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Research Paper

Cellular and Histopathological Changes in BALB/c Mice Infected With Live Attenuated *Leishmania major* Parasite

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ABSTRACT

Introduction: Cutaneous leishmaniasis is a common form of leishmaniasis caused by infection with the *Leishmania major* parasite in the world and in Iran. The lack of an effective vaccine and drug resistance prompted this study to investigate the invasive mechanisms caused by the live attenuated strain of *L. major* parasite in BALB/c mice.

Materials & Methods: *Leishmania* promastigotes which have been attenuated after one, four, nine and twenty passages in a special culture medium were injected into the tail base of 6-8w BALB/c mice. The size of the wound was measured, the phagocytosis ability of macrophages in the peritoneal cavity was evaluated by NBT (nitroblue tetrazolium test), the proliferation of splenocytes was measured by the MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide] test and histopathologic changes of skin, spleen, lymph nodes and liver were examined at day 15, 30, and 45 after inoculation.

Results: The severity of tissue damage to the skin, spleen, and liver was higher at passages 1, 4 and 9 of live attenuated parasite on days 15 and 30 and increased at passage 1 of parasite on day 45. However, the intensity of the cellular response was lower at passage 20 of attenuated parasite, the size of the wound showed a significant decrease and a relative increase in phagocytosis on day 30 of the infection period; despite the infiltration of mononuclear cells and macrophages in all organs, parasites were only visible in the skin on day 45, and no *Leishmania* parasites were observed in the lymph nodes, spleen and liver tissue.

Conclusion: The results of histopathologic changes, phagocytosis and proliferation of splenocytes showed that as the virulence of the *L. major* parasite were decreased, the invasive potency of the parasite also decreased, which will be useful as initial findings for the study to develop a live attenuated vaccine.

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1. Introduction

Leishmaniasis is a neglected vector-borne disease; *Leishmania* parasites are obligate intracellular protozoa that live in mononuclear phagocytes of humans and other vertebrates. *Leishmania* cause a wide spectrum from self-healing cutaneous to fatal visceral diseases. The most common form of the disease is cutaneous leishmaniasis, both anthroponotic cutaneous leishmaniasis (ACL) and zoonotic cutaneous leishmaniasis (ZCL) are an important health problem in Iran [1]. The drugs currently available to treat leishmaniasis are not satisfactory because they are administered over a long period of time and their cost and toxicity are high, there is no effective vaccine against leishmaniasis, but there is promising evidence that leishmaniasis is one of the parasitic diseases that could be controlled by vaccination, numerous studies have been conducted on the efficacy of attenuated vaccines in mouse and dog models of *Leishmania mexicana* and *Leishmania major* against cutaneous leishmaniasis [2]. The results of some studies have shown that long-term protection against reinfection develops after natural infection, these results have encouraged scientists to use attenuated *Leishmania* parasites to induce protective immunisation in humans [3]. Various examples of attenuated strains are produced by different subcultures, cultivation under drug pressure, selection for temperature sensitivity, chemical mutagenesis and gamma attenuation [4]. Undefined and defined genetic strains are two forms of live attenuated parasites based on the type of attenuation method. Defined strains are generated by mutation through knockout of one or more virulence-specific genes, while undefined strains are obtained by long-term in-vitro culture using chemicals or irradiation mutagenesis. Live-attenuated *Leishmania* parasites obtained by long-term in-vitro culture are being considered for the development of an effective vaccine [5]. The studies conducted by some researchers have shown that a low-dose of *L. major* parasite elicited different types of immune responses from CD8+T cells for primary immunity in C57BL/6 mice [6]. Immunological insights into leishmaniasis have mainly been obtained through studies in animal models such as C57BL/6 and BALB/c mice, these findings have shown that resistance to *Leishmania* infection is related to the activity of Th1 lymphocytes [7]. The interaction between host and parasite immune responses determines the outcome, including resistance or susceptibility. Activation of Th1 leads to limitation of infection, but activation of Th2 is associated with disease progression. The immune response was different in C57BL/6 and BALB/c mice which indicates the different susceptibility of mice to *Leishmania* parasites [8]. Identification of the pro-and anti-inflammatory genes expressed during parasite infection will help to elucidate the mechanisms of gene regulation and intracellular survival of *Leishmania* parasites [9]. Identification of the expressed genes and the molecular mechanisms underlying their regulation could be considered as therapeutic targets. Pro-inflammatory genes play an important role in the elimination of *Leishmania* infections. The main mechanism of parasite destruction in these cells is the production of free radicals from oxygen and nitric oxide due to activation. In addition, IFN-γ released by T lymphocytes stimulates macrophages to produce TNF-α a chemical mediator in both innate and acquired immune responses and an important communication factor between acquired immune responses and acute inflammation [10]. Most studies on *Leishmania* parasites focus on the immunogenicity and pathogenicity of the parasite to know the mechanisms for vaccine development and infection prevention. We decided to determine phagocytosis reactions, cellular as well as tissue changes in *Leishmania* infection with live attenuated *L. major* promastigotes at passage 1, 4, 9 and 20 in BALB/c mice.

