

Evaluation of New *Leishmania* Major Vaccine Against Immune Responses (Serum's IL-17 and IL-23 and Spleen White Pulp Changes) Post Challenging with *Leishmania* Amastigotes in Balb/c Mice

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Abstract

Introduction: *Leishmania* is a protozoan parasite that has a life cycle in the form of an amastigote (in the mammalian body and in the middle of the macrophage) and promastigote in the mosquito saliva and culture medium with different antigenic determinant sites.

Aim: Evaluation of new *leishmania* major vaccine against serum's IL-17 and IL-23 and spleen white pulp changes post challenging with *leishmania* amastigotes survival rate of live mice was also evaluated for the second time after re-exposure.

Result: Number of pulp spleen had almost significant differences between doses 100 and 200 µg/ml. LB group had lowest levels of IL-17, IL-23 and mouse weight and highest MPS, PSW/MW, number of pulp spleen and spleen weight was almost significant. LT group had highest levels of IL-23, whether, had lowest levels of mean pulp size, percent of spleen weight/mouse, and spleen weight was almost significant. LBT group had highest levels of IL-17. Lowest levels of IL-23 belonged to LB and control group and highest levels of percent of spleen weight/mouse weight and number of pulp spleen belong to control group also.

Conclusion: In LT group, with adjuvant-*Teucrium polium*, weight of spleen and number of pulp spleen were at lowest level and had highest IL-23. It can be argued that this adjuvant was better than BCG and causes the mice to exhibit stronger immune responses that are likely to go Th1 immune response and protective effect after challenge with live *leishmania* major.

Keywords: *Leishmania*; Vaccine; Challenge; Amastigote; Spleen; Cytokines; Immune system

Introduction

It is thought that the protozoa promastigote phase passes to amastigote and vice versa to be accompanied with the incidence of some antigenic markers or loss associated others [1]. It is unknown that, why in some individuals, such as resistant mice within 6 to 8 weeks of wound is healing, while, in Balb/c mouse leads to leishmaniasis and involvement of the liver, spleen and bone marrow, and at least death [2,3]. Many vaccines have been made in different ways, which may have been somewhat effective on animal models, but have not been able to provide protection in humans yet [4]. The lipophosphoglycan, which is on the surface of the *leishmania* promastigotes, can cause the parasite to die during the mechanisms of phagocytosis killing. There are several unknown mechanisms for parasite escape from the fork of the immune system in the body, which can help to survive the parasite in the macrophage [5]. *Teucrium polium* is used in Iran for abdominal pain, dyspepsia, colds, and type 2 diabetes, and it seems that this adjuvant has an anti-inflammatory and

antioxidant effect and helps the immune system to fight against infections and fungi and cancers [6]. In this study, the new leishmaniasis vaccine with two adjuvants BCG and *Teucrium polium* after re-exposure with *leishmania* amastigote that was isolated from the *leishmania* ulcer interleukins 17 and 23, mouse weight, spleen weight, percentage of spleen weight/mouse measured weight of the mouse, the number of white pulp and the increase in the size of the white pulp evaluated in sensitive Balb/c mice. The results were analyzed after statistical analysis and survival rate of live mice was also evaluated for the second time after re-exposure.

Material and Methods

This study was conducted in accordance with the Helsinki Declaration. The protocol is supported by both the research fellows of the School of Medicine and Deputy of Research of Tehran University of Medical Sciences. Tehran, Iran.

Culture and isolation of leishmania parasites

Leishmania parasites and promastigote antigens from the *L. major*, WHO strain prepared from the Pasteur Institute. It was cultured and grown in medium NNN (Novy-Mac Neal Nicoll) medium and/or 5%-10% heat inactivated fetal calf serum. The harvested parasites were washed three times with normal saline (0.9%) or phosphate/salt serum. The parasites were counted using a Neubauer chamber and then kept at -70°C until use. Then the collected parasites reached the concentration of 5.92×10^8 , and were divided into five equal volume tubes. They were ready to prepare antigen for vaccine preparation like before [7-15].

Vaccine preparation

The five batches were mixed and centrifuged, and the sediments were dispensed into sterile vials. *Leishmania* components vaccines were tested for complete parasitic infections. The culture was carried out on a plate of blood agar and injected into the footpads Balb/c mice.

Detailed procedures have already been described [7-15]. In short, just before the injection, the BCG vaccine "SSI" was suspended in a suspension of SST and 0.1 mg BCG (first dose) or 0.01 mg BCG (consecutive dose) was added to each bottle containing promastigote.

Based on previous studies, 100 mg/0.1 ml or 200 mg/0.1 ml of *leishmania* protein was selected at each dose of the temporary vaccine to formulate and prepare the vaccine. The protein content of each dose was estimated by the Lowry method [16]. The vaccine was kept at 4°C until injected. BCG adjuvant for each injection dose is included 2×10^8 CFU/0.1 ml. To prepare *Teucrium polium* adjuvant : 400 mg of *Teucrium polium* alcoholic extract in 1 ml distilled water without endotoxin deionized, 205 mg/0.1 ml was used for each of the doses of the *leishmania* antigens given above, and two injection doses containing 100 µg/0.1 ml antigens or 200 µg/0.1 ml containing adjuvants [7-15].

Animal model

They included 48 Balb/c mice kept at the Faculty of Medicine's animal House. For detailed procedures please refer to Latifynia and collaborations [7-15]. In brief, Balb/c and traditional white laboratory mice (n=48) were obtained at three months old from the Pasteur Institute.

Vaccination

All doses of *leishmania* vaccine were injected intra dermally to the tail (or legs) of mice. Dosages (100, 200 µg protein) were used as follow: Group LB (100,200) received 100, 200 µg/0.1 ml antigen combined with BCG. Group LT (100, 200) received 100, 200 µg/ 0.1 ml antigen combined with alcoholic extract of *Teucrium polium*. Group LBT (100, 200) received 100, 200 µg/0.1 ml antigen combined with BCG and alcoholic extract of *Teucrium polium*. The control group did not receive any injection of *leishmania* vaccine.

All groups were injected subcutaneously one week later using similar doses (booster dose), and seven days after booster they challenged with amastigote that removed from *leishmania* lesions from

mouse that had leishmaniasis infection and solved in 2 ml normal saline, until a thick solution was obtained, and as in latest step, it was inoculated 0.1 ml of this solution to each mouse as challenge.

Sampling

Twenty five days (4-5 weeks) after challenge, all injected animals, and also control group, were weighted, Blood sampling and spleens removed and weighted and then were performed all of the following experiments and statistical analysis.

