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Immunity response of mice infected with laser irradiated protoscoleces of *Echinococcus granulosus*

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Abstract

The present study investigated the effect of the Neodymium- Doped Yttrium Aluminum Garnet (Nd: Y3AL5G12) Laser eradiation 1000 mw, against infection with secondary hydatid disease in BALB/c mice by inoculating the animals with protoscoleces of *Echinococcus granulosus* exposed to laser irradiation, for various periods 40,60,120 and 240 minutes, with viability of 70%,57%,50% and 40%, respectively, contrasted to the control set (mice inoculated with unexposed protoscoleces) along three months, depending on the evaluation of the total count of WBCs and assessment of acquired cell- mediated immunity, demonstrated by delayed type hypersensitivity test (DTH).

The results showed considerable increase ($p<0.01$) in the total WBCs counting, in mice injected with irradiated protoscoleces, up to 13962 cell/cm^3, in comparison to the control group 5420 cell/cm^3, after 120 minutes, two months post infection. Moreover, there was considerable excess ($p<0.01$) in the foot pad thickness in treated mice, 2.94 mm after 120 minute (24hour post antigen injection), two months post infection, 2.86mm after 120 minutes (24 hours post antigen injection), 1 month post infection and 2.64 mm, 120 minutes (24 hours post antigen injection), three months post infection, compared with the control group 1.18mm, 1.80mm and 1.72mm, respectively.

Results revealed that irradiation of *Echinococcus granulosus* protoscoleces with laser had a significant influence on the production of WBCs and stimulation of delayed-type hypersensitivity reaction in mice.

Keywords: Hydatid disease, *Echinococcus granulosus*, laser eradiation

الاستجابة المناعية في الفئران المصابة بالرؤيسات الأولية للمشوكة الحبيبية *Echinococcus granulosus* المشعة بالليزر

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الخلاصة:

تحرت الدراسة الحالية عن تأثير اشعة الليزر Neodymium- Doped Yttrium Aluminum Garnet (Nd: Y3AL5G12 بقوة 1000 ملي وات في الفئران سلالة BALB/c ضد الإصابة بداء الأكياس العدرية الثانوي بواسطة إعطاء الفئران الرؤيسات الأولية لدودة المشوكة الحبيبية *Echinococcus granulosus* والمعرضة لأشعة الليزر، ولفترات مختلفة 40، 60، 120، و 240 دقيقة، ذات حيوية 70%، 57%، 50% و 40%، على التوالي ، مقارنة مع مجموعة السيطرة (الفئران المعطاة الرؤيسات الأولية غير المعرضة)، طيلة ثلاثة أشهر، بالاعتماد على تقدير التعداد الكلي لخلايا الدم البيض والمناعة المكتسبة الخلوية المتمثلة باختبار فرط الحساسية المتأخر(DTH).

أظهرت نتائج الدراسة ارتفاعا معنويا ($P\leq0.01$) في التعداد الكلي لخلايا الدم البيض في الفئران المحقونة بالرؤيسات الأولية المشعة بالليزر الى 13962 خلية/ سم^{3}مقارنة مع مجموعة السيطرة 5420 خلية/ سم^3 لفترة 120 دقيقة، لمدة شهرين من الإصابة. فضلا عن لك، ظهر ارتفاع معنوي ($P\leq0.01$) في سمك وسادة القدم في الفئران المعاملة بلغ أقصاه 2.94 ملليمتر في الفترة 120 دقيقة (بعد 24 ساعة من حقن المستضد) لمدة شهرين من الإصابة، 2.86 ملليمتر في الفترة 120 دقيقة (بعد 24 ساعة من حقن المستضد) لمدة شهر من الإصابة و 0.02 ملليمتر في الفترة 120 دقيقة (بعد 24 ساعة)، لمدة ثلاثة أشهر من الإصابة، مقارنة مع مجموعة السيطرة التي بلغت قيمها 0.98 ملليمتر، 1.18 ملليمتر و 1.42 ملليمتر، على التوالي.