2. Material and Methods

2.1. Parasite culture

L. major strain (MHOM/IR/75/ER) were obtained from Pasteur Institute of Iran. Late log phase parasites were collected by centrifugation, washed with phosphate buffer saline (PBS) and were cultured in a T25 flask containing RPMI-1640 medium, 5000 IU/mL penicillin and 5000 µg/mL streptomycin antibiotics and 10% fetal bovine serum (FBS) by incubation at 24 ˚C±1.

2.2. Animals and infection

Eighty female BALB/c mice, weighing 25-30 g, aged 6–8 weeks were purchased from Pasteur Institute (Karaj, Iran). The study protocol is done based on the Helsinki declaration and was confirmed by the Ethics Committee of the Deputy of Research of Ahvaz Jundishapour University of Medical Sciences, Ahvaz, Iran (No:IR.AJUMS.ABHC.REC.1398.074). All animals were kept in a controlled environment (24±1 ˚C and 12:12 h light-dark cycle) and had free access to food and water. Animals were divided into four equal groups and each group was injected intradermally into the tail base with 2×10^6/100 uL of the live attenuated *L. major* strain at 1, 4, 9 and 20 passages.

2.3. Sample collection, imaging and measuring the diameter of the wound

When the lesions appeared, the animals were euthanized and samples of the surrounding wound, spleen, liver, and lymph nodes were collected on days 15, 30, and 45 respectively. Samples preserved and fixed in 10% buffered formalin for pathological examination. The wound size was photographed weekly using a Canon digital camera (Canon, Japan) mounted on a tripod at a fixed distance.

2.4. MTT assay

The MTT [3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyl-tetrazoliumbromide] assay was used to evaluate the proliferation rate of splenic lymphocytes. MTT test used to evaluate the rate of splenic lymphocyte proliferation. The spleen samples were flushed out under sterile conditions using a syringe and 2×10^5 splenocytes of each spleen were cultured (triplicate) in 96-well tissue culture plates containing RPMI-1640 medium & 7% FBS. Spleen cells were treated with 50 uL soluble *leishmania* antigen (SLA, 40 ug/mL) as a stimulus and 50 uL bovine serum albomin (BSA, 40 ug/mL) as a control. After 72 h incubation at 37 ˚C with 5% CO_2, 20 uL of MTT (5 mg/mL) solution added to all wells. Plates were incubated again for 3 h, then measured the reduction of MTT dyes (tetrazolium) in to formazan by added isopropanol solution (0.04M HCL) with optical absorbance of the treated and untreated samples (490 nm) [11].