Spleen hematoxyline and eosine staining

All removed spleens were cut in equal length and width and fixed in 10% formaldehyde buer solution. Stable protein tissue was processed in tissue processing. Paraffin blocks were made and tissue sections of 4 to 5 microns were prepared and stained with Harris Hematoxyline and Eosine. The fixed spleen was stained in paraffin blocks and stained in a tissue processor .Tissue sections (5-6 µm thick) and stained with Hematoxyline and Eosine. The number and diameter of the white pulp of the spleen (lymphoid spleen follicles) were examined using a microscope of light with the fragment of the eye. The diameter of the spleen white pulp sections was measured and compared with each other and with the controls.

Serum cytokine assay

To evaluate cytokine levels in the sera of the animals, at most 2 ml of blood sample was taken from each mouse, and its serum was separated by using the routine standard method. The levels of the IL-17 and IL-23 in the six injection groups and the control group mice were determined by the sandwich ELISA method according to the recommendations of the manufacturers. Mice serum levels of the IL-17 and IL-23 in the subjects were measured by using an automated micro plate reader set at 450 nm. The sensitivity limit was 20 pg/ ml for the IL-17 and IL-23.

Statistical analysis

Data were obtained using statistical software (SPSS Inc., Chicago, IL, USA). The means were analyzed using standard variance analysis simple factorial test with two-way Student-Newman-Keuls methods. The correlation coefficient was determined using a Pearson bivariate, two tests.

Results

IL-17: According to Figure 1, the highest serum levels of IL-17 were obtained between the control group that did not receive any vaccine and the three injection groups (LB, LT, LBT) that received the vaccine before challenged with amastigotes emission from the wound, with a 95% confidence interval belong to the control group. The results of analysis variance (ANOVAs) for serum level IL-17 between three injection groups (LB, LT and LBT) no considering two injection doses (100 and 200 µg/ml) and also two injections doses (100 and 200 µg/ml) no considering to three injection groups (LB, LT and LBT) did not show significant differences (Tables 1 and 2).

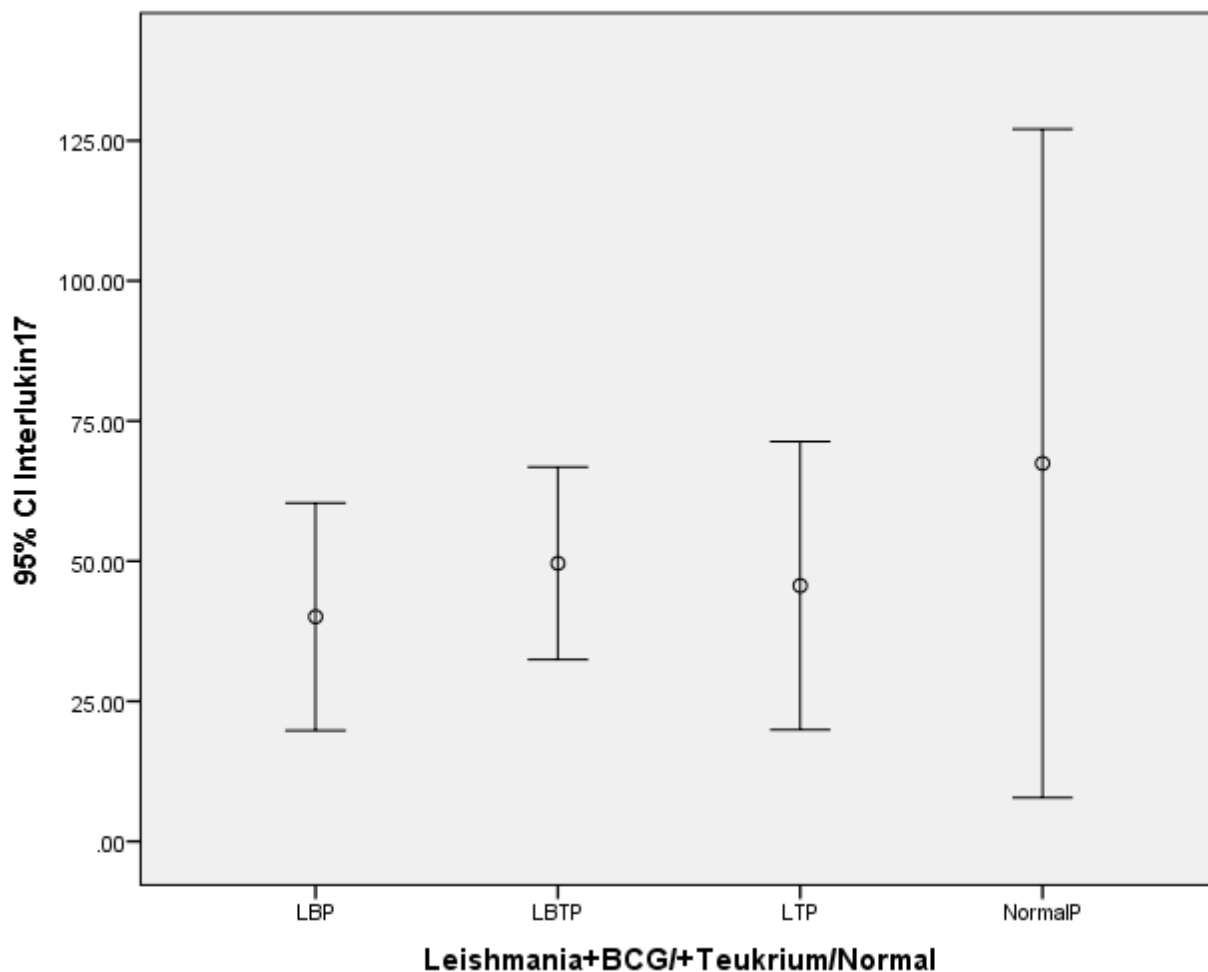


Figure 1: The post challenge with live amastigote results of serum levels of IL-17, for three injection groups (LB, L and LBT) and normal group with 95% confidence interval.

IL-23: According to Figure 2, compared to control group that did not receive any vaccine and three injection groups (LB, LT, LBT) that received the vaccine after being exposed to re-exposure to amastigotes emission from the wound, with a 95% confidence interval showed that highest serum levels of IL-23 related to LT group and lowest belonged to control and LB groups which were equal together. The results of

analysis variance (ANOVAs) for serum level IL-23, mouse weight, spleen weight, between three injection groups (LB, LT and LBT) no considering to two injection doses (100 and 200 µg/ml) (Table 2), and also two injections doses (100 and 200 µg/ml) no considering to three injection groups (LB, LT and LBT) did not show significant differences (Table 1).

