Available online on www.ijddt.com
International Journal of Drug Delivery Technology 2019; 9(2); 236-241
doi: 10.25258/ijddt.9.2.19

ISSN: 0975 4415

Research Article

Employing Gamma Ray Irradiated *Giardia lamblia* as Trialed Vaccine in Experimental Animal

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Received: 2nd May, 19; Revised: 29th May, 19, Accepted: 16th Jun, 19; Available Online: 25th Jun, 2019

ABSTRACT

This is an experimental trial to prepare a vaccine from gamma-irradiated *Giardia lamblia* which is evaluated in experimental animals. The study was conducted from December 2015 to April 2016. The field survey of the parasite was conducted from those patients attending the laboratories of the Alawi Children's Hospital in Rusafa and the Al-Yarmouk Teaching Hospital in Karkh, through which 1250 stool samples of different age groups were examined. Five groups of mice were used in the study; the first was injected with normal saline and considered as a negative control group, the second was injected with cystic form of non-irradiated *Giardia lamblia* and considered as a positive control group, whereas the other three groups were injected with gamma irradiated *Giardia lamblia* at three different doses 10, 15 and 25 rad respectively. *Giardia lamblia* was primarily cultivated in liver infusion agar for ten days to obtain the active phase. On the sixth day, the cystic phase was purified and standardized to be used in the infection of mice with or without the exposure of gamma rays. Mice showed high sensitivity to parasitic infestation, in the gamma non-irradiated and the irradiated with gamma 10 rad, and 15 rad irradiated groups which was 100%. The results expressed an excystation process of the depleted phases and the release of the feeder phases. The results of the three irradiated groups consisted of histopathological changes of the small, and the rectum by dissection after two weeks of infection, with intestine amputation lesions, as well as ulceration and inflammation of the inflammatory cells represented in small numbers of neutrophil, lymphocytes, and eosinophils. The presence of ulceration and fall of epithelial cells in the intestinal cavity has been shown, and different forms of the parasite have been observed. Mice which was injected with irradiated *G lamblia* at high dose (25 rad), not show and sensitivity to the challenge infection and no excystation of thy parasite had been done. After 2 wreaks, a comparison was achieved between all study groups in which no histopathological changes were noticed in the mice irradiated with dose of25 rad. After another two weeks, a challenge dose was given (un-attenuated *G lamblia*) and mice were dissected after another two weeks, no changes on the level of histopathology of intestinal tissue were noticed the results suggested that mice acquire an immunity against the parasite infection.

Keywords: Giardia, radiant gama, weakened parasite, contamination, challenge.

INTRODUCTION

Giardia is one of the most common intestinal parasites that cause diarrhea worldwide. Many names that have been given to species that infect humans such as G.lamblia, G.intistinalis and G.doudinalis[1]. The life cycle of this species consists of two consecutive stages, the trophozoite stage, which lives in the small intestine and causes intestinal giardiasis and the cyst stage, which is the resistant stage as it can stay in the small intestine for several months in the surface water and in the sewage water where it is transmitted to humans through direct and indirect ways[2]. The rate of contamination with Giardia vary depending on the degree of fecal contamination and the extent of contaminated surface water use Most sources consider Giardia cysts to be immediately infectious[3]. Fresh leafy vegetables, particularly vegetables which are unclean and irrigated with sewage water, are the main sources of the parasite[4]. In the few last years, several studies stated an increased prevalence of diseases associated with food contamination with increased rate of infection with intestinal parasites, particularly in the developing countries[5,6]. Infection with Giardia ranges between 2-7% in developed countries and reaches 40% in developing countries and tropical and subtropical regions, where conditions of sterilization and hygiene are low[7]. Their prevalence depends on the environmental conditions and on the hygiene level, where it is highly prevalent in poor and overcrowded places that lack health and public conditions, lack or unavailability of drinking water, and not washing fresh vegetables well as well as discharge and use of human wastes and sewage water as fertilizer in many farms, therefore, tropical and subtropical regions are considered to be the most suitable areas for the survival of this parasite because of the climatic conditions suitable for the survival and development of cysts such as temperature, humidity and soft soils[8]. Most infections are concentrated in middle and south America, Africa and Asia, low socio-economic conditions and popular traditions help in the spread of this parasite Chronic

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Table 1: Shows the numbers and percentages of mice death during the activity test for attenuated and unattenuated parasite doses with Gamma rays.