2.5. Nitroblue tetrazolium test (NBT)

NBT test was performed to evaluate the phagocytosis function of macrophages in the peritoneal cavity. Peritoneal macrophages were collected by injection and aspiration with cold physiology serum (with pen/strep 5%). 2×10^6 cells were cultured in a 96-well cell culture plate contains RPMI-1640 with FBS 10%. The cells were treated with 20 uL of zymosan (5 mg/mL) as stimulus, then 15 uL of NBT (1 mg/mL) solution was added after incubation for 24 hours at 37 ˚C with 5% CO_2. The plate were incubated for 1 h incubation at 37 ˚C with 5% CO_2 then 70 uL dimethylformamide was added to it. Optical absorbance of the wells was measured by spectrophotometer at 450 nm (Biotek, American, ELX808).

2.6. Histopathology

Full thickness skin samples of skin, spleen, lymph nodes and liver tissue of mice were collected and fixed in 10% buffered formalin on days 15, 30 and 45. The tissue samples were then dehydrated, cleared, paraffin embedded and sectioned at 5-7 µm thickness followed by mounting on glass slides and tissue sections were deparaffinized in xylene-I & II (for 5 min each), 100 % alcohol, 95 % alcohol, 80 % alcohol & 70% alcohol (8 min each) to staining with Hematoxylin and Eosin (H&E). The sections were then examined using a light microscope (Olympus CX41, Japan) equipped with a digital camera (Olympus DP25, Germany).

2.7. Statistical analysis:

The data were statistically analyzed using SPSS software, version 20, and GraphPad prism software, version 8. Due to the normal distribution of the data, one-way analysis of variance (ANOVA) followed by post hoc Tukey's test were performed, $P\leq0.05$ were considered significant. The mean diameter of the wound was calculated using the image J application.

3. Result

3.1. Measuring the wound size

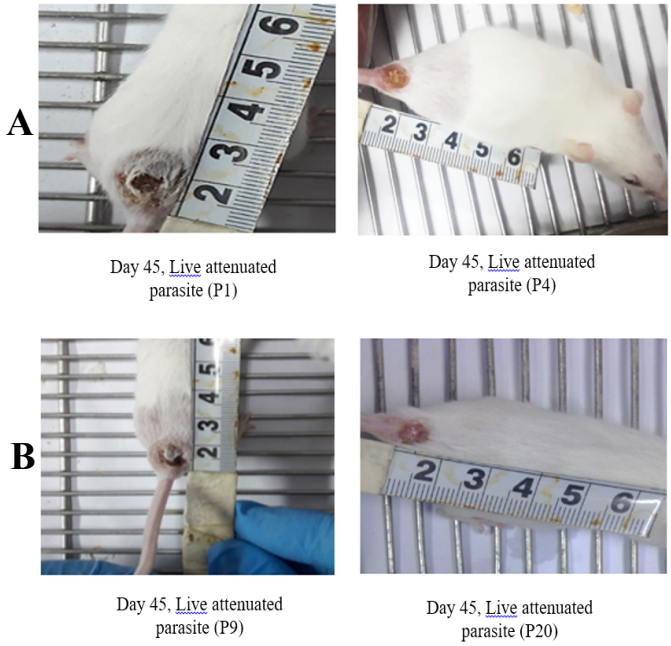
Wound size change was observed with infection of live attenuated parasite (passages 1, 4, 9 and 20) on days 1 to 45 after parasite inoculation. Wound appear was faster and the lesion progression rate was higher in injection of passage 1 of *L. major* parasite compared to the other groups ($P\leq0.05$). The progression of the lesion and the size of the wound decreased by the reduction of parasite virulence, respectively (Figures 1A and 1B) (Table 1).

3.2. Proliferation assay (MTT)

The proliferation rate of the splenocytes from each group were evaluated using MTT colorimetric assay. The splenocytes collected from the *Leishmania*-infected animals stimulated in-vitro with SLA, the proliferation rate of splenic lymphocyte proliferation the mice infected with passage 1 of the attenuated parasite was higher than other virulences except on day 1 and 15 ($P\leq0.05$). The reaction of splenic cells to SLA showed the lowest proliferation was in passage 20 of the attenuated parasite (significantly $P\leq0.05$) (Figure 2).