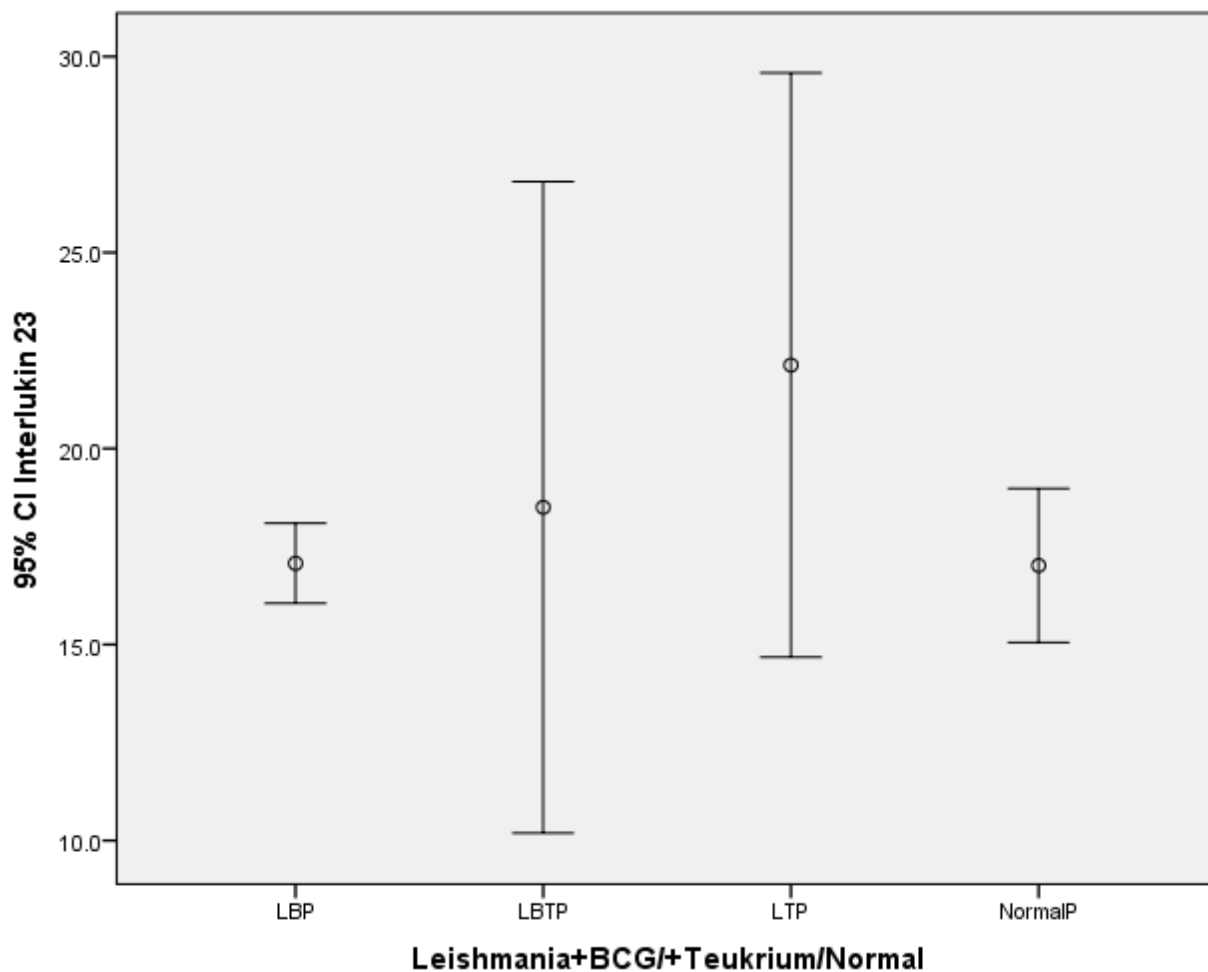


Figure 2: The post challenge with live amastigote results of serum levels of IL-23, for three injection groups (LB, LT and LBT) and normal group with 95% confidence interval.

Spleen weight: According to Figure 3, the highest levels of spleen weight were obtained between the control group that did not receive the vaccine and the three injection groups (LB, LT, LBT) that received the vaccine before being exposed to re-exposure to amastigotes emission from the wound, with a 95% confidence interval for the LB group, and lowest belonged to LT group. The results of analysis

variance (ANOVAs) for level of SW between three injection groups (LB, LT and LBT) no considering to two injection doses (100 and 200 $\mu\text{g}/\text{ml}$) showed almost significant differences ($P < 0.071$) (Table 2). While ANOVA test, between two injections doses (100 and 200 $\mu\text{g}/\text{ml}$) no considering to three injection groups (LB, LT and LBT) did not show significant differences (Table 1).