أظهرت نتائج الدراسة ان تشعيع الرؤيسات الأولية لدودة المشوكة الحبيبية *Echinococcus granulosus* بالليزر كان له تأثيرا معنويا في تكاثر خلايا الدم البيض وتحفيز تفاعل فرط الحساسية المتأخر في الفئران.

الكلمات المفتاحية: داء الاكياس العدرية ، المشوكة الحبيبية *Echinococcus granulosus* ، اشعة الليزر

1. Introduction

E. granulosus is the causative agent of cystic echinococcosis in human beings and other animal hosts. This disease is a zoonosis that usually infects domestic animals and livestock and creates a general health and economic problem (1, 2). The disease spreads in the Middle East, Germany, Central Asia, Spain, Australia, and regions central and southern regions of the former Soviet Union, northern and southern regions of China, East Africa and the semi-desert regions of Africa and South America (3). The hydatid cyst grows slowly and can be located in different places in the human body, the infection may be asymptomatic, especially in its early stages, and there are many unregistered cases in the affected areas (4). Diagnosis of hydatid disease is based on immunoassay methods including indirect hemagglutination, indirect immunofluoresence, counter-current immunoelectrophoresis, radioimmunoassay, and enzyme-linked immunosorbent assay (ELISA). (5-7). In addition to imaging techniques ultrasound, Computed Tomography (CT), Magnetic resonance Imaging and x ray (8, 9).

The percentage of infection distribution in the organs varies according to the host and the methods of transmission of the parasite, the infection develops mainly in humans in the liver 70%, lungs 20%, and to a lesser extent in the heart, brain, bones, spleen, kidneys, central nervous system and adrenergic gland (4). There are many ways to treat cystic echinococcosis (8). Recently, there are three options for treating hydatid cysts in different organs: surgery, puncture-aspiration-injection, and medical treatment. (6, 10), for the medical treatment, Albendazole is treatment of choice, rather than Mebendazole or Benzimidazole Carbamates (6).

Researchers investigated better therapeutic non-invasive alternatives, as safe killers for the protoscoleces and stimulative of the immune system, including electromagnetic radiation techniques, ultrasound, ultraviolet, Gamma ray, laser (11-18), electrical current and nanoparticles (19, 20).

The current research pursued to reveal the ability of radiation exposed protoscoleces to stimulate the production of WBCs, and the cellular acquired immunity represented by delayed- type hypersensitivity in mice infected with secondary hydatidosis.

Materials and Methods

Experimental animals

One month old experimental mice, animal house bred (in the Scientific Research, College of Education for Pure Sciences), worm free males of BALB/c mice were used

Collect of hydatid cysts and estimation of protoscoleces Vitality

Larval stages of E. granulosus were gained from livers of infected sheep slaughtered in Mosul city massacre. Protoscoleces were isolated from the larval stages, under disinfected conditions (21). Vitality was assessed (22). 2000 irradiated protoscoleces/mouse were inoculated into the peritoneal cavity of mice, treated group. 2000 not radiated protoscoleces/ mouse were injected into the peritoneal cavity of mice, control group, (23).

Laser device

The laser device, (ND / Y3AL5G12) 1000 milliwatt and a wavelength of 532 nm (2), was used (figure 1).