3.3. Phagocytosis potency assay (NBT)

The phagocytosis rate of macrophages was different at passages (1, 4, 9 and 20) of live attenuated *L. major* on day 15, 30 and 45. Phagocytosis in mice infected with passage 4 of parasite was higher than other infected mice on day 45 ($P\leq0.05$). Phagocytosis was significantly higher in mice infected with passage 20 of parasite on day 15, but in passage 4 of parasite, phagocytosis was more on days 45 (Figure 3).



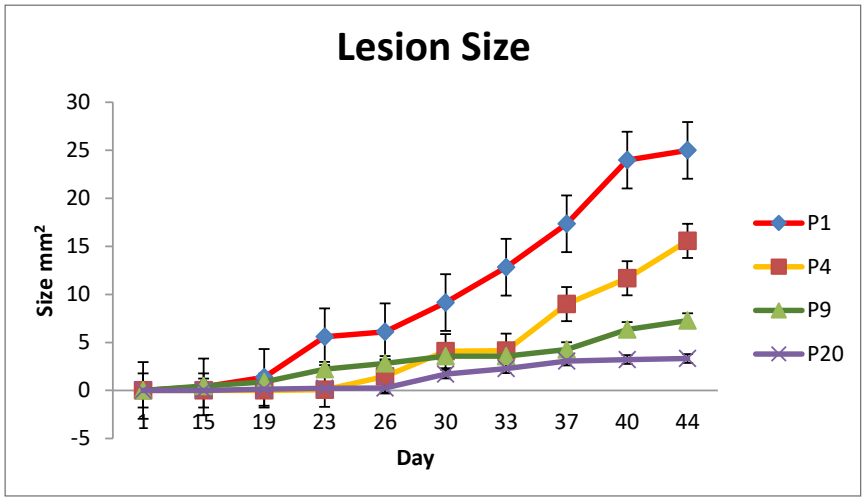


Figure 1. Lesion size in treatment groups

A) Wound caused by live attenuated *L. major* at different passages on day 1-45, B) Wound size in the four groups of BALB/c mice infected with live attenuated *L. major* at different passages (1, 4, 9 and 20)

P: Passage.

Table 1. Different parameters of wound measurement in infection with live attenuated *L. major* at passages (1, 4, 9 & 20)

Groups	Mean	Std. Deviation	Std. Error of Mean	Tukey's Multiple Comparisons Test	Mean Diff.	Sig.	P
P1	10.17	9.33	2.95	P9 vs P20	1.41	**	0.0075
P4	4.6	5.61	1.77	P1 vs P4	4.6	**	0.0094
P9	3.14	2.4	0.76	P1 vs P9	3.14	*	0.0460
P20	1.41	1.44	0.45	P1 vs P20	1.41	*	0.0293

P: Passage. *, **Significance level.