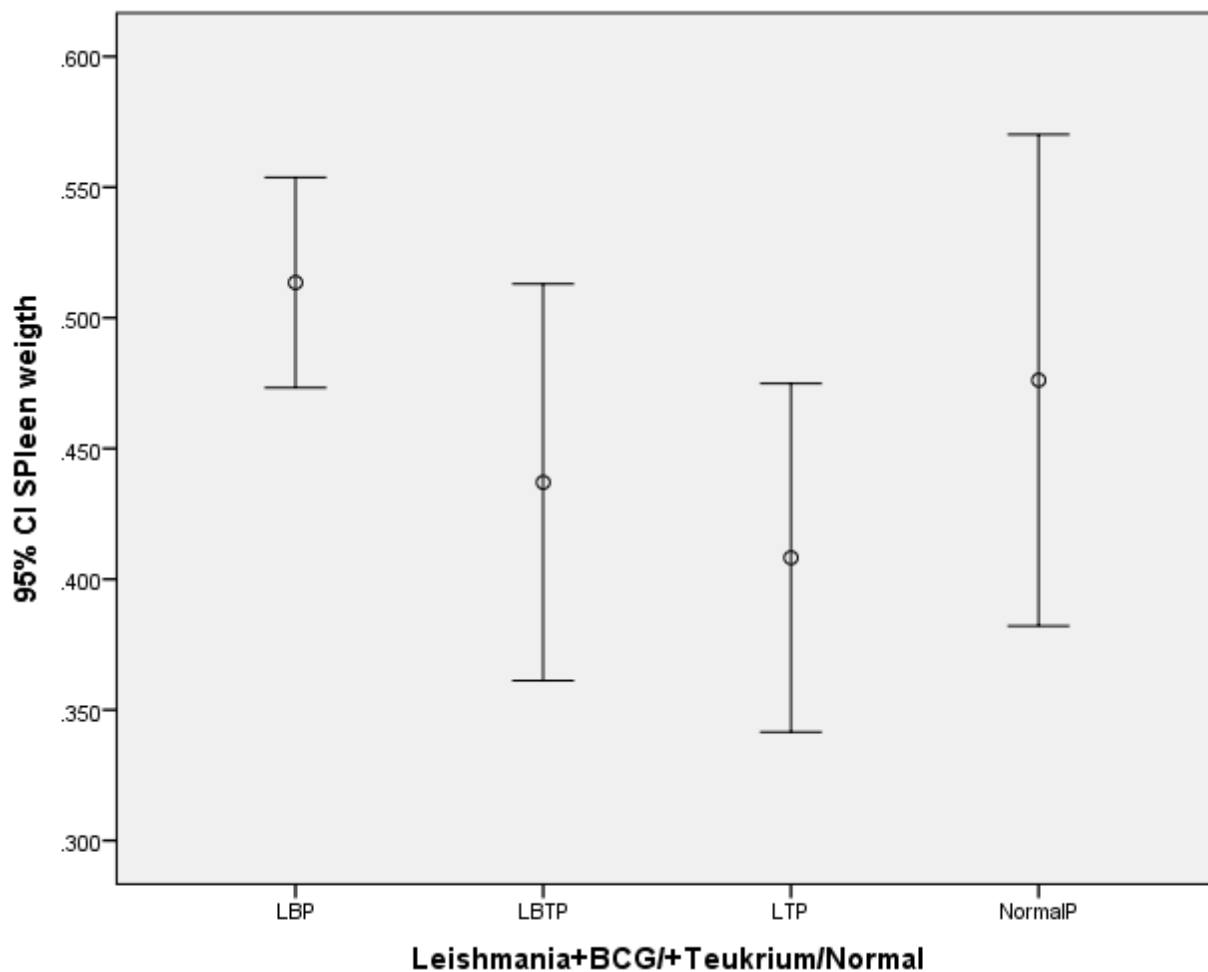


Figure 3: The post challenge with live amastigote results of serum levels of spleen weight, for three injection groups (LB, LT and LBT) and normal group with 95% confidence interval.

Mouse weight: According to Figure 4, the highest levels of mice weight were obtained between the control group that did not receive the vaccine and the three injection groups (LB, LT, LBT) that received the vaccine before challenged with amastigotes emission from the wound, with a 95% confidence interval belong to LB group, and, and lowest belonged LBT group. The results of analysis variance (ANOVAs)

for level of MW, between three injection groups (LB, LT and LBT) no considering to two injection doses (100 and 200 $\mu\text{g/ml}$) (Table 2), and also two injections doses (100 and 200 $\mu\text{g/ml}$) no considering to three injection groups (LB, LT and LBT) did not show significant differences (Table 1).

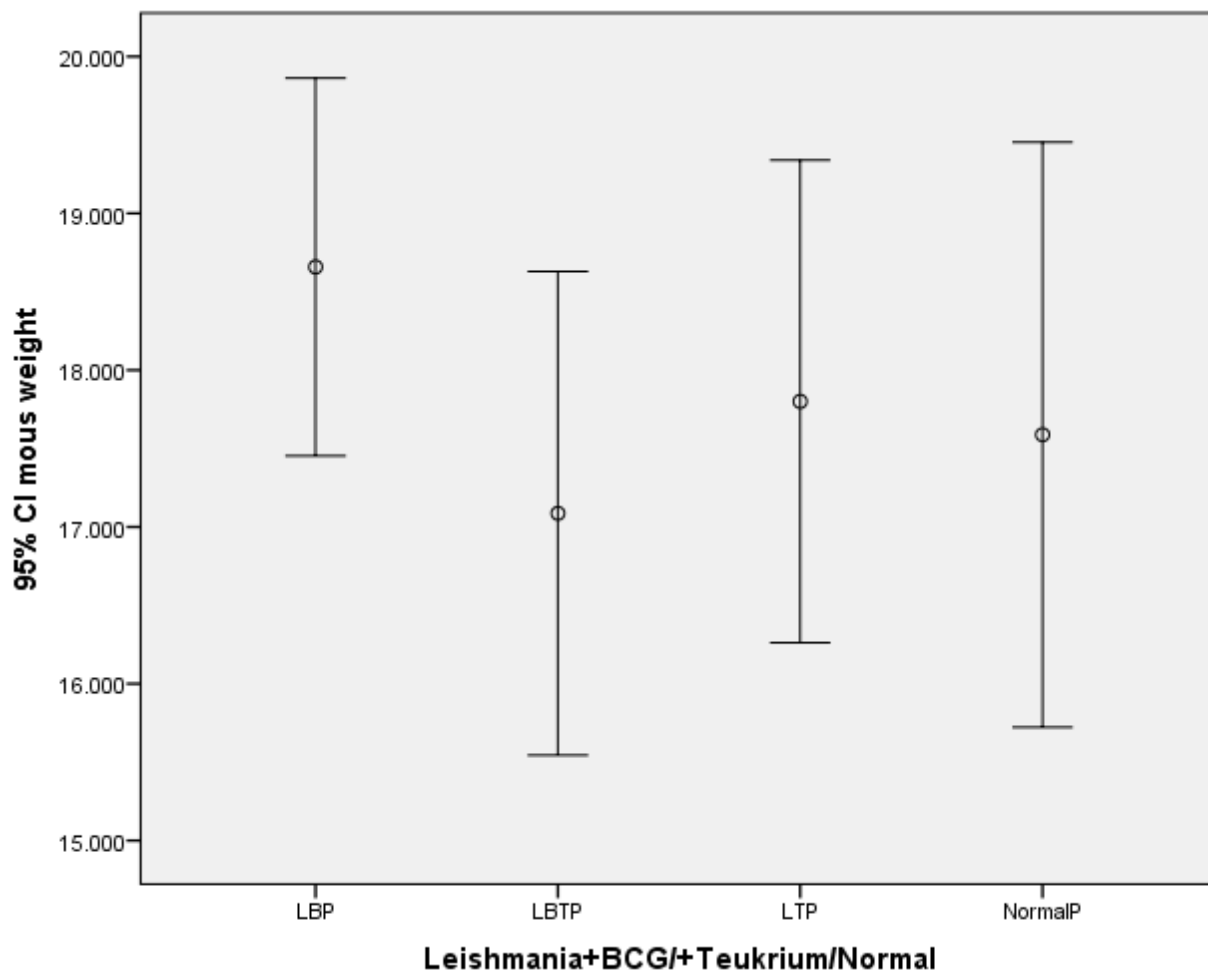


Figure 4: The post challenge with live amastigote results of serum levels of mouse weight, for three injection groups (LB, LT and LBT) and normal group with 95% confidence interval.

Percent spleen weight/mouse weight: According to Figure 5, the highest levels of mice weight were obtained between the control group that did not receive any vaccine and the three injection groups (LB, LT, LBT) that received the vaccine before challenged with amastigotes removed from the wound, with a 95% confidence interval belong to LB group, and, and lowest belonged LT group. The results of analysis

variance (ANOVAs) for level of PSW/MW, between three injection groups (LB, LT and LBT) no considering to two injection doses (100 and 200 $\mu\text{g/ml}$), did not show significant differences But the difference is contemplative ($P=0.186$) (Table 2) while two injections doses (100 and 200 $\mu\text{g/ml}$) no considering to three injection groups (LB, LT and LBT) did not show significant differences (Table 1).