Dose 1000/ml	Total number of mice (number of deaths)		Death percentage
	First week	Second week	
Control mice	----	----	----
Unattenuated mice	1	3	80%
10 rad attenuated mice	----	2	40%
15 rad attenuated mice	1	2	40%
25 rad attenuated mice	----	1	10%

infection can lead to weight loss and malabsorption[9]. Metronidazole (flagyl) derivatives are the drugs of choice for the treatment of giardiasis which invades tissues. The drug Emitene Hydrochloride Diodoqinin is given to patients who are not cured by flagyl. Recent studies showed that Amitine and Chloroquine gave best results in the treatment. Immunization program against diseases by vaccination is the most important method to avoid their effects on humans and animals and reducing losses resulting from the problems associated with these infections such as drug resistance and side effects on the host's body[10]. Several methods were used to prepare parasitic vaccines such as using low doses of sexual stages of the parasite or using the antigens of the parasite directly or following the genetic engineering methods to prepare these antigens, as well as attenuation of the pathogens to stimulate the immune system by chemical and physical methods or by irradiation which is the most important method used to attenuate pathogens and production of vaccines because it is easy and inexpensive, and proved to be effective in many uses[11]. The use of radiation has come after many studies and knowledge of its effect on the living and organic materials of the parasite, which causes a series of physical and structural changes that lead to weaken the ability of the parasite to cause infection while maintaining its ability to stimulate the immunity of the host[12].

The current study is designed to achieve the following objectives:

Growing the parasite on modified HSP-1 medium, then accumulation of cysts and irradiating them with three doses (10,14, and 25) of gamma rays.

Giving irradiated and un irradiated doses of the parasite to the white mice.

Study of histopathological changes in albino mice infected with giardiasis, to identify the virulence of the parasite comparing it with infected with attenuated giardia group that

Try to find out the best dose of gamma radiation to select it as a vaccine

MATERIALS AND METHODS

stool samples were collected from males and females children and adult patients who were suffering from diarrhea in two hospitals in Baghdad city, Al-Elwiya pediatric and Al-Yarmook teacing ,taking into account the difference of geographical area, being one of the hospitals located in Al-Rusafa and the other in Al-Karkh area. Microscopic examination showed six cysts stage of giardia per field. The parasite was cultured by taking a small amount (loop full) of feces and placing it on the previously prepared culture medium, and adding 0.5 ml of sterile stains and 0.07 microliters of antibiotics and a loop full of sterile rice starch to the culture media. The culture was incubated at $37C^0$ and then transferred to a new culture medium every day until trophoziotes of *G. lamblia* had been detected. After the appearance of the parasite, the transfer a new culture media was repeated twice a week while maintaining the amount of antibiotics as the number of the bacteria was suitable for the growth of the parasite, culture was transferred from the medium containing it by aspiration of the fluid that covered the solid part by means of a sterile Pasteur pipette. The culture was then removed and the fluid part at the bottom of the tube was mixed with the remaining deposits on the surface of the solid part, then it was transferred to a new culture medium[13].