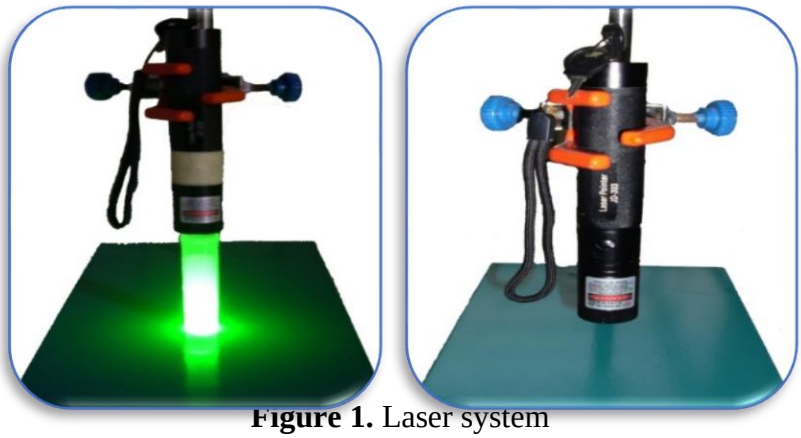

Figure 1. Laser system

Treatment Schedule

Six groups of mice were used, five for each group, animals of the 1st, 2nd, 3rd and 4th groups were injected intraperitoneally with 2000 protoscoleces exposed to laser radiation, with viability 70%, 57%, 50%, 40%, the fifth positive control group was injected intraperitoneally with unexposed protoscoleces with 100% viability (15). The sixth negative control group was neither injected with protoscoleces nor exposed to laser radiation.

Blood

The experimental animals of the five groups were anesthetized with Diethyl ether, blood was withdrawn from the ophthalmic venous plexus according to Waynforth (24), one, two, three months post infection, for total count of WBCs according to Dacie and Lewis (25).

Delayed-type hypersensitivity

Elaboration of antigen and assessment of foot pad response

Protoscoleces antigen was elaborated in accord with Dottorini et al. (26). Protein was assessed in accord with Schacterle and Pollack (27). Foot pad swelling to the antigen was measured (Ali-Khan 28). The antigen was injected into the right foot of the mouse at a concentration of 139 micrograms / ml after sterilizing the injection area with alcohol. The left foot was injected with the same volume of sterile PBS; the foot thickness was measured using the Vernier after 3, 24 and 48 hours of injection. The difference between the thickness of the left and right foot pad is a measure of hypersensitivity.

Statistical analysis

Results analyzed statistically by Complete Randomized Analysis CRD and by Duncan's Multiple Range Test to detect the significant differences between treated (mice injected with protoscoleces that exposed to laser radiation) and not treated positive and negative control groups (29).

2. Results And Discussion

Total White Blood Cells Count

Table (1) reveals alterations in the overall WBCs counting in mice injected with radiated protoscoleces in contrast with not exposed positive and negative mice for 1, 2, 3 months. A significant increase ($P < 0.01$) in the total white blood cells count was observed in the treated mice, reported 13962 cell/cm3 after 120-minute of exposure, two months post infection, followed by 9837cell/cm3 after 240 minute, three months post infection and 9010 cell/cm3 after 60 minutes, one month post infection, respectively, in comparison with the positive control group 5420, 6370 and 5350 cell/cm3, respectively, which in turn showed a significant decrease($P < 0.05$) in the Total white blood cells count when compared to the negative control 6690, 7110 and 6850cell/cm3, respectively.

Table 1. changes in total white blood cells count in mice injected with laser radiated protoscoleces in comparison with positive and negative control groups for 1, 2, 3 months

Exposure period (min) / vitality	Total count of leukocytes cell / cm3 (1stmonth) ± SD mean	Total count of leukocytes cell / cm3 (2ndmonth) ± SD mean	Total count of leukocytes cell / cm3 (3rdmonth) ± SD mean
40 /70%	8380 ab ±1118.369	8570 c b ±903.188	9012 ab ±636.76
60 /57%	9010 a ±810.401	8760 b ±1346.013	9562 ab ±3236.00
120 /50%	6450 c ±1479.442	13962 a ±5437.644	9475 ab ±1121.10
240 /40%	6750 c ±882.468	9650 b ±389.935	9837 a ±790.86
C+	5350 c ±1426.972	5420 c ±590.127	6370 c ±2050.18
C-	6850 bc ±1243.483	6690 c b ±768.44	7110 c b ±1317.85

Delayed-type hypersensitivity

Table (2) shows the changes in the rates of foot pad thickness in mice injected with irradiated protoscoleces, contrasted to the positive set for 1, 2, 3month post infection. A significant increase ($P<0.01$) was observed in the rates of thickness in the treated mice reported a maximum thickness 2.94 mm followed by 2,86mm and 2.64mm, after120 minute exposure, 24 hours post antigen injection, two months, one month and three months post infection, respectively, compared to the positive control 1.18 mm, 1.80 mm and 1.72 mm, after120 minute exposure, 24 hours post antigen injection, two months, one month and three months post infection, respectively.

Table 2. Changes in the foot pad thickness (mm) in mice injected with laser radiated protoscoleces in comparison with positive and negative control groups for 1,2,3 months

Exposure period (min) / vitality	Foot pad thickness(mm) (1st month)			Foot pad thickness(mm) (2nd months)			Foot pad thickness(mm) (3rd months)		
	3 hr. ± SD mean	24 hr. ± SD mean	48 hr. ± SD mean	3 hr. ± SD mean	24 hr. ± SD mean	48 hr. ± SD mean	3 hr. ± SD mean	24 hr. ± SD mean	48 hr. ± SD mean
40 /70%	1.94 a ±0.782	2.16 ab ±0.461	1.62 a ±0.334	1.86 c b ±0.403	2.62 a ±0.334	1.84 a ±0.350	1.82 a ±0.449	2.02 a ±0.723	1.54 a ±0.296
60 /57%	1.82 a ±1.077	2.54 ab ±0.433	1.46 a ±0.698	2.42 a ±0.286	2.72 a ±0.618	2.02 a ±0.438	2.06 a ±0.240	2.46 a ±0.466	1.60 a ±0.644
120 /50%	2.14 a ±0.512	2.86 a ±0.384	1.74 a ±0.680	2.22 ab ±0.402	2.94 a ±0.541	1.70 a ±0.463	1.58 a ±1.089	2.64 a ±0.606	1.46 a ±0.826
240 /40%	1.52 a ±0.311	1.94 b ±0.531	1.42 a ±0.414	1.36 cd ±0.320	1.82 b ±0.649	1.54 a ±0.378	1.74 a ±0.861	2.38 a ±0.756	1.28 a ±0.697
C+	1.46 a ±0.572	1.80 b ±0.994	1.38 a ±0.521	0.98 d ±0.511	1.18 b ±0.708	1.42 a ±0.471	1.16 a ±0.409	1.72 a ±0.715	1.00 a ±0.244

The outcome of the current research demonstrated a significant increase in the total count of WBCs in mice injected with radiated protoscoleces (table 1) Which leads to stimulating the immune system, as Al-Ghamdi et al. (30) stated that LLLT enhances rapid multiplication of different types of cells in the culture, including stem cells, which are the source of all white blood cells, and radiation therapy stimulates the production of high levels of Adenosine Tri Phosphate, Ribonucleic Acid and Deoxyribonucleic acid in stemmed cells and another types of cells. Consequently, treatment with this radiation improves cell reproduction without causing any toxic effects. The results of low-intensity irradiation also differ according to the intensity of the energy and the wavelengths to which target cells are exposed (30).

The study has shown that the energy density value that ranges between 0.4-0.5 joules / centimeter square and the visual spectrum extending from 600-700 nanometer of eradiation, were good enhancements for the reproduction of different cell types. This effect includes several mechanisms, one of which is that the laser power is absorbed by the intracellular chromophores and transformed into metabolic energy. Therefore, cellular Adenosine Triphosphate scales augment approximately twice next helium-neon eradiation (31). ATP works by the diverse P2 nucleotide receptor subtypes to rise calcium concentration within the cell (32-34). ATP simultaneously regulates protein construction, DNA construction, and gene expression (33, 35). One theory regarding the effect of LLLT states that the laser has the ability to affect photoreceptors in cells. This mechanism is called photobiology, or biostimulation, It has been provided in details that photobiostimulation happens by enzymes of the electron transfer chain in mitochondria, including higher rates of cell respiration, either via endogenous porphyrins in the cell or by cytochrome C (36), which increases cellular and functional metabolism. The bio stimulating influence of the low level laser therapy results in an augmentation in microcirculation, high levels of ATP production, RNA and DNA building, leading to improved cellular oxygenation, nutrition, and renovation, and enhancement of the electron transport system in the mitochondria.