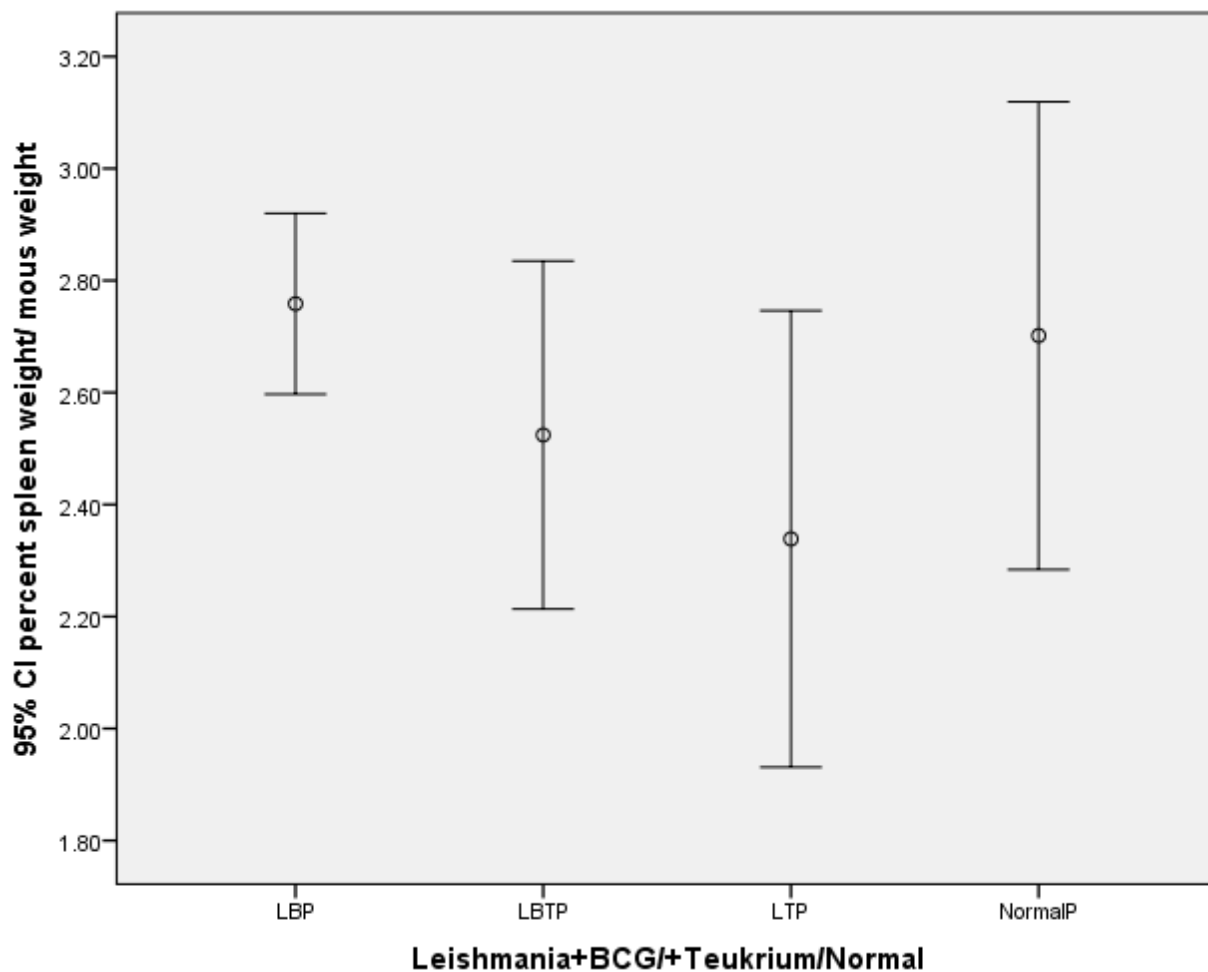


Figure 5: The post challenge with live amastigote results of serum levels of percent spleen weight/mouse weight for three injection groups (LB, LT and LBT) and normal group with 95% confidence interval.

Number pulp spleen: According to Figure 6, the highest levels of mice weight were obtained between the control group that did not receive any vaccine and the three injection groups (LB, LT, LBT) that received the vaccine before challenged with amastigotes emission from the wound, with a 95% confidence interval belong to LB group, and, and lowest belonged LT group. The results of analysis variance (ANOVAs) for level of (NPS) between two injection doses (100 and

200 µg/ml) no considering to three injection groups (LB, LT and LBT) showed almost significant differences ($P < 0.067$) (Table 1). While ANOVA test, between three injection groups (LB, LT and LBT) no considering to two injection doses (100 and 200 µg/ml) did not show significant differences but the difference is contemplative ($P = 0.291$) (Table 2).