From each culture medium, only the cyst stage was transferred and saved in sterile tubes with a small amount of normal saline at $4C^0$ until it was injected into the albino mice. The number of cysts was estimated per/cm3 in the suspension by using the improved Neubauer Haemocytometer then divided into three parts in tightly-closed sterile tubes for the purpose of irradiation with Gamma rays, where it was transferred to the Department of Physics - University of Baghdad, and the parasite was irradiated with three doses (10,15 and 25 Rad) and directly given orally to the experimental mice. The animals used in this study were the male white mice Balb/c males which were obtained from the animal house of the College of Science / Department of biology / University of Baghdad. The mice were placed in animal breeding cages in the laboratory during the study period to make it easy to observe them several times a day. The feces of these animals were examined by the direct method to ensure that they were free of intestinal parasites, and then distributed regularly at the rate of 6 mice per cage. These animals in our study were used at the age of (1-3) months, with keeping them in suitable conditions of temperature and food suitable for their growth.

Experimental animals

The total number of animals used in the study was 30 white mice divided into five groups.

Group I (control group) six mice inoculated with saline only.

Group 2 six mice, inoculated with parasites which were un attenuated by radiation.

Group 3 six mice inoculated with parasite attenuated by15 rad of Gamma rays.

Group 4 six mice inoculated with the parasite attenuate d by10 rad of Gamma rays.

Table 2: Percentage of mice which showed changes in behavior within 48 hours from giving different doses of attenuated and unattenuated parasite with Gamma rays.

Notes	Control group	Unattenuated dose	Functioning groups		
			10 rad attenuated dose	15 rad attenuated dose	25 rad attenuated dose
Hypoactivity	---	100%	100%	80%	20%
Motor weakness	---	100%	70%	40%	10%
Anorexia	---	100%	80%	70%	20%
Generalized tremor	---	100%	100%	100%	30%

Group 5 six mice inoculated with parasite attenuated by25 rad of Gamma rays.

Inoculation of laboratory animals

In all experiments, experimental mice were inoculated via intra-esophageal route by the bag suspension of normal saline, solution containing 10,000 cyst/cm3 and for each group using a micropipette attached to the tephlon tube, which is inserted orally into the esophagus, then the stomach, and the cyst-containing fluid is then pushed directly into the stomach.

After inoculation the animals with the attenuated suspension by Gamma rays and inoculation the other group with the parasite un attenuated by Gamma rays was done , the control group was inoculated with only normal saline. The daily production of the parasite cysts was estimated by placing the mice in clean, sawdust-free cages and then stool was collected, and examined to determine the extent of the infection. The examination was done daily for six days from the beginning of the infection.After the infection was confirmed by stool examination and observing the parasite, the infected mice were killed two weeks later, and a piece (1 cm) of the small intestine was taken and used to study the histopathological changes during the infection period .

Group of mice that were inoculated with the suspension irradiated by 25 rad to make sure they were free of infection after two weeks, where they were given the challenge dose, and two weeks later, they were killed and a piece (1 cm) of the small intestine was taken. The histological sections were prepared using the tissue processing according to[14]

Laboratory growth on special medium for Giardia lamblia

HSP-1 is a liquid media according to[15]

Lugol's-Iodine preparation method

a drop of Lugol's iodine solution on the other end, and taking a small amount of feces from multiple places with a clean wooden stick. Small amount of stool was taken and mixed with the Lugol's-iodine solution until it became homogeneous, then the cover slide. Hematoxylene - Eosin stains were prepared according to[14].

Preparation of human serum

Venous blood from a healthy person was obtained and then put in a sterile tube containing no anticoagulant, left at room temperature for 30 minutes, then rapidly centrifuged at 2000 RPM for 10 minutes and kept at -20°C until use.

Preparation of tissue fixative

The tissue section taken from the small intestine was fixed in Formal saline solution then processing according to (Bancroft etal.,2007)

General stool examination

All stool samples were examined by the naked eye prior to microscopy, for color observation and stool consistency (Diarrhea, soft, or semi-solid). noting that the presence of fatty substances in the sample, often indicates infection with *Giardia lamblia* examined under 10X, then 40X

RESULTS

Test of mice activity for attenuated and unattenuated parasite doses with Gamma rays .The percentage of mice death and the attenuated and unattenuated parasitic dose ranged from (80%-10%). The results showed that the unattenuated dose was the lethal dose for the majority of mice as shown in table (1).

