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Estimating the Nature of Leishmania Major Different Immune Reactions in Laboratory Model Animals After Alum-Alm Injection

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Abstract

Introduction & Objective: This study is to determine how to react the vertebrate immune system after receiving or injecting Autoclaved leishmania Major with Alum-AluM and the main propose of conducting this study is that the immunity in leishmaniasis is often cellular, so it is expected that Vaccine-induced immunity formed by the predominance of cellular immunity.

Materials and Methods: In this regard some statistical review and number of research samples in 5 groups is considered. Each group included 5 BALB/c mice and each group consisted of two replicates.

Results: Titer challenges with Alum-Alm vaccine (i.e. new injected) with 3 booster dosage that equal to P >1:100 which means the test is negative. Titer challenge with Alum-Alm Vaccine (new injected) with 4 boosters that equal with P >1:100. Titer from mice vaccinated (i.e. past month after last injection that was sampled) with 3 boosters (P >1:100). The titer challenge with Alum vaccine with 4 boosters (after past month of their injection) P > 1:100. The titer for group 1 of vaccinated with two boosters as (P>1:100).

Conclusion: Based on this study it was revealed that the immunity orientation after injection of the same doses of Alum-ALM in BALB/c mice progressed to TH1 and IFN-Y stimulation and not seen any meaningful differences in IL-4 ELISA test (color reaction against 1: 400 dilution) and Alum-ALM injection failed to stimulate the TH2 response thus the results was as (P>1:100) so it was considered as negative test.

Keywords: Leishmaniasis, Immune, Laboratory Model, Alum-ALM.

Introduction

Almost 2 million new cases of Leishmaniasis seen as Annually around the world while Cutaneous Leishmaniasis (CL) covers about two-thirds of the cases. Although with the geographical spread of this disease, cutaneous Leishmaniasis (CL) as a disease, ignored without any effective intervention strategy specially in tropical area (1). In this study, in order to remove this challenge, we have used a kind of experimental model based on the application of BALB/c mice. In this regard due to its simplicity and flexibility, we attempted to use the CL model to provide significant information on cellular safety as well as

to detect and evaluate various vaccine excipients. Leishmaniasis includes a group of infectious diseases caused by various species of the protozoan parasite called Leishmania, which are found in 88 countries. In overall, almost 350 million people around the world are at risk of these parasites, in fact, prevalence of leishmaniosis is high, and it is only estimated 12 million cases infected around the world with 60000 deaths as annually (2). First generation vaccines for Leishmaniasis. In this field, the first history of vaccination against Leishmaniasis back to several hundred years ago, means when this type of disease was practiced in the Middle East. Bedouin tribal communities have traditionally exposed their children to sand fly bites to protect against skin damage. In some cases, pus from active lesions is also inoculated by removing the recipient's arm or thigh (3). This technique was modified in the early 1900s when culture conditions were created for Leishmania promastigotes, so used to prevent and treat active diseases (3,4-11). These studies formed the basis for large-scale vaccination trials that were carried out in the Soviet Union, Israel, and Iran and the success of these experiments depends on the survival and infectivity of the injected organisms (3,12). The results obtained show that many organisms which lost their virulence cause some particular delayed sensitivity, but they cannot prevent anything against the next usual infection. Furthermore, using survival vaccines has many problems such as uncontrolled skin lesions, exacerbation of psoriasis and other skin diseases, which paved the way for testing using killed parasites, but the contradictory results made this approach unfavorable (6). Vaccination of patients, that they defined as clinically even in severe cases, can induce high rates of treatment that it is with creating Immunity Type 1 (TH1) response in recipients. However, preventive vaccinations have been formulated with parasites killed with or without BCG (13-15). Since Leishmania parasites are intracellular pathogens, cell mediated immune responses are needed to restriction of the infection. Adjuvants selected for Leishmania vaccination it should be able to induce cell-mediated immune responses (16). Alum is the only adjuvant licensed by the WHO & US FDA to use in human vaccines (15). Aluminum based adjuvants used in vaccines mainly include aluminum hydroxide, aluminum phosphate and alum. Among these, aluminum hydroxide is the most commonly used chemical as adjuvant (17). Aluminum hydroxide-based adjuvants can recruit hemocytes, promote dendritic cell (DC) differentiation and accelerate local inflammatory reactions independently of Toll like receptors (TLR) (10). Aluminium containing adjuvants induce strong innate immune responses that consist of an influx of Neutrophils, Eosinophils, NK cells, CD11b+ monocytes and Dendritic cells (DCs) to the site of injection. Alum markedly improves IgG1 and IgE response against coinjected antigens and suppresses production of antibody isotypes associated with Th1 responses such as IgG2a/c (18,19).

Materials and Methods

Animals and parasites

In this study used many female BALB/c mice that Were obtained from the Research, Production and Breeding Center for Laboratory Animals of Razi Vaccine and Serum Research Institute. First of all, 30 mice (BALB/c) between 3-4 weeks were exposed to in vitro at 22° C to 25° C for 1 week. The reason for this matter is the identification of laboratory resistant mice and the weak mice which would be destroyed. Animal experiments were carried out according to Tehran University of Medical Sciences, Ethical Committee Acts.