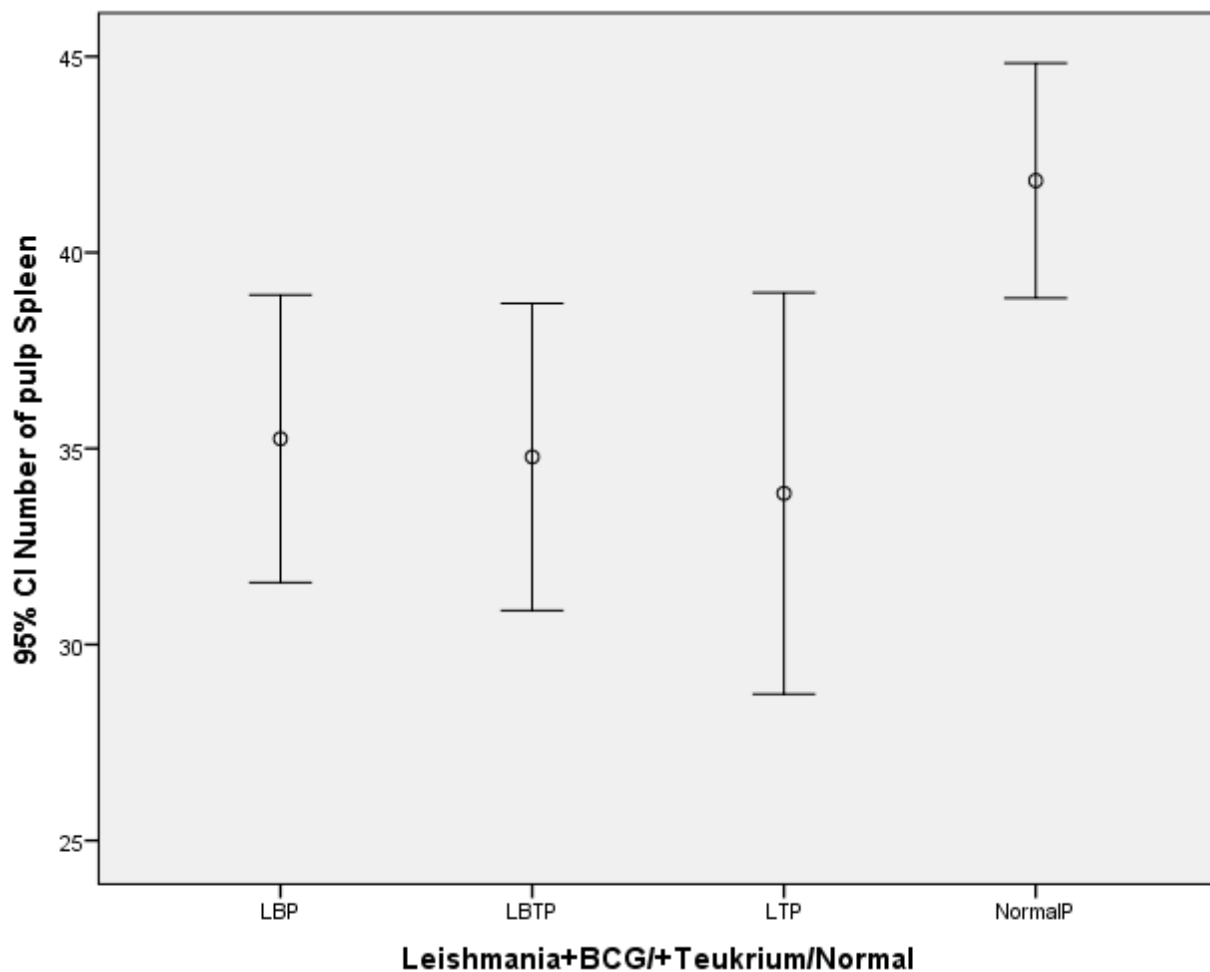


Figure 6: The post challenge with live amastigote results of serum levels of number of pulp spleen for three injection groups (LB, LT and LBT) and normal group with 95% confidence interval.

Mean of pulp size: According to Figure 7, the highest levels of mice weight were obtained between the control group that did not receive any vaccine and the three injection groups (LB, LT, LBT) that received the vaccine before challenged with amastigotes removed from the wound, with a 95% confidence interval belong to LB group, and, and lowest belonged LT group. The results of analysis variance (ANOVAs)

for level of MPS, between three injection groups (LB, LT and LBT) no considering to two injection doses (100 and 200 $\mu\text{g/ml}$) did not show significant differences. But the difference is contemplative ($P=0.291$) (Table 2) while two injections doses (100 and 200 $\mu\text{g/ml}$) no considering to three injection groups (LB, LT and LBT) did not show significant differences (Table 1).

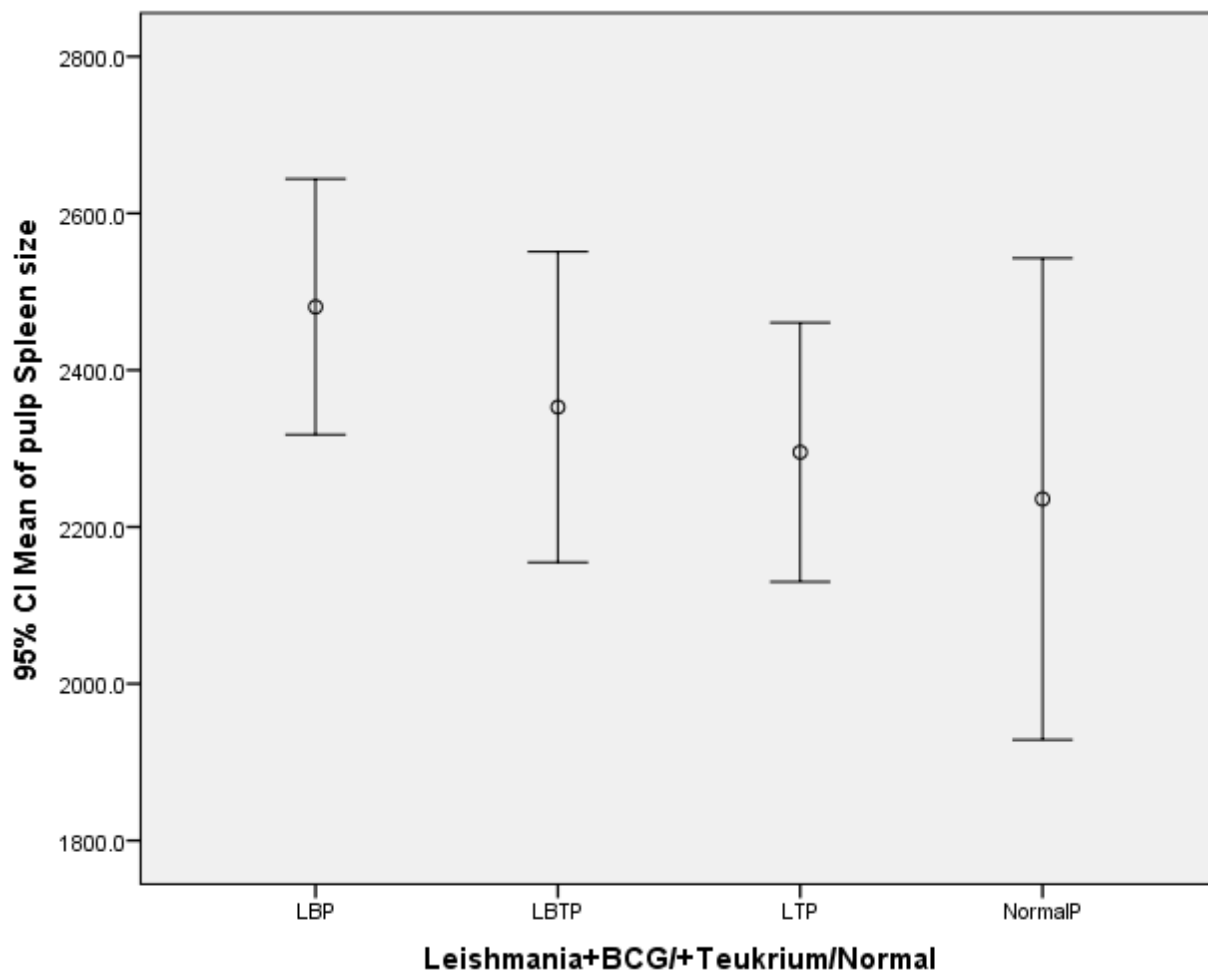


Figure 7: The post challenge with live amastigote results of serum levels of mean of pulp size, for three injection groups (LB, LT and LBT) and normal group with 95% confidence interval.