The mice showed changes in behavior during the test. These changes were associated with the dose given and represented by: hypoactivity, motor weakness, anorexia and generalized tremor. These changes were severe in unattenuated doses of Gamma rays and within 24 hours after inoculation, but the changes decreased gradually after that, while these changes were few and were determined in the early hours of inoculation with the doses attenuated by 25 rad as seen in table (2).

The results of the laboratory infection of the mice inoculated with a dose of 10,000 cyst/ml orally showed that these animals were susceptible, as the rate of infection with unattenuated and attenuated by 10 rad and 15 rad was (100%) during the microscopic examination of intestinal wastes, showing an increase in the numbers of cyst stage during two weeks of infection.

The daily microscopic examination also showed intestinal excystation and releasing of actively motile trophozoites with the presence of active bacteria , as well as the presence of parasite phases in the contents of the small intestines of the dissected mice see photos (1) and 2).

The results of mice inoculated with attenuated parasite with 10 rad and 15 rad Gamma rays were close to the results of the mice which were inoculated with unattenuated parasitic rays, but they were different from the results of the group of mice inoculated with attenuated 25 rad Gamma rays during the microscopic examination of intestinal wastes, in which no parasite was found. Two weeks later, the attenuated, unattenuated and control groups of mice were dissected.The laboratory infection of one of the orally inoculated with unattenuated mice in one sample showed a small abscess in the right

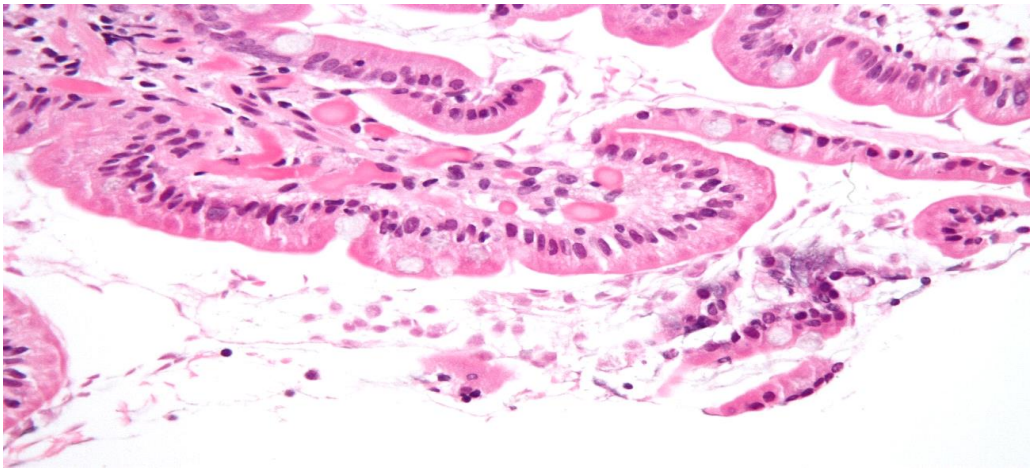

Figure 1: Cross section of the small intestine of mice inoculated with 25 rad attenuated parasite showed normal tissue structure(H&E X40).

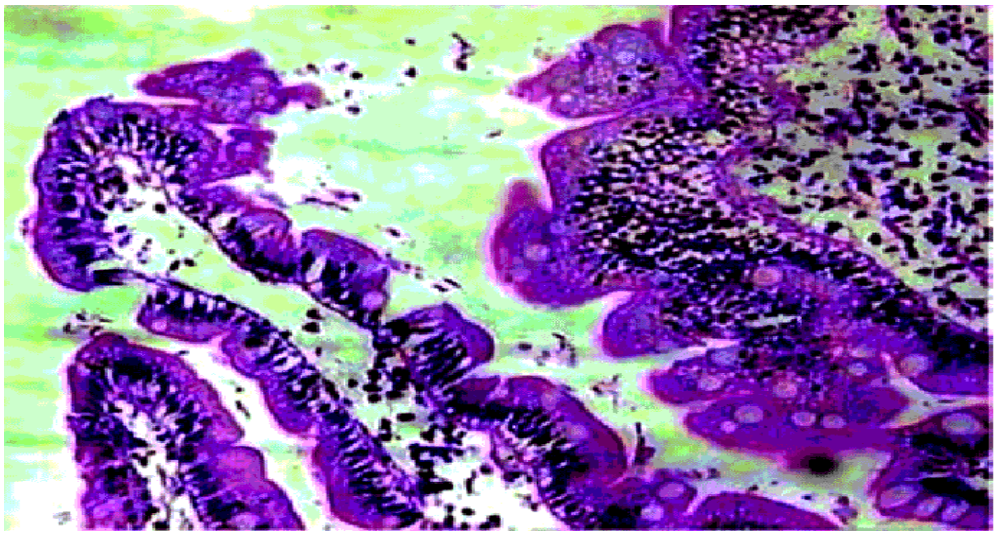

Figure 2: Cross section of the small intestine of mice inoculated with 10 rad attenuated parasite showed numerous trophozoite forms of *Giardia Lamblia* on the surface of mucosa.

lobe of the liver 35 days after infection, and when a direct microscopic examination of the liver contents was done, the trophozoite stage was observed with a brown necrotic material and pus cells

Histopathological study

The histopathological effects of the attenuated and unattenuated parasite on the small intestine of the orally inoculated mice were studied. Microscopic examination of histopathological sections the presence of trophozoites in the small intestine layers showed after different periods of infection (15-35days). histopathologic sections showed the presence of trophozoites in mucosa, sub mucosa and serosa, necrosis and inflammatory response as shown in the figure (1). With regard to mice inoculated with 10 rad attenuated Gamma rays, inflammatory response with presence of defensive cells were observed included: Neutrophils, lymphocytes, monocytes and eosinophils.

Histopathological sections taken from the small intestine of mice inoculated with 15 rad attenuated parasite revealed the presence of trophozoites of the parasite in the in mucosa, sub mucosa and serosa while the sections of the intestinal tissue of mice inoculated with 25 rad attenuated parasite showed all the layers appear to be in good condition

DISCUSSION

The orally infected mice showed the occurrence of infection and infiltration of some of the trophozoites into the intestinal tissues and causing various histological impacts. The mechanism of the tissue invasion by the parasite includes four stages: The first is the adhesion of the trophozoites to the mucous layer and the intestinal epithelial cells, the second penetrating or destroying the epithelial barriers of the intestines by the trophozoites, the third is the lysis of intestinal epithelial cells by the trophozoites with the inflammatory response of the host cells, and the fourth is the resistance of the trophozoites to the immunological defenses of the host deep in the tissues and their access to different organs such as liver[16,17]. The adhesion of the parasite to the intestinal cells of the host is mediated by N-acetyl-D-galactosamine (Gal / Gal Nac) inhibitory lectin Galactose, which binds to the exposed lateral Galactose / N-acetyl-D-galactosamine found in glycoproteins of the target cell.

Gal / Gal Nac lectin is very necessary against the parasitic virulence. Lactin is a protein composed of hea vy chains and light chains with molecular weight of 170 and 35 kDa respectively[18]. The parasite may settle in the glandular crypts for a long time to feed and grow to the normal size of a trophozoite and then divide and multiply[19]. On adhesion, the parasite penetrates the intestinal epithelial barrier, causing damage to the intestinal epithelial cells by its proteolytic enzymes known as Cysteine proteases ,the proteins on the parasite surface which are present on the surface of the parasite which explains the role of the enzyme in destroying the cellular structure of the intestines, and facilitating the parasitic penetration or a result of cytotoxic released directly from the lysed white blood cells or from toxins released from the white cells after digestion of the parasite, this pathogenicity, according to the opinion of many researchers[20].

Al-Kubaisi[22] also showed in his comparative study of different culture media that HSP-1 was the most efficient medium for Giardia lamblia growth because the parasite could survive for eleven days on this medium. The results of the present study agree with what was previously mentioned regarding the presence of these stages when the mice are infected with the parasite, due to the observation of the parasite in the different layers of the small intestine tissues and causing different necrotic changes there. occur.

Our study also agree with[23] who found the occurrence of necrosis and corrosion on the surface of the mucous layer and the arrival of trophozoites to the deep glands of the intestine, as well as to the sub mucosal and muscle layers and *Giardia*-mediated increases in intestinal permeability result from alterations to the apical tight junctional complexes.

Ghosh et al.[24] in their histopathological study of small intestine of the mice inoculated with trophozoites at different times observed the corrosion of the epithelial surface and the destruction of the glands associated with the inflammatory response. Moreover, the results of the current study showed that the inflammatory response in the tissue sections of parts of the small intestine is a physiological process to protect the tissue, and it is a complex overlap between the vascular system, the immune system, and the repair mechanism,the inflammatory response may affect the infected organ or host by causing certain physical effects, such as change in body temperature regulation, and leukocytosis by bone marrow, which is due to chemical mediators, which result from white blood cells and platelet lysis[25]. The inflammatory response begins with the emergence of defensive cells such as white blood cells, especially polymorpho nuclear granulocytes, followed by monocytes and lymphocytes and accompanied by platelets and red blood cells accumulation, which is called cellular exudates. The appearance of the inflammatory response is attributed to the chemical mediators that affect the vascular endothelium where vascular permeability increases, and these mediators act as chemotactic agents to white blood cells, as well as activation of these cells as it is useful in the process of phagocytosis and killing of microorganisms[26].

The penetration of the trophozoites of the parasite to the mucous layer stimulates the inflammatory response by infiltrating of the neutrophils to these layers, which is observed in the histological sections prepared of the rectal biopsy of a patient infected with this parasite ,*Giardia*-induced diffuse shortening of epithelial brush border microvilli represents a key factor in the production of diarrhoeal disease *via* malabsorption and maldigestion[27]. Whether or not the diffuse loss of microvillous border surface area associated with giardiasis is related to the release of a "toxin" by the parasite, a phenomenon similar to the release of proteases in the bacterial overgrowth syndrome. Recent laboratory studies have shown the ability of the epithelial cells of the small intestine to produce a group of pro inflammatory cytokines in response to culture with Giardia parasites and in the presence of Escherichia coli bacteria. These substances act as host signals for chemical attraction and neutrophil activation leading to an inflammatory reaction in the mucous layer maldigestion[28,27].

In addition, the intestinal mucosal cells represent the body's first line of defense against pathogens, and the immunological mechanism also contributes to the presence of antibodies that are represented by secretory IgA, which can pass through epithelial membranes and prevents the passage of pathogenic microorganisms[29]. After adhesion with the parasite, the lymphocytes are activated in the Peyer's patch of the intestines and migrate to the lymph nodes to mature and then return to the digestive tract through the blood and lymphatic vessels[27].

CONCLUSIONS

The infection rate with *Giardia lamblia* among patients studied was high (24.0%).

1-The results of the current study showed high susceptibility of mice to infection with Entamoeba histolytica parasites when they are orally inoculated with the parasite, which may be explained because theyof different global strains and high virulenc

2-Radiation has an important effect on the activity of parasites and determining their ability to cause infection by direct influence on the (DNA) or the rest of its organic substances, and this effect is most severe in the divided phases of the cell.

3- Demonstration the ability of gamma rays in the attenuation of Giardia lamblia parasite for the purpose of anti-giardia vaccine .

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