P≤ 0.05

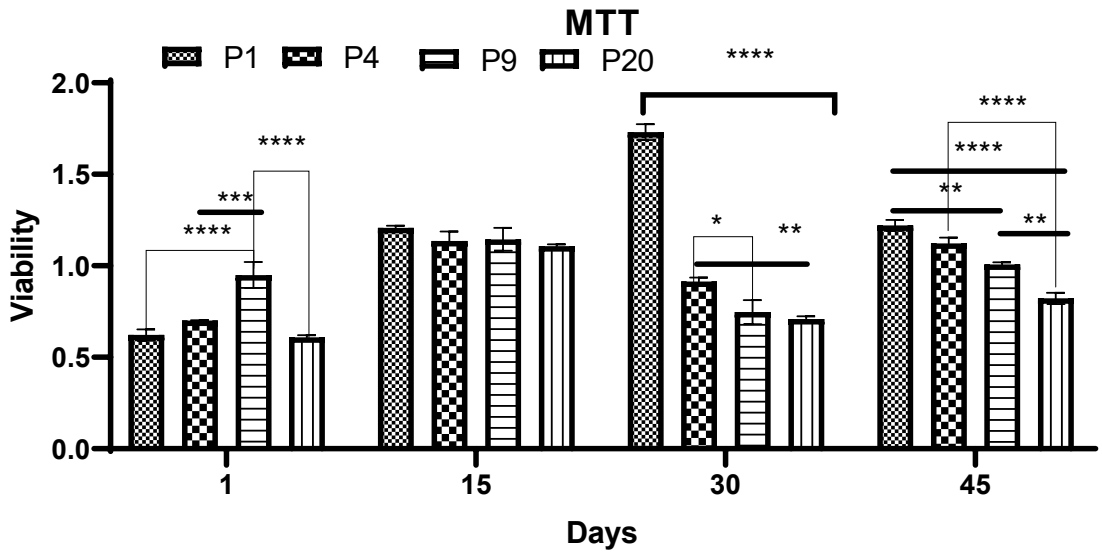


Figure 2. Proliferation of splenocytes of *Leishmania*-infected mice with different passages (1, 4, 9 and 20) of live attenuated *L. major*

P: Passages of live attenuated *L. major*.

3.4. Histopathology

After parasite injection with different passages 1, 4, 9, and 20 of live attenuated *L. major*, samples were taken from the tissues around the lesion, liver, spleen, and lymph nodes.

3.4.1. Day 1

At passage 1, 4, 9 and 20 from live attenuated *L. major*, the skin, liver, spleen, and lymph nodes had normal tissue structure and similar to the control group samples (Figure 4).

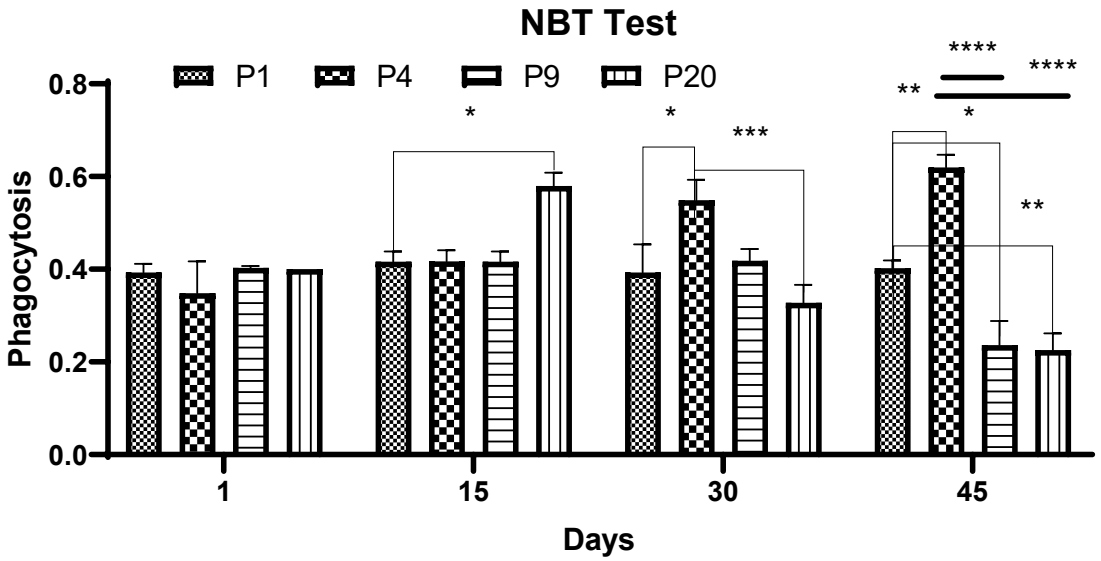


Figure 3. Phagocytic potency of macrophages isolated from the peritoneal cavity of *Leishmania*-infected mice with different passages (1, 4, 9 and 20) of parasite

P: Passages of live attenuated *L. major*.