Doses 100 and 200 µg/ml: According to Table 1, MW (P=914), SW (P=0.786), PSW/MW (P=0.619) and IL-23 (P=0.653), MPS (P=0.340), IL-17 (0.353) had not significant differences and only NPS (P=0.067) had almost significant differences between doses 100 and 200 µg/ml.

		Sum of Squares	df	Mean Square	F	Sig.
Mouse weight (MW)* <i>Leishmania</i> injection doses 100/200	Between Groups (Combined)	1.095	2	0.548	0.09	0.914
	Within Groups	274.092	45	6.091		
	Total	275.187	47			
Spleen weight (SW)* <i>Leishmania</i> injection doses 100/200	Between Groups (Combined)	0.006	2	0.003	0.242	0.786
	Within Groups	0.581	45	0.013		
	Total	0.587	47			
Percent spleen weight/ mouse weight (PSW/MW)* <i>Leishmania</i> injection doses 100/200	Between Groups (Combined)	0.283	2	0.141	0.484	0.619
	Within Groups	13.144	45	0.292		
	Total	13.426	47			

Number of pulp spleen (NPS)* <i>Leishmania</i> injection doses 100/200	Between Groups (Combined)	273.657	2	136.829	2.884	0.067
	Within Groups	2039.756	43	47.436		
	Total	2313.413	45			
Mean of pulp spleen size (MPS)* <i>Leishmania</i> injection doses 100/200	Between Groups (Combined)	206851.703	2	103425.9	1.105	0.34
	Within Groups	4210763.19	45	93572.52		
	Total	4417614.893	47			
Interleukin17* <i>Leishmania</i> injection doses 100/200	Between Groups (Combined)	3285.315	2	1642.658	1.065	0.353
	Within Groups	69435.637	45	1543.014		
	Total	72720.953	47			
Interleukin 23* <i>Leishmania</i> injection doses 100/200	Between Groups (Combined)	96.257	2	48.129	0.43	0.653
	Within Groups	5035.061	45	111.89		
	Total	5131.318	47			

Table 1: The results of analysis variance (ANOVAs) for serum level IL-17, IL-23, MW, SW, PSW/MW, NPS, MPS, between two injection doses (100 and 200 µg/ml) no considering to three injection groups (LB, LT and LBT) (P<0.01).

LB group: According to Figures 1-7 and Table 2, this group had lowest levels of IL-17 (P=0.594) and MW (P=0.399), IL-23 (0.590) which were not significant, whether, highest levels of SW (P=0.071) almost significant and MPS (P=0.291), PSW/MW (P=0.186), NPS (P=0.132) which were not significant but contemplative also belonged to this group.

		Sum of Squares	df	Mean Square	F	Sig.
Mouse weight* <i>Leishmania</i> +BCG/+Teukrium/Normal	Between Groups (Combined)	17.686	3	5.895	1.007	0.399
	Within Groups	257.501	44	5.852		
	Total	275.187	47			
Spleen weight* <i>Leishmania</i> +BCG/+Teukrium/Normal	Between Groups (Combined)	0.086	3	0.029	2.507	0.071
	Within Groups	0.501	44	0.011		
	Total	0.587	47			
Percent spleen weight/ mouse weight* <i>Leishmania</i> +BCG/+Teukrium/Normal	Between Groups (Combined)	1.377	3	0.459	1.676	0.186
	Within Groups	12.05	44	0.274		
	Total	13.426	47			
Number of pulp spleen* <i>Leishmania</i> +BCG/+Teukrium/Normal	Between Groups (Combined)	286.258	3	95.419	1.977	0.132
	Within Groups	2027.155	42	48.266		
	Total	2313.413	45			
Mean of pulp spleen size* <i>Leishmania</i> +BCG/+Teukrium/Normal	Between Groups (Combined)	356033.371	3	118677.8	1.286	0.291
	Within Groups	4061581.522	44	92308.67		
	Total	4417614.893	47			

Interleukin17* <i>Leishmania</i> +BCG/+Teukrium/Normal	Between Groups (Combined)	3259.009	3	1086.336	0.688	0.564
	Within Groups	69461.943	44	1578.681		
	Total	72720.953	47			
Interleukin 23* <i>Leishmania</i> +BCG/+Teukrium/Normal	Between Groups (Combined)	216.113	3	72.038	0.645	0.59
	Within Groups	4915.205	44	111.709		
	Total	5131.318	47			

Table 2: The results of analysis variance (ANOVAs) for serum level IL-17, IL-23, MW, SW, PSW/MW, NPS, MPS, between three injection groups (LB, LT and LBT) no considering to two injection doses(100 and 200 µg/ml) (P<0.01).

LT group: According to Figures 1-7 and Table 2, highest levels of IL-23 (0.590) which was not significant, whether, lowest levels of SW (P=0.071) almost significant, and MPS (P=0.291), PSW/MW (P=0.186) which were not significant but contemplative also belonged to this group.

LBT group: According to Figures 1-7 and Table 2, highest levels of IL-17 belong to this group but was not significant (P=0.594).

Control group: According to Figures 1-7 and Table 2, lowest levels of IL-23 (0.590) belonged to LB and control group which was not

significant, whether, highest levels of PSW/MW (P=0.186), NPS (P=0.132) which were not significant but contemplative also belonged to this group.

Correlations: According to Table 3, there were significant correlations between MW with SW (P=0.001) and MPS (P=0.023), doses 100 and 200 µg/0.1 ml with NPS (P=0.023), SW with MW (P=0.001) and MPS (P=0.012), PSW/MW with SW (P=0.000) and MPS (P=0.091) near significant. There was no significant correlations between IL-17 and IL-23 with together and another parameters.

		<i>Leishmania</i> injection doses 100/200	Mouse weight	Spleen weight	Percent spleen weight/ mouse weight	Number of pulp spleen	Mean of pulp spleen size	IL-17	IL- 23
<i>Leishmania</i> injection doses 100/200	Pearson Correlation	1	0.062	-0.103	-0.145	-0.266	0.216	-0.072	-0.029
	Sig. (2-tailed)		0.677	0.486	0.326	0.074	0.14	0.624	0.846
	N	48	48	48	48	46	48	48	48
Mouse weight	Pearson Correlation	0.062	1	.455**	-0.038	0.086	.328*	0.153	-0.162
	Sig. (2-tailed)	0.677		0.001	0.8	0.569	0.023	0.299	0.272
	N	48	48	48	48	46	48	48	48
Spleen weight	Pearson Correlation	-0.103	.455**	1	.865**	0.177	.359*	0.19	0.06
	Sig. (2-tailed)	0.486	0.001		0	0.24	0.012	0.196	0.683
	N	48	48	48	48	46	48	48	48
Percent spleen weight/ mouse weight	Pearson Correlation	-0.145	-0.038	.865**	1	0.124	0.247	0.13	0.168
	Sig. (2-tailed)	0.326	0.8	0		0.411	0.091	0.378	0.253
	N	48	48	48	48	46	48	48	48
Number of pulp spleen	Pearson Correlation	-0.266	0.086	0.177	0.124	1	0.152	0.017	-0.113
	Sig. (2-tailed)	0.074	0.569	0.24	0.411		0.315	0.908	0.455
	N	46	46	46	46	46	46	46	46