Yu et al. (37) claimed that Photons enter the cell and are absorbed directly by chromophore carriers that are situated either in the mitochondria or the cell membrane. These color carriers interact powerfully with lasers. The energy of photons is transformed into chemical energy within a cell, in the shape of Adenosine Triphosphate, which improves cellular activities and cell reproduction rates. The ability to be permeated of the cell membrane changes, following by physiological alterations in the objective cells. The degree of bio stimulation of laser radiation relies on the wavelength at the same time the physiological status of the cell during the irradiation period (38).

The infected mice showed a decrease in the total white blood cell count because of the infiltration and migration of the cells to the sites of infection, where the parasite is present in order to control the growth the evolution of secondary hydatid cysts.

Concerning cell-mediated immunity, the current research exhibited a significant increase in an acquired cellular immune response represented by the delayed-type hypersensitivity test (table 2) in mice injected with irradiated protoscoleces, in contrast with the positive set. This increase may be attributed to the ability of laser irradiation to induce cellular immunity represented by swelling in the thickness of the pad that continued after 24 hours of antigen injection, by inducing division, reproduction, division and differentiation of immune cells (39, 40) boosting and modifying the immune system (41). The increase in the thickness is also attributed to the effect of the laser on the differential activation of the antigen presenting cells, which include macrophage cells, dendritic cells, and B lymphocytes leading to the release of a number of pro inflammatory cytokines in the cells that attract and activate macrophages (42- 44), this result is consistent with Kivity et al. (45) in patients with Echinococcus cyst. The researchers also

explained by the histological examination of the pad, that the growth is due to the infiltration of neutrophils, macrophages mononuclear and lymphocytes, and this infiltration, which is a characteristic of the typical late hypersensitivity reaction, continues to the seventh day, as it was observed on the sixth day of neutrophil cells and mononuclear cells by Ryu & Kim (46). Czuprinski et al. (47) suggested that the immunized mice were able to recruit a large number of macrophage cells at the site of infection due to the release of lymphokine by lymphocytes signaling the role of cellular immunity.

The most characteristic feature of the hypersensitivity reactions is the accumulation of eosinophils in the tissues preceded by the process of accumulation of TH2 lymphocytes (46,47). The Bacillus Chalmette Guerine (BCG) vaccine was used with the complete Frunze's adjuvant or with S. mansoni eggs in the delayed hypersensitivity test in mice, it was concluded that the delayed hypersensitivity reactions stimulated the production of attracting chemicals and binding molecules to endothelial cells necessary for eosinophilia migration, and the eosinophil migration process was inhibited by depleting TH2 cells or the use of anti-INF-, which indicates the vital role played by TH2 cells and -γ INF in delayed hypersensitivity reactions (48, 49), the increase in thickness of foot pad in the present study may also be attributed to laser stimulation of-INF and INF-alpha, TNF-alpha. Willey et al. (50) indicated that IL-2, INF-, and TNF- detachment of helper T -Helper cells are responsible for delayed hypersensitivity reactions. Untreated infected mice showed a low depressed Cell-Mediated Immunity (CMI) response, and this is similar to what be observed by Khan (28) in mice infected with E. multilocularis, and in mice infected mice with E. granulosus (45).

3. Conclusion

It could be well concluded that laser irradiated protoscoleces of *Echinococcus granulosus* has a significant impact on the total count of WBCs by induction division and proliferation of the cells, and on cell-mediated immunity, by stimulation of delayed-type hypersensitivity reactions in mice, This technique may well be used in the future as an alternative remedy against this disease.

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