

المنهجيـــة:: استعملت أربع مجموعات من الفئران البيضاء الذكور للخمج التجريبي بالروزيسات البدائية للأكياس العدرية كجرعة تحدي ضد أربعة تراكيز لصبغة **(1-هيدروكسيفينازين)** مقارنة مع مجموعة السيطرة سالبة (المحلول الملحي الفسلجي الدارئ) ومجموعة السيطرة الموجبة **(مشطر لا نوعي).**

النتائـــج: أظهرت النتائج بأن التراكيز المستعملة قيد البحث وخصوصاً العالية للصبغة **(75,100)** مايكرومول/مل لها تأثيراً مثبطاً وبمعنوية عالية في عيوشية الخلايا اللمفاوية التائية مقارنة مع مجموعة السيطرة السالبه والموجبة.

التوصيـــات: أوصت الدراسة باستخدام تراكيز واطئة (أقل من 25 مايكرومول/مل) التي قد تفعل الخلايا اللمفاويه التائية ضد الخمج التجريبي بالروزيسات البدائية.

Abstract

Objectives: To find out the effect of 1-hydroxyphenazine (1-HP) on viability of T-lymphocytes and the reflects of this effect on experimental hyadatidosis on hydatid cyst protoscoleces infectivity *in vivo.*

Methodology: Four groups of white male *Blab/*C mice were experimentally infected with four concentrations of (1-HP) with challenge dose of 2000 protoscoleces /1 ml with negative (P.B.S) and positive (P.H.A) control groups.

Results: It has been found that the higher concentrations (75,100) µmole/1ml of the (1-HP) causes significant decrement in the lymphocytes viability in comparison with negative and positive control groups. ($P<0.01$).

Recommendations: The study recommended using concentrations lower than 25 µmole/1ml which may be able to activate the T-lymphocytes against experimental hydatidosis.

Key Words: ***Pseudomonas*, 1-hydroxyphenazine, T cell viability, *Echinococcus*.**

Introduction

Hydatid cyst is the larval stage of the tiny dog tapeworm *Echinococcus granulosos* hatches from eggs in the intestine of the intermediate host(Human) and claws its way through the intestinal mucosa and transported through the lymphatic and blood vessels to sites in different organs in which it grow and developed to hydatid cysts [(1)]. Ultimate growth of the cyst depends on location inside the body of the host, so in some organs of the body they are unable to expand freely, whereas in others, most growth results in serious impairments to the function of vital structures or even in death [(2)].

Pseudomonas aeruginosa is an opportunistic pathogen in human, causing many adverse effects, like Urinary Tract Infection [(3)].

Many virulence factors produced by this pathogen effect the immune system during infection causing both acute and chronic diseases, some of these factors that are poisons, toxic and suppressive to the host immune response are phenazine pyocyanine [(4)] and 1-hydroxyphenazine [(5)] pigments.

* Assistant Professor, Department of Basic Medical Sciences, College of Nursing, University of Baghdad

** Assistant Professor, Department of Medical Pathological Technology, College of Health and Medical Technology-Foundation of Technical Education, Baghdad- Iraq

*** Instructor, Department of Basic Medical Sciences, College of Nursing, University of Baghdad

Many of these products have biological or immunological effects on some of the specific immune response cells like T-lymphocytes [6], while others may effects some of the innate immune response elements like complement [7].

T-lymphocytes are specific immune cells that are differentiated primarily in the thymus gland and they accordingly elaborates a majority of soluble molecules that mediate interaction between different types of immune and non-immune cells called interleukins [8] which they are biologically active molecules released to control and regulates the immune response [9]. These cells are playing very important role against parasitic infection, especially with *Echinococcus granulosus* whether this role is pathogenic or protective in the immune response [10].

Methodology:

Six groups of white male *BABL/C* mice (each group with 10-12 mice; age 8-10 weeks; weight 21-24gm) were used for experimental infection. All hydatid cysts were collected from residents patients in some of Baghdad hospitals In Iraq, and Protoscoleces were isolated from cysts in very sterile conditions according to [11] 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 [12] method using eosin Stain (their viability must be more than 98%).

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

Four groups were inoculated intraperitoneally (I.P) with four purified concentrations of 1-hydroxyphenazine (25, 50, 75, 100) µmole/1ml [13], and after seven days, they were given the same concentrations as a booster dose of the pigment, and after same period, they were infected (I.P) with 2000 protoscoleces/1mL (P.B.S) as a challenge dose, then the fifth group was inoculated (I.P) with (1) ml of sterile (P.B.S) and used as negative control group and the sixth group was inoculated (I.P) with100µgm/ml mitogen Phytohaemagglutinin (PHA, Sigma, England) challenge dose with same number of protoscoleces and used as positive control.

After (2 ,4 ,6) weeks, T-lymphocytes were separated according to [14] method and their viabilities were tested by dye exclusion using (0.2%) trypan blue stain, then one hundred cells were counted and percentage of lymphocyte viability was calculated by using haemocytometer under compound microscope. After 25 week, all mice groups were killed and dissected under dissecting microscope and the infectivity of protoscoleces was investigated and recording cysts numbers and their diameters using vernier micrometer.

The usual statistical analysis methods were done to analyze and asses all results obtained using SPSS vers.10 under windows XP as follow:

1- Mean. 2-Standerd Deviation (S.D). 3-Standerd Error (S.E). 4-Analysis of Variance (ANOVA) 5-Least Significant Difference (LSD), and results were expressed as follow:

Highly Significant if $P<0.01$, Significant if $P<0.05$, Non significant if $P>0.05$.

Results:

After two weeks, all mice groups which were exposed to protoscoleces as a challenge dose, 1-hydroxyphenazine caused decrement in lymphocytes viability which was highly significant ($P<0.01$), especially among mice groups which exposed to high concentrations (75,100) µmole/ml of pigment which were (50.75± 3.775; 37.50± 3.342) respectively, while low concentration (25) µmole showed no significant difference ($P>0.05$)(85.25 ±3.098) in comparison with negative control group PBS (86.25 ±3.864) and with positive control group P.H.A (88.25±1.125) (Table 1).

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

Pigment concentrations µmole/ml	Lymphocytes viability			
	Mean	±	S.D	#
P.B.S (-control) *	86.25	±	3.864	ae
25	85.25	±	3.098	ae
50	65.25	±	1.531	b
75	50.75	±	3.775	c
100	37.50	±	3.342	d
P.H.A(+control)**	88.25	±	1.125	e

P.B.S=phosphate buffer saline, P.H.A..= phytomaemoagglutinin SD= standard deviation

This decrement was highly significant P<0.01 after (4) weeks from mice groups exposure to challenge dose with protoscoleces which exposed to (75,100) µmole/ml in comparison with negative control group PBS (86.00 ±2.432) and with positive control group P.H.A (87.50 ± 3.221) (Table 2).

Table 2. Effect of purified 1- hydroxyphenazine on Lymphocytes viability *in vivo* after (4 weeks) from protoscoleces infection

Pigment concentrations µmole/ml	Lymphocytes viability			
	Mean	±	S.D	#
P.B.S (-control) *	86.00	±	2.432	ae
25	84.25	±	3.321	ae
50	53.00	±	1.225	a
75	40.25	±	3.333	b
100	31.25	±	4.663	bc
P.H.A (+control) **	87.50	±	3.221	e

P.B.S=phosphate buffer saline, P.H.A..= phytomaemoagglutinin SD= standard deviation

Pigment concentration (75,100) µmole/ml showed high significant decrement P<0.01 after 6 weeks of the challenge dose which were (30.00 ± 1.562; 17.75 ± 1.343) respectively in comparison with negative and positive group (84.25 ± 1.437; 86.75 ± 3.162) respectively (Table 3).

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

Pigment concentrations µmole/ml	Lymphocytes viability			
	Mean	±	S.D	#
P.B.S (-control) *	84.25	±	1.437	fc
25	82.35	±	1.550	c
50	44.25	±	2.432	b
75	30.00	±	1.562	e
100	17.75	±	1.343	d
P.H.A(+control) **	86.75	±	3.162	f

P.B.S=phosphate buffer saline, P.H.A..= phytomaemoagglutinin SD= standard deviation

This decrement in the lymphocytes viabilities causes highly significant ($P<0.01$) increment of Protoscoleces infectivity (Cyst numbers and diameters) in comparison with P.H.A and P.B.S groups (Table-4), but the infectivity was not significant ($P>0.05$) between some each two concentrations especially between (75) & (100) µmole/ml.

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
P.B.S(-control)*	# 0	±	0	# 0	±	0
25	a3.88	±	1.46	c1.088	±	0.380
50	b9.25	±	3.69	da1.813	±	0.577
75	c15.63	±	5.50	d1.875	±	0.420
100	c 16.13	±	3.00	d2.213	±	1.792
P.H.A(+control)**	a 1.75	±	0.71	c0.738	±	0.250

***One way ANOVA of P.B.S p<0.01 High Significant**

****One way ANOVA of P.H.A p<0.01 High Significant, P.B.S**=Phosphate Buffer Saline, **P.H.A..= phytomaemoagglutinin SD= standard deviation**

 # Different letters indicated significant difference between each two groups

Same letters indicated non significant difference between each two group. From all the above, the results showed that the higher concentrations of 1-hydroxyphenazine had suppressive effects on the viability of lymphocytes, while PHA is a good mitogenic able to stimulate and proliferate T-lymphocytes [15].

Discussion:

No studies were done previously about the effect of this pigment (1-hydroxyphenazine) which isolated and purified from *Pseudomonas aeruginosa* on T-lymphocytes viability as immunomodulators, especially against secondary experimental hydatidosis and the results of this effect on hydatid cyst development, but some of these studies said that some toxic factors secreted by this pathogen, especially phenazine pigments and its derivative cause direct damage to host tissue by impacting the host immune response [16], while, others said that this toxic effect causes the inhibition of cyclooxygenase response and inhibition of leukotriens production from the cell [5], while, (Nutman *et al.*, 1987) demonstrated that phenazine pigment pyocyanine caused local suppression of T-lymphocytes proliferation and may interfere with cellular immune responses and this pigment inhibits the production one of the essential lymphokines, Interleukin-2 (IL-2) and it's receptor on the T-cell membrane [17], whereas, [18] said that *Pseudomonas aeruginosa* phenazine pigment causes inhibition of human lymphocytes proliferation *In Vitro* in presence of killed *Pseudomonas aeruginosa* and purified phenazine is strongly inhibitor for T-cells proliferation more than crude pigment.

Moreover, (Risan, 1998) said that this pigment which is produced by the bacterium *Pseudomonas aeruginosa* had toxic effect on T-lymphocytes activity and functions in *Vitro* against different species of bacterial growth [13]. This pigment like all phenazines pigments secreted by *Pseudomonas aeruginosa* may effects on the Interleukin-2 (IL-2) production from T-helper cells which are responsible for T-lymphocytes activation, especially in higher concentrations [19], and

inoculation of mice with alive protoscoleces manifested high markers (IL-4, 5, 10) which they are responsible for the cyst progressions and establishment [12].

In our conclusion, 1-hydroxyphenazine is toxic pigment (dose dependent) causing decrement of T-cells viabilities, especially at higher concentrations which let protoscoleces to develop and growth. Finally, the mechanism of phenazine pigment was not well known and till now numerous questions regarding this mechanism remain unanswered [20].

Recommendations:

More studies should be done to see the direct effect of this pigment on different types of tissues and cells, especially the activities and functions of immune cells and the indirect effects on these cells organelles.

References:

1. McKerrow, J.: Parasitic Disease. In: (Stites, D.P., Terr, A.I and Parslow, T.G ed). **Medical Immunology,** 9th Ed, Middle East Edition, 2004, P.P. 725-738.

2. Markell, E., John, D. and Krotoski, W.: **Medical Parasitology,** 1999, **8th ed.** Philadelphia: W.B. Saunders Company, P.P. 253-261.

3. Buonanno, A. and Damweber, B.: Review of Urinary Tract Infection. **U.S. Pharmacist**, 2006, 31 (6), P.P.HS 26-HS 36.

4. Price-Whelan, A., Dietrich, L. and Newman, D.: Pyocyanine Alters Redox Homeostasis and Carbon Flux through Central Metabolic Pathways in Pseudomonas Aeruginosa PA14. **J. Bacteriol**, 2007, 189 (17), P.P. 6372-6381.

5. Muller,M. and Sorrell, T.: Inhibition of the human platelet Cyclooxygenase response by the naturally occurring Phenazine derivative 1-Hydroxyphenazine. **Prostaglandines**, 1995, 50, (5-6), P.P. 301-311.

6. Epelman, S.; Neely, G.; Ma, L.; Gjomarkaj, M.; Pace, E.; Melis, M.; Woods, D.; and Mody, C.: Distinct Fates of Monocytes and T-Cells Directly Activated by Pseudomonas Aeruginosa Exoenzymes, **S. J. Leukoc. Bio**, 2002, 71, P.P. 458-468.

7. Kindet, T.; Goldsby, R. and Osborne, B.: **Immunology**, 6th ed., New York: W.H. Freeman and Company2007, P.P. 447-472.

8. Druet, P.; Sheela, R. and Pelletier, L.: Th1 and Th2 cells in Autoimmunity. In: Upadhyay, S. ed., **Immunomodulation.** New Delhi, Madras, Mumbai, London. Narosa Publishing House, 1997, P.P. 19-31.

9. Stewart, J.: Innate and Acquired Immunity. In: (Greenwood, D., Slack, R.C.P and Peutherer, J.F. ed). **Medical Microbiology**.16th ed. New Yorko, Churchill Livingstone, 2002, P.P. 121-145.

10. Romagnani, S.: Induction of Th1 and Th2 Responses: A Key Role for the 'Natural' Immune Response? **Immunology Today,** 1992, 13, P.P. 379-381.

11. Smyth, J.: In Vitro Culture of Echinococcus Spp. In: Proceedings of 13th International Congress of Hydatidology. Madrid,1985, P.P. 84-89.

12. AL-Qaoud, K. and Abdel-Hafez, S.: The Induction of T-helper Type-1 Response by Cytokine Gene Transfection Protects Mice Against Secondary Hydatidosis. Original Paper, **Parasitology Research,** 2008.

13. Risan, F.: Immunological and Physiological Study of Purified Pyocyanine and 1-Hydroxyphenazine Produced by Pseudomonas Aeruginosa Isolated from Human Urinary Tract Infection. PhD Thesis Submitted to the College of Science, University of AL-Mustansiriya, 1998, Baghdad-Iraq.

14. Hassan, B.: Study of Some Immunological Aspects in Pregnant Women during Three Trimesters of Pregnancy. M.SC Thesis Submitted to the College of Health & Medical Technology, 2004. Baghdad-Iraq.

15. Male, D.; Brostoff, J.; Roth, D. and Roitt, I.: Immunology. 7th ed, UK, Mosby, 2006, P. 533.

16. Buret, A. and Cripps, A.: The Immunoevasive Activities of Pseudomonas Aeruginosa Relevance for Cystic Fibrosis. **Amer. Rev. Respir. Dis**, 1993, 148, P.P. 793-805.

17.Nutman, J.; Berger, M.; Chase, P.; Dearborn, D.; Miller, K.; Waller, R. and Sorensen, R.: Studies on the Mechanism of T-Cell Inhibition by Pseudomonas Aeruginosa Phenazine Pigment Pyocyanine. **J. Immunology**, 138 (10), 1987, P.P. 3481-3487.

18. Sorensen, R.; Klinger, J.; Cash, H.; Chase, P. and Dearborn, D:. In Vitro Inhibition of Lymphocytes Proliferation by Pseudomonas Aeruginosa Phenazine Pigments, **Infect. Immun,** 1983, 41 (1), P.P. 321-330.

19. Ulmer, A.; Pryjma, J.; Tarnok, Z.; Ernst, M. and Flad, H.: Inhibitory and Stimulatory Effects of Pseudomonas Aeruginosa Pyocyanine on Human T-and B-Lymphocytes Human Monocytes. **Infect. Immun**, 1990, 58 (3), P.P. 808-815.

20. Look, D., Stoll, L., Romig, S., Humlicek, A., Britigan, B. and Denning, G.: Pyocyanin and Its Precursor Phenazine-1-Carboxylic Acid Increase IL-8 and Intercellular Adhesion Molecule Expression in Human Airway Epithelial Cells by Oxidant-Dependent Mechanisms. **J. Immunol**, 2005, 175, P.P. 4017-4023.