Mouse immunization

In overall, only 28 mice remained and were divided into five groups inclusive 5 mice and one group with 3 mice, as follows:

[1]. Alum-ALM group with 3 boosters, [2]. Alum group with 4 boosters, [3]. Alum-ALM group with 3 boosters at two-week intervals, [4]. Alum group with 4 boosters at two-week intervals, [5]. Alum-ALM group with 2 boosters as a control group (PBS injected only once).

Table 1: Grouping mice and how to inject them.

Injection Volume	4 Boosters	3 Boosters	2 Boosters	1 Booster	Treatment Group
0.1*ml*	-	-	-	-	Control group or witness
0.1*ml*	-	Has	Has	Has	Group 1
0.1*ml*	Has	Has	Has	Has	Group 2
0.1*ml*	-	Has	Has	Has	Group 3
0.1*ml*	Has	Has	Has	Has	Group 4
0.1*ml*	-	-	Has	Has	Group 5

Challenge with Alum precipitated autoclaved L.major (Alum-ALM)

To each mouse in the experimental groups injected some Alum-Alm suspension (0.1ml) in the groin of the left leg as muscularly (IM) but it is notable that the standard WHO strain vaccine was provided by Razi Vaccine and Serum Research Institute. Each vial had a volume of 0.9 ml and contained 2.2 mg of Leishmania, and the Alum adjuvant was Al (OH) 3 hydroxide. Two weeks after the last injection for spleen sampling, from each group a mouse selected to euthanize by using cotton soaked in diethyl ether (C4H10O) (painless death). Mice died within 40 seconds to 1 minute. Then the carcasses of the mice were transferred to the laboratory and autopsied under completely sterile conditions.

Parasite burden

After removing the spleen in order to separate the capsule, the spleen is placed inside the cell strainer and placed on 150 ml Falcon tube. The spleen is pounded well by using the plunger tip of the syringe and wash

the cell strainer with 5 ml RPMI 1640 10X and transfer the contents of the Falcon to a 15 ml Falcon. In following the suspension prepared in this way centrifuged at 2500 RPM for 3 minutes at 4° C.

Then the supernatant is taken Out and 1% ammonium chloride added to the precipitate as a lysis buffer and placed at room temperature for 5-10 minutes. In coming up, we vortex the falcons to homogenize the suspension and the formed suspension was cultured by adding RPMI 10X culture medium containing FBS (we increased the concentration of the medium to 10% for better cell growth and enrichment) in a 50 ml Falcon tube. And the volume is adjusted in 10 ml. At this stage, mitogen (Con-A) is added. It is notable that the ratio of suspension to culture medium is equal to 20% (1 to 5). Then, a certain volume of sample (about 10µl) mixed with trypan blue dye is counted. Cell counts were performed under a light microscope and most of the cells were monocytes and macrophages.

The average number of cells counted is as follows:

- Tube 1 (Alum-Alm group 3 injected reminder): 68
- Tube 2 (Alum group): 83
- Tube 3 (Alum-Alm group 3 injected reminder): 59
- Tube 4 (Alum group): 93
- Tube 5 (Alum-Alm group with two reminders): 68
- Tube 6 (PBS group): 410

Cytokines evaluation

The suspension made in this way, was poured into a plate containing 24 wells 1CC. this well contains RPMI 10X and considered as a positive control to control the operation. Therefore, the plate incubated at 37° C and 5% CO_2 for 72 hours and the plate was checked daily to record the growth rate of the parasite in the environment. Eventually in order to separate the supernatant and measure the cytokines, we pour the contents of each well into 2 ml tubes and centrifuge for 5 minutes at 12,000 RPM. The levels of cytokines were assessed by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (R&D System Elisa kits) with protocols provided by the manufacturer.

Statistical analysis

One-way ANOVA analysis (Multiple comparisons Tukey’s post hoc test) was performed using the GraphPad Prism software.

Blood sampling from mouse hearts to measure IL-4 and IFN-Y cytokines in the blood

In this study, in addition to spleen extraction, blood samples were taken from the hearts of BALB/c mice to

measure animal serum IgM. To do this, after anesthetizing the animal, an insulin syringe was inserted into the ventricle while the heart was still beating, and blood was drawn into the heart. The tubes placed in Ben Marie at 37° C for 5 minutes and then centrifuged at 2500 RPM for 5 minutes.

Antibody response

Then the serum separated and poured into other tubes to place in a freezer at -80° C. In the following, the IgM level of the groups assessed by ELISA method. So according to this matter, almost 96 house plates (Microtiter) with circular bed and mouse IgM measuring kit were used. But first the plate was coated with trapping antibody and then the system was supplemented with conjugated antibody. in this regard by determining the minimum and maximum sensitivity of the kit, the samples were diluted and placed inside the plate in duplicate All steps were performed according to the kit's agenda. OPD (Orthophenylene Diamine) was used as a substrate and H2SO4 as a reaction stop. The OD of each sample was read by the ELISA reader at 492 nm for OPD and 620 nm as the source wavelength. So, the number of injections of mice varies and given the grouping and its type of the test performed (ELISA and direct agglutination) was so different. In order to do this type of test (ELISA TEST) Alum-ALM was injected 4 times with one week interval and 3 injections were performed for direct agglutination test with 21 days interval. All injections were Intramuscular (IM) with a volume of 0.1 ml and performed by using a sterile insulin syringe. That’s why the usage of 1640 RPMI medium is to produce monoclonal antibodies, a type of mouse myeloma cell line that can grow in the laboratory but does not produce antibodies is needed. The most famous of these cells is called SP2/O, which originates from BALB/c mice. One of the characteristics of these cells is that they are not able to grow in an environment containing aminopterin and die in this culture medium, which is also called HAT medium.

But in normal culture media such as RPMI 1640 and DMEM, they grow easily in the presence of penicillin and streptomycin antibiotics and 10% fetal bovine serum (FCS). The obtaining suspension was placed in an incubator at 37° C with 5% CO_2 for 72 hours. Then the supernatant was then separated under sterile conditions by centrifugation and the samples from each group, which included Alum alone, Alum-ALM with 3 reminders, and Alum-ALM with two reminders and PBS, were poured into separate tubes. In order to do a direct agglutination test, blood samples were taken from the heart of mice after the first injection for a period of 21 days. Therefore, the next two injections performed with a period of 21 days and blood samples were taken after blood sampling, the samples were centrifuged at 2500 RPM for 5 minutes, and the final serum was separated and poured into other tubes to place in a freezer at -20° C. Then in order to do the ELISA test we need to isolate and culture of spleen cells to perform this mentioned test.

The cells were isolated by cell strainer then washed by RPMI 1640 without FBS serum with a mixture of

penicillin and streptomycin and the suspension that at the end all of them centrifuged. In this field, the final sediment contains spleen cells and in following it reaches the sediment then 1% ammonium chloride was added to the precipitate as a lysis buffer, RPMI 10X culture medium containing FBS and Concanavalin A as a growth stimulant.

By adding 1% ammonium chloride, stem cells were isolated from red blood cells and cell purity was increased. This type of suspension made in this way then should be kept in an incubator at 37° C and 5% CO_2 for 72 hrs. With particular consideration as microscopic examination, can be seen these purified spleen cells which were mostly monocytes and macrophages. This method performed separately for each group of mice (Alum, Alum-ALM and PBS). For long-term storage, the purified cells were amplified several times and stored in deep freezing. In order to do this, we have to add to each cryotube vial about 6 million cells with a cell viability of more than 95% in a volume of less than 100 microliters and again we can add to them one milliliter of cryoprotectant solution (solution containing 70% medium). Cultivation is 20% FCS and 10% dimethyl sulfoxide (DMSO). Therefore, these vials will be packed to keep it at -70° C, in this way the temperature is reduced by about one degree Celsius per minute. After overnight storage at -70° C, these vials can transfer to liquid nitrogen at -196° C. The main goal of doing this is to store for a long time.

Results

In this study, the data were analyzed by using SPSS 5 software as standard Error mean (SEM). And in following, the data which obtained by behavioral tests and biochemical evaluations are compared using one-way ANOVA followed by comparative test after LSD. Statistical significance was considered at $P < 0.05$**.** The main propose of this type of study is to evaluate the effect of leishmaniasis vaccine on cutaneous leishmaniasis changes in TH2 cells and the rate of cutaneous leishmaniasis in infected mice. In this chapter, the collected information is analyzed using descriptive and inferential statistical methods and is presented in two parts:

The first section is related to the information of the subjects and in the second section is about the inferential statistical methods which are used to test the research hypotheses and analyze the data and finally based on them the results reported. Scenarios (groups): It is divided into 5 groups. Each group consisted of 5 BALB/c mice and two replicates (groups one, three, two and four received equal doses of the vaccine), which are as follows:

0 = Negative witness.

1 = Vaccine injection with three boosters.

2 = Alum injection alone with four boosters at 1-week intervals.

3 = Inject the vaccine with three boosters at two-week intervals.

4 = Alum injection with four boosters at two-week intervals.

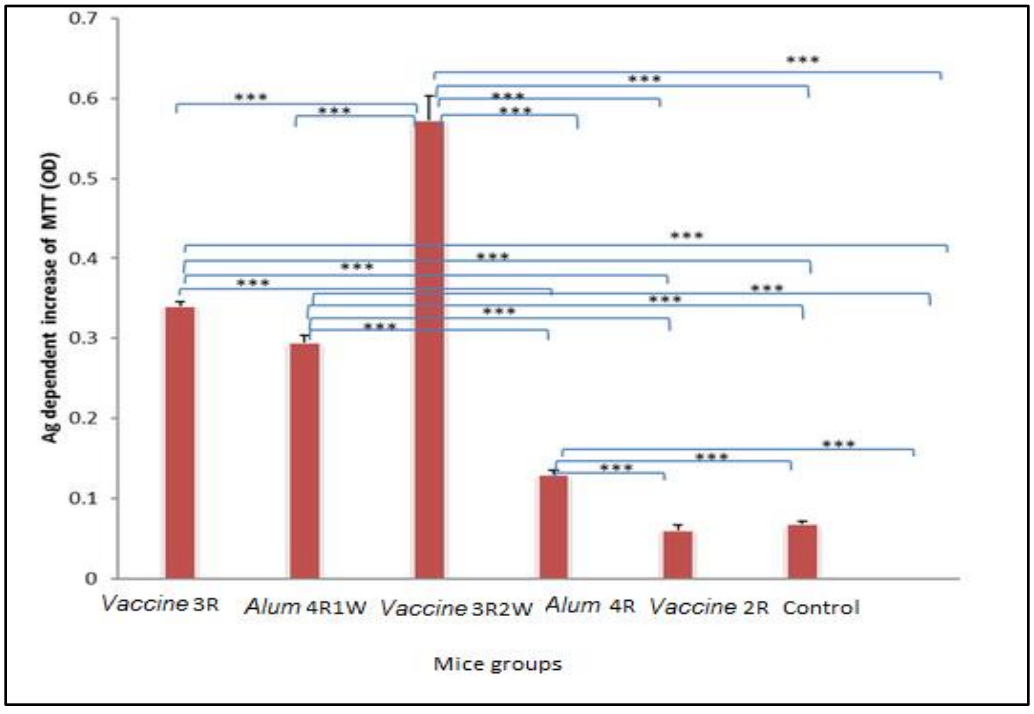
5 = Vaccine injection with two boosters.

Reviewing the final results by using killed Leishmaniasis vaccine on cutaneous Leishmaniasis rate (IL-4 and IFN-Y secreted cytokine levels) of BALB/c mice with cutaneous Leishmaniasis Obviously, in the table below we can see the results of analysis of variance test, but the final results released that there is no significant difference between the means in the five groups studied (P = 0.000). In this case, H0 is rejected. This means that the use of killed Leishmaniasis vaccine has a significant effect on the rate of cutaneous Leishmaniasis (levels of cytokine IL-4 and secreted IFN-Y) in BALB/c mice with cutaneous Leishmaniasis.

Table 2: Indicated the results of Analysis of Variance (*ANOVA*) test for vaccine injection on cutaneous leishmaniasis (cytokine level *IL-4* and secreted *IFN-Y*) *BALB/c* mice with cutaneous leishmaniasis.

Source of change	Sum of squares	Degree of freedom	Mean of squares	F	P
Intergroup	614.1	1	541.2	1.14	0
Intragroup	293.6	4	712.3		
Total	9.07.07	5	1253.5		

Figure 1: *MTT* is a colorimetric study to evaluate cellular metabolic activity and in the figure above the existing groups are examined. In fact, the values of light density obtained from stimulation of mouse spleen cells by vaccine injection and based on this grouping we try to show.



Post hoc test result: Given the meaningful test, Tukey test is used for post hoc test.

Table 3: Tukey test results to compare means with *SPSS* output.

	Groups average	DF	Mean square	F	Sig.
Between groups	44.67	1	7.232	56.34	0
Whitin groups	13.93	4	0.144		
Total	58.6	5			

Table 4: In Table 4 indicated some final results in this field of killed Leishmaniosis vaccine on cutaneous leishmaniasis rate (injection of identical doses of *Alum-ALM*) in *BALB/c* mice with cutaneous leishmaniasis.

Treatments	N	Subset for alpha = 0.05		
		1	2	3
Vaccine injection with 3 boosters	5	-	0.766	-
Alum-injection alone with 4 boosters as a week apart	5	-	0.798	-
Alum-injection with 3 boosters as two weeks apart	5	-	0.703	-
Alum injection with 4 boosters	5	-	0.822	-
Vaccine injection with 2 boosters	-	-	-	0.617

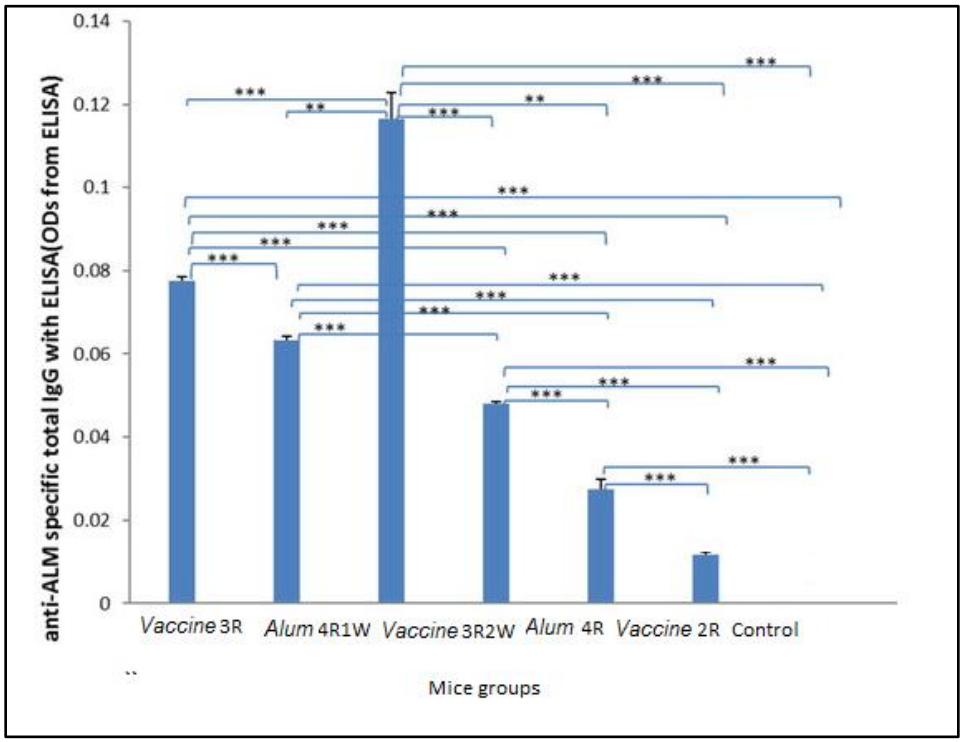
Table 5: Summary of ANOVA results for vaccine injection period on cutaneous leishmaniasis (same doses of Alum-ALM) in BALB/c mice with cutaneous leishmaniasis.

Source of change	Sum of squares	Degree of freedom	Mean of squares	F	P
Intergroup	614.1	1	296.8	-	-
Intragroup	369.42	4	52.14	1.17	0
Total	983.62	5	48.94	-	-

Obviously, what we can see in table above is based on the results of analysis of variance but the not seen any significant difference between the means in the five groups studied (*P = 0.000*); So, in this case *H0* is rejected.

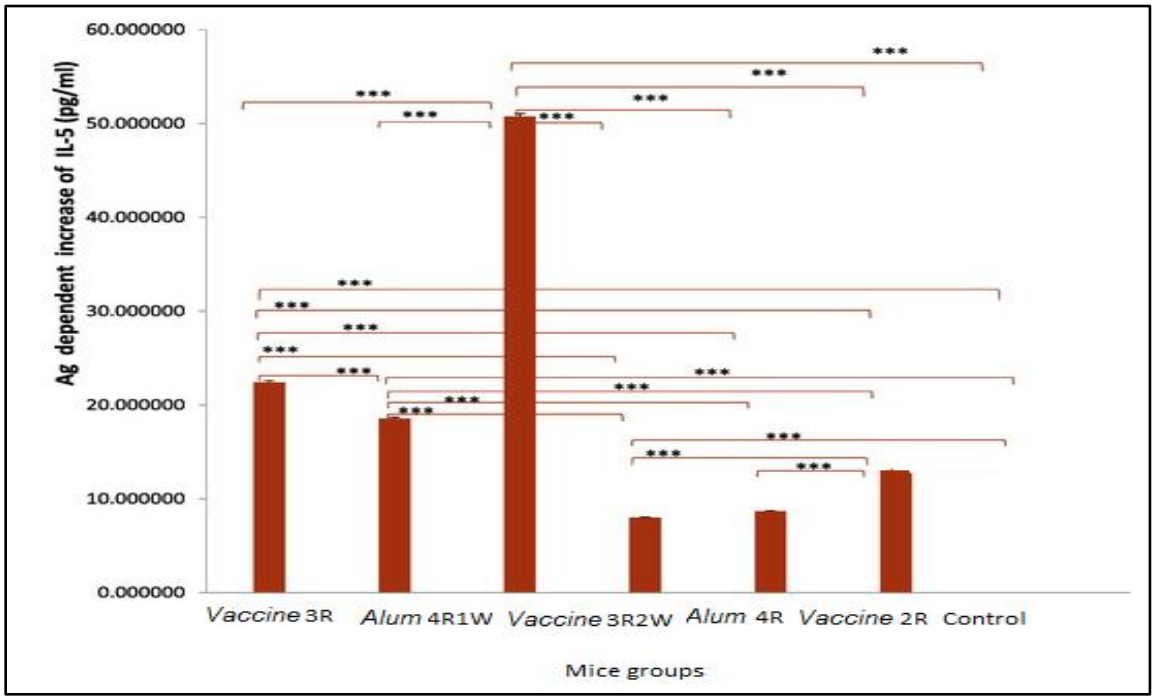
Which means the use of the killed leishmaniasis vaccine has a significant effect on the rate of cutaneous leishmaniasis (injection of the same doses of *Alum-ALM*) in *BALB/c* mice with cutaneous leishmaniasis.

Figure 2: what we can see in this figure is about a kind of Comparison of light densities by ELISA for IgG-specific *anti-Alum* serum among different groups of mice.



According to the study, vaccinated mice produced more anti-Alum-specific *IgG* than Alum-only and control groups.

Figure 3: As we seen in figure 3 is about the results of *IL-5* spleen production in different groups of mice.



Based on some studies, spleen IL-5 values were higher in the vaccine groups than in the Alum only groups and this type of increasing seen in groups that receiving vaccine.

Table 6: The result of post hoc test for mice in groups one, four and control, and based on the significance of Tukey test it was used for post hoc test.

	Group average	Df	Mean square	F	Sig.
Between groups	39.12	1	8.46	49.8	0
Intergroup	18.37	4	0.117	-	-
Total	54.49	5	-	-	-

The results for all scenarios included: the control group indicated a negligible effect of vaccine injection on BALB/c mice with cutaneous leishmaniasis.

Table 7: What we can see in table 7 is about the result of Leishmaniasis vaccine which used on immune responses to TH1-TH2 cells and TH1-TH2 cells of BALB/c mice with cutaneous leishmaniasis.

Treatments	N	Subset for alpha = 0.05		
		1	2	3
Vaccine injection with 3 boosters	5	-	0.756	-
Alum injection alone with 4 boosters as one week apart	5	-	0.704	-
Vaccine injection with 3 boosters with 2 weeks apart	5	0.86	-	-
Alum injection with 4 boosters	5	0.89	-	-
Vaccine injection with 2 boosters	-	-	-	0.608

Examination of the results of the Tukey test shows that a group of animals that exercise and receive *STZ* had the greatest effect of vaccine injection on *TH1* cell changes and the level of treatment of *BALB/c* mice with cutaneous leishmaniasis was higher in this group.

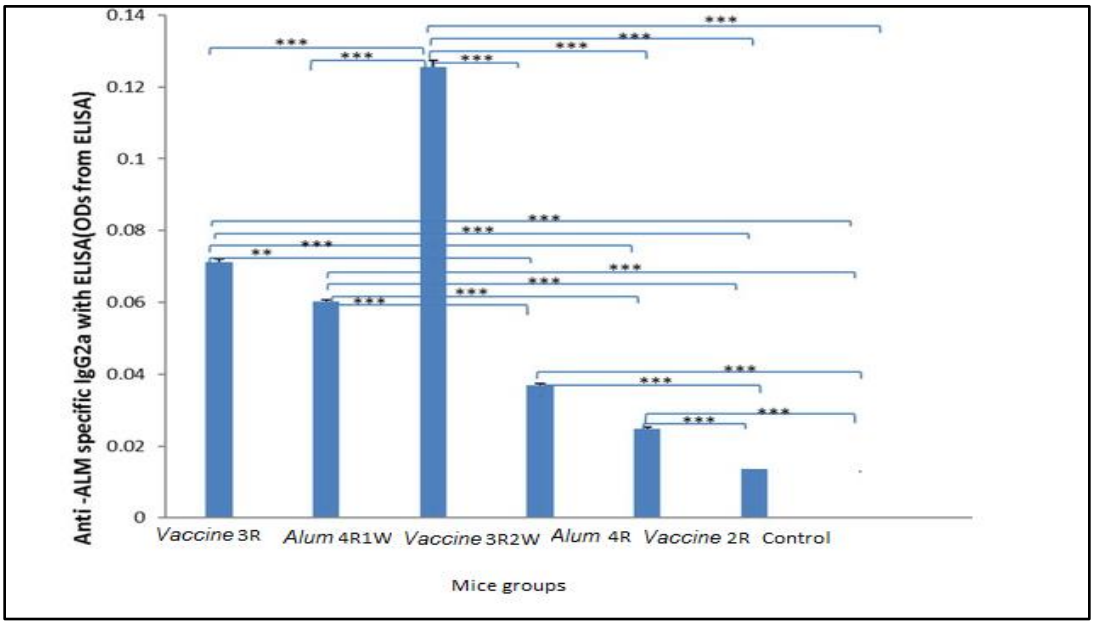
Table 8: Summary of ANOVA results to evaluate the effect of vaccine injection on immune responses to *TH1-TH2* cells in the hippocampus.

Source of change	Sum of squares	Degree of freedom	Mean of squares	F	P
Intergroup	804.75	1	488.12	-	-
Intragroup	416.91	4	279.4	0.98	0
Total	1221.96	5	727.31	-	-

As can be seen in Table 8, based on the results of analysis of variance, there is a significant difference between the means in the five groups (P = 0.000); In this case, H0 is rejected. This means that the use of

killed leishmaniasis vaccine has a significant effect on immune responses to TH1-TH2 cells in TH1-TH2 cells of BALB/c mice with cutaneous leishmaniasis. The test results showed that at a significant level of 0.000 there is a significant difference between the average of twelve weeks of pre-test and post-test pre-test aerobic exercise in all five groups (P = 0.000).

Figure 4: Comparison of optical densities from the ELISA test for *IgG2a-specific anti-Alum* serum in different groups of mice. (p value < 0.05/: p value < 0.01/***: p value < 0.001).**



Two weeks after the last booster injection samples were isolated from mice and the sera were collected to evaluate IgG2a antibodies by ELISA method. The study indicated that in all scenarios, the control group had a negligible effect of vaccine injection on BALB/c mice with cutaneous leishmaniasis. It is noteworthy that according to the study, and given the above figure, groups of vaccinated mice produced more IgG2a-specific anti-Alum antibody than only Alum and control groups.

Table 9: Tukey test results to compare means with spss output.

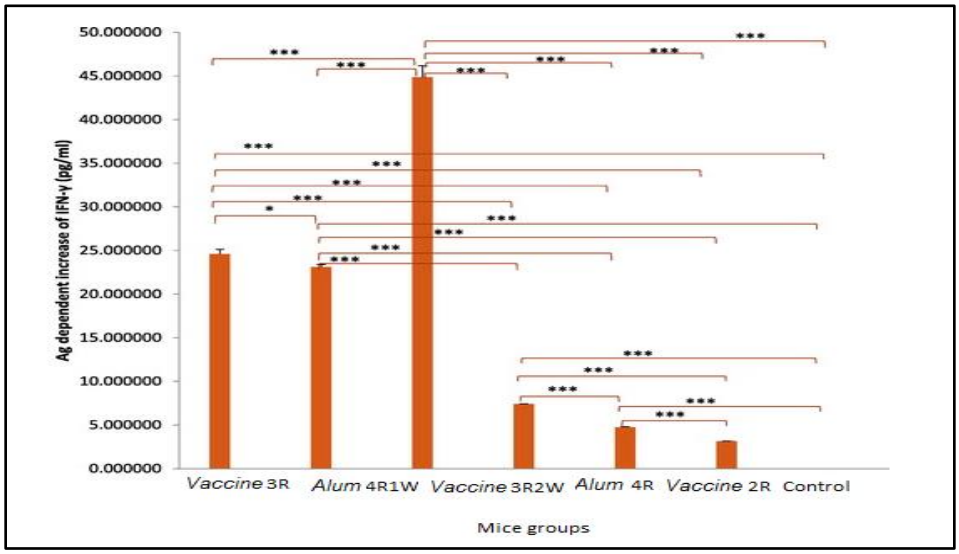
	Group average	Df	Mean square	F	Sig.
Between groups	49.05	1	8.98	61.44	0
Within groups	10.45	4	0.253	-	-
Total	59.5	5	-	-	-

To assess the significance of the differences between various groups One-way ANOVA statistical test was used. In the case of significant F value, the Tukey–Kramer multiple comparisons test was carried out as a post-test to compare the means in different groups of mice. P<0.05 was considered as statistically significant.

Table 10: Results of leishmaniasis vaccine use on immune responses.

Treatments	N	Subset for alpha = 0.05		
		1	2	3
Vaccine injection with 3 boosters	5		0.687	
Alum-injection only with 4 boosters a week apart	5	0.743		
Vaccine injection with 3 boosters with 2 weeks apart	5	0.798		
Alum injection with 4 boosters	5	0.814		
Vaccine injection with 2 boosters				0.554

Figure 5: It is about Evaluation of the immunogenic effect of mice by IFN-Y secretion in the spleen and by injection of vaccine and Alum at intervals of one and two weeks. (p value < 0.05/: p value < 0.01/***: p value < 0.001)**



According to the figure above, the *IFN-Y* secreted in the *Alum-Alm* vaccine groups was higher than in the other groups, and this amount was much higher in the group that received the vaccine 2 weeks later. Reviewing of this study indicated that in all scenarios, the control group showed a negligible effect of vaccine injection on BALB/c mice with cutaneous leishmaniasis.

Discussion

Leishmaniasis is counted as one of the most important health problems in the world. The occurrence of leishmaniasis depends on some indicators such as: Leishmania species virulence, genetics and immune cell response. Although with many advances in disease control and laboratory facilities for the confirmation and diagnosis of parasitic infections, leishmaniasis is still one of the most common infections due to various

reasons, especially the increase in drug resistance and resistance of vectors to insecticides. They are considered in developing countries. In recent decades, studies on the appropriate vaccine have been extensively conducted, and much information has been published about the activities performed in the past few decades. Nowadays, vaccination is one of the most important methods of prevention and protection against infectious diseases and malignancies.

Thus, the main goal of vaccination is to establish an appropriate, strong and long-lasting immune response against the pathogen, therefore, sounds it is necessary to use the ability of compounds such as adjuvants that enhance these responses. In fact, Glenny.et al. in 1920s discovered the effects of Aluminum salts as Adjuvants in vaccines, stating that Aluminum Adjuvants act as a reservoir and release antigens slowly over a long period of time. Releases and subsequently induces more intense immunity (20-25), But in this study, in addition to Alum, *Alum-ALM* was also examined, and it was found that in groups containing *Alum-ALM*, there is better safety than *Alum* alone.

Mineral-based adjuvants actually contain mineral salts, the most well-known of which are aluminum salts (*Alum*) and calcium phosphate. *Alum* adjuvants are among the most widely used adjuvants ever presented. In this field the Aluminum hydroxide and Aluminum phosphate are chemically different (26). A comparative study examined the ability of both types of alum to induce humoral immune responses in *C57BL/6* mice. The strongest inducer of antibodies as well as Th2-type cytokines like *IL-4* and *IL-5* was related to aluminum hydroxide (27,28).

Different studies indicated that the alum adjuvant enhances the humoral immune response by *Th2* motility resulting in B lymphocyte proliferation. Other features of this adjuvant are the ability to stimulate the production of monocytes and granulocytes and also increase the differentiation of monocytes into dendritic cells. In addition, alum adjuvant is able to induce a type II inflammatory response that is associated with the accumulation of eosinophils at the injection site and increased production of specific *IgE* and *IgG1* antigens, and this was clearly observed during this study. The main advantage of *Alum* is its ability to increase hemoral immunity and produce protective antibodies with minimal side effects. Other advantages of this method are a kind of stabilization of *Alum*-associated antigens as well as large-scale production (7), but this study found that the immunity induced by Alum was not very long.

In a study, the immunogenicity of *Autoclaved L. major* (*Alum*) with *ALM-BCG* adjuvant against *ZVL* was assessed using *LST* test. A very significant difference was observed between the vaccinated groups and the control group ($P = 0.001$). The general findings in this study showed that the *Alum-ALM + BCG* and Imiquimod vaccines were safe and tolerable and had no significant side effects. It is notable that adverse reactions were seen only in people who were injected with *BCG* with the vaccine in the form of mild to

moderate pain, nodule formation, ulceration and scaling. The results showed that immunization with these compounds could stimulate the cellular response of *TH1*, *IFN-Y* secretion and *IL-12*. some researching indicated that *CD4* and *CD8* are essential for disease control (9).

Heat-killed leishmaniasis used as an experimental vaccine against various forms of leishmaniasis in Iran and Sudan (8). In another study, the immune response was examined in *BALB/c* mice immunized with the *ALUM-ALM* vaccine at different doses. Low-dose immunization of *Alum-ALM* mixed with an adjuvant appears to elicit an *TH1* immune response in *BALB/c*-susceptible mice (7). Evaluation of humoral immune response in another study showed that *IgG1* and *IgG2a* antibody titers were significantly *(P <0.05*) low in mice containing *Alum_ALM* mixed with *BCG*. On the other hand, *IgG2a* and *IgG1* antibody titers were significantly higher *(P <0.05*) in the group of mice that received *Alum-ALM* or M. Vaccine alone.

The effect of a dose of 1 mg of *ALM* with 0.1 of *BCG* dose was tested in an endemic region as well as in Iran. The immunization of the vaccine was greatly reduced and only 16.5% succeeded in providing protection. Three injections of the vaccine had no greater protection than the two injections in Iran (unpublished observations) and it was decided that the vaccine was not sufficiently immunogenic and that other adjuvants were needed to increase immunity. To enhance the immunogenicity of the vaccine, *ALM* was added to *Alum* (aluminum hydroxide) and the resulting *Alum-ALM* was mixed with *BCG* prior to injection.

In fact, the addition of *ALUM* to *ALM* enhances the body's immunity as a killed injection of leishmaniasis in alum with the production of *IL-12* and increases the type 1 cellular immune response and protection against cutaneous leishmaniasis, and this was observed in this study. killed leishmaniasis used as an experimental vaccine against various forms of leishmaniasis in Iran and Sudan. Other studies revealed that *ALM* alone cannot produce a protective immune response against leishmaniasis and should be injected with effective adjuvants.

This antigen has been studied in combination with *BCG* and Alum and the results have shown that immunization with these compounds can stimulate the *TH1* cellular response, *IL-12* secretion and *IFN-Y* secretion. Dolati et al., Following previous studies, compared the safety and immunogenicity of the *BCG* vaccine with *ALM-Alum* and found that it had side effects such as nodules and ulcers. In a study, Mudaber et al. Compared the effect of injecting the *Alum-ALM + BCG* vaccine against BCG alone against anthroponotic cutaneous leishmaniasis (human-to-human transmission). The announced results showed that *Alum-ALM + BCG* has more immunogenicity than *BCG* alone. *ALM* combined with Mycobacterium *vaccae* and *BCG* then all of them evaluated. And based on this study, injection of *M. vaccae* with low dose of *Alum-ALM*, although it was not able to induce immunity in *BALB/c* mice but changed the cytokine pattern in favor of *Th1* response. Also, different doses of *Alum-ALM* combined with *BCG* to change the immune system in favor of *Th1*

response and in this case, there was no significant difference between *BCG* and *M. vaccae* helpers. On the other hand, injection of high dose of *Alum-ALM* together with high dose of *M. vaccae* stimulated the immune system response to *Th2* type stimulation.

In fact, many studies in mouse models shown that the acquired resistance to Leishmania major infections is highly dependent on the induction and formation of *Th1* responses, which is associated with the *IFN-Y* production response, which can Provide immunity to subsequent infections by stimulating macrophages to kill parasites. Thus, the role of cytokines in inducing an immune response has been demonstrated in a number of studies. In humans, *IL-4* has been shown to be associated with the non-curable form of cutaneous and visceral leishmaniasis. Based on this type of study it was determine that immunity orientation after injection same dosage (*Alum-Alum*) in *BALB/c* mice goes toward *TH1 & IFN-Y* but not seen any meaningful differences in *IL-4* ELISA test (color reaction against 1: 400 dilution) *Alum-ALM* injection failed to stimulate the *TH2* response and the results indicated this matter as *p >1:1:00* and it was considered as a negative reaction.

Conclusion

Based on this study it was revealed that the immunity orientation after injection of the same doses of Alum-ALM in BALB/c mice progressed to TH1 and IFN-Y stimulation and not seen any meaningful differences in IL-4 ELISA test (color reaction against 1: 400 dilution) and Alum-ALM injection failed to stimulate the TH2 response thus the results was as (P>1:100) so it was considered as negative test.

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Ethics approval

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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