Mean of pulp spleen size	Pearson Correlation	0.216	.328*	.359*	0.247	0.152	1	0.063	0.027
	Sig. (2-tailed)	0.14	0.023	0.012	0.091	0.315		0.669	0.853
	N	48	48	48	48	46	48	48	48
Interleukin 17	Pearson Correlation	-0.072	0.153	0.19	0.13	0.017	0.063	1	0.272
	Sig. (2-tailed)	0.624	0.299	0.196	0.378	0.908	0.669		0.061
	N	48	48	48	48	46	48	48	48
Interleukin 23	Pearson Correlation	-0.029	-0.162	0.06	0.168	-0.113	0.027	0.272	1
	Sig. (2-tailed)	0.846	0.272	0.683	0.253	0.455	0.853	0.061	
	N	48	48	48	48	46	48	48	48

Table 3: The results of correlations between serum levels of IL-17, IL-23, MW, SW, PSW/MW, NPS, MPS for two injection doses (100 and 200 µg/ml) no considering to three injection groups (LB, LT and LBT) (P<0.01).

Discussion

It is important that not only the parasite entry route is consideration, but also parasite life cycle (promastigote, amastigote) has many and different antigenic determinants. It is also important that consider to antigenic determinants and the period of the protozoan life cycle which exposed to the immune system. In order to ensure proper intracellular immunity, immune memory, and cytokines of the type I (TNF- α , IFN- γ , IL-12, IL-23,...) in response to new vaccine, we prepared new vaccine for fourth times that had successful immune response in this article. In the current study, re-exposure was observed at a rate of 0.1 ml with a suspension of amastigote, removed from the wound and diluted in the physiologic serum almost one month after a vaccine inoculation and 4-5 weeks after a reminder dose. Mice were killed almost one month after the re-exposure and statistical results were obtained (an important point was that, all of the mice were killed). In a considering to dose study, the new vaccine was able to activate and increase the number of cells in the 100 and 200 µg/0.1 ml doses of the spleen (Figure 7) (Tables 1,2). Spleen is a secondary lymphoid tissue and has an antigen and antibody encounter. It suggests that the spleen has identified as secondary lymphoid tissue and has given different responses and the largest of number of pulp size is related to LB and the lowest was related to LT group. Spleen has an antibody production site and interleukin 10 [10,17-19]. The spleen has production sites for produce antibodies and interleukin 10. It indicates that after challenge with amastigote the best group LT has the smallest number of white pulp and is predicted to advance the immune system to Th1 and increase its cytokines. As the same way group LB increases in the number of white pulp and is expected to advance the immune system to Th2 and increase the corresponding cytokines and eventually led to leishmaniasis, but no significant difference was observed. But the interesting point is that control group without any memories of the vaccine had a large increasing in their white pulp number. Control mice would receive an ulcer with leishmaniasis after ingestion and would be died at least 4 months later. Here it can be suggested that although this time amastigote was selected for re-exposure but the new vaccine contains many anti-genetic markers and has been able to create a good immune response that will even show an adequate response to the re-encounter with amastigote. As shown in Figure 2,

the highest of IL-23 is observed in group LT whether, group B and control were lowest and approximately equal. According to Table 1 and 2, no significant differences are observed, but the control group, which had no safety memory, had similar results with group LB. Here's a hypothesis that: IL-23 has a common chain with IL-12 (P40). In control and LB group probably production of IL-12 prevented by an increasing of IL-10. According to Figure 1, interleukin 17 as an inflammatory cytokine was produced faster in the control group that did not receive any vaccine and had no memory of primary immune response. It is reasonable. The *leishmania* parasite is intracellular and when it enters the body, the first defensive line is neutrophils and macrophages which take to eliminate the intracellular parasite. On the other hand neutrophil is one of the cells that is synthesized and secreted interleukin 17A and interleukin 17F. It can be suggested that when neutrophils firstly exposure with *leishmania*, they simultaneously enter the phase of the primary immune response as phagocytosis (the first line of intrinsic immune system and intrinsic immune response or innate immunity), as well as synthesizing and increasing the synthesis of interleukin 17. For this reason the body of the mice entered the inflammatory phase and more elevated than other three injection groups. However, according to Tables 1 and 2 do not have a significant difference between them. It can only be said that the vaccine has been successful and has the many antigenic determinants, so that the safety record could reduce the inflammatory phase in the three injection groups against re-exposure to *leishmania* amastigote. According to Figure 3, the highest spleen weight were observed in the control group, which did not receive any vaccine, and therefore did not have a safety memory, and group LB, which had adjuvant BCG. According to Table 1 and Table 2, this difference was almost significant (P=0.071). This indicates that the weight of the spleen in control group is rapidly increased and it is suggested that the immune response be carried out in the direction of humoral and inflammatory responses and Th2 immune response. Consideration to Figure 4 and Tables 1 and 2, the highest mice weight was in group LB and lowest belong to LT but did not differ significantly. As shown in Figure 5, the highest percentage of percent spleen weight/mouse weight belongs to the control and LB, and the least belongs to LT groups. It again suggested that the control group which had not been received vaccine and had no safety memory and LB group that had BCG as adjuvant will definitely go towards

leishmaniasis, because they lead quickly to changes in the spleen. While in the LT group, with adjuvant-*Teucrium polium*, the weight of the spleen is at its lowest amounts. It can be argued that this adjuvant is better than BCG and causes the mice to exhibit stronger immune responses that are likely to go toward Th1 immune response. Our results were satisfactory and confirm our previous results [7-15]. In this experiment all of mice in all groups were alive and killed before just the end of experimental step of study. We present satisfactory results in this article and response to many assumptions mentioned above will be given in future studies.

Conclusion

This study shows that: 1) This vaccine has many antigenic markers that when tested with amastigote form of *Leishmania*, it can regain the memory of this immune memory system, and also has been able to increasing in interleukin 23 in group LT. 2) It can prevent of induce inflammatory cytokine (IL-17) in the group LT. 3) The effects of the vaccine on the spleen weight were also appropriate response .4) Responses to vaccine in group LB, which had adjuvant BCG and the control group that did not received vaccine were approximately same responses. 5) The hypothesis is that BCG with vaccine may have not an effective immune response and leading toward leishmaniasis will be addressed in future. Questions will be answered in subsequent studies.

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