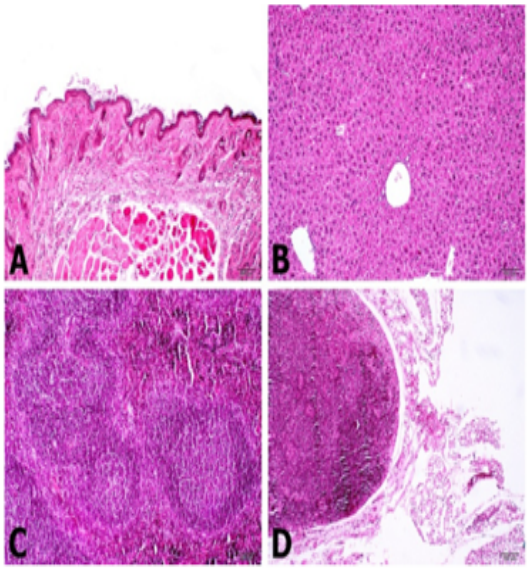


Figure 4. Histopathological changes of skin, liver, spleen, and lymph nodes with normal tissue structure on day one from mice infected with live attenuated parasite

3.4.2. Day 15

In infected mice with passage 1 of the live attenuated parasite, the lesion tissue with severe inflammation and extensive infiltration of inflammatory cells in the dermis and hypodermis, the liver with infiltration of inflammatory cells in the portal area together with an inflammatory reaction and microgranuloma formation, a hyperemic spleen with small foci of necrosis in the white pulp, and mild hyperemia in the cortical region of the lymph node (Figure 5-asterisk) were observed.

In infected mice with passage 4 of the live attenuated parasite, severe swelling and inflammation of the lesion tissue was observed along with infiltration of inflammatory cells in different parts of the dermis as well as the infiltration of inflammatory cells in the portal area (Figure 5-arrow), liver with inflammatory reaction and microgranuloma formation were seen in this organ. Hyperemic spleen with red pulp and the formation of necrosis and inflammatory foci in the white pulp with the presence of parasites in the macrophages present in the inflammatory foci, lymph nodes with brief hyperemia in the cortex of the organ were seen.

In infected mice with passage 9 of the live attenuated parasite, it was observed that the lesion tissue showed severe inflammation and extensive infiltration of inflammatory cells in the dermis and hypodermis, the liver had an infiltration of inflammatory cells in the portal area along with an inflammatory reaction and microgranuloma formation, in the hyperemic spleen along with the formation of small necrotic foci in the white pulps of the organ, lymph nodes with brief hyperemia were seen in the cortical area of the organ (Figure 5-arrows).

In infected mice with passage 20 of the live attenuated parasite, the lesion tissue showed inflammation and infiltration of inflammatory cells in the dermis and hypodermis and the proliferation of Kupffer cells was observed in the liver (Figure 5-arrowheads), after parasite injection, the spleen tissue had a normal appearance and lymphocytes in the follicles of the cortex (Figure 5- asterisk).

3.4.3. Day 30

In infected mice with passage 1 of the live attenuated parasite, severe inflammation and necrosis of the dermis and extensive infiltration of inflammatory cells in the dermis and hypodermis, central venous hyperemia, proliferation of Kupffer cells, widespread inflammation and necrosis of liver cells, and the formation of multiple foci of granuloma in the liver, in the spleen the development of white pulp, tissue necrosis and the formation of granulomatous lesions, lymph nodes with moderate hyperemia and scattered foci of necrosis in cortical organs were seen (Figure 6-arrows). In infected mice with the passage 4 of the parasite were observed the lesion tissue severe inflammation and the emergence of necrotic foci in the dermis along with extensive infiltration of inflammatory cells, in the hyperemic liver of the central vein, the presence of numerous foci of necrosis of hepatocytes and inflammation along with granulomatous reaction

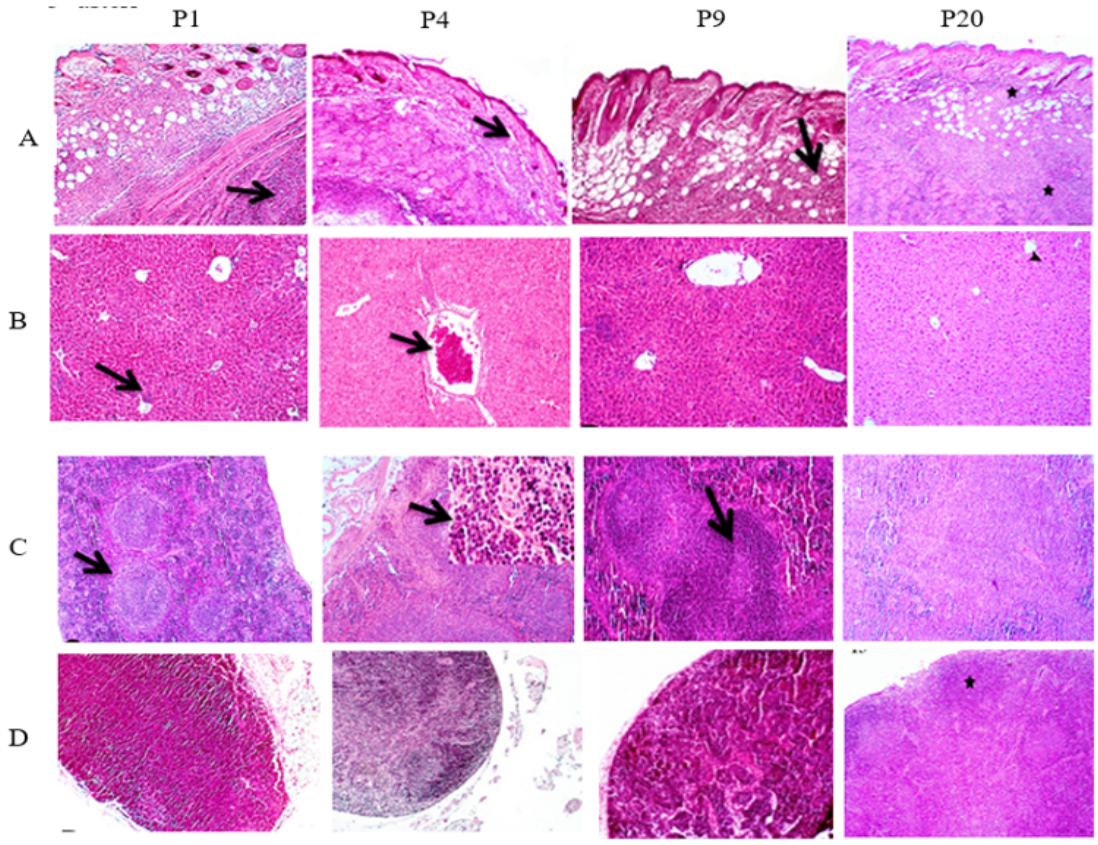


Figure 5. Histopathological changes of skin tissues, liver, spleen and lymph nodes on day 15

A) Skin, B) Liver, C) Spleen, D) Lymph node from mice infected with live attenuated parasite

P: Passages of live attenuated *L. major*.

and formation of microgranuloma, pathological manifestations in the spleen including hyperemia of red pulps, development of white pulps along with tissue necrosis and creation of granulomatous lesion, lymph nodes were swollen and hyperemic of the organ cortex with extensive inflammation and scattered foci of necrosis (Figure 6-arrows).

In infected mice with passage 9 of the live attenuated parasite showing severe inflammation and necrosis of the dermis and extensive infiltration of inflammatory cells in the dermis and hypodermis (Figure 6-arrow) and central venous hypertension. There was the proliferation of Kupffer cells, widespread inflammation and necrosis of liver cells, and the formation of multiple foci of granuloma in the liver, the development of a white pulp in the spleen, tissue necrosis and the formation of granulomatous lesions, moderate hyperemia of the lymph nodes and the formation of scattered foci of necrosis in the cortical organs.

In infected mice with passage 20 of the live attenuated parasite, it was showed severe inflammation and necrosis of the dermis and extensive infiltration of inflammatory cells in the dermis and hypodermis that in the livers, an accumulation of inflammatory cells and the formation of granuloma foci in the liver parenchyma that the low proliferation of lymphocytes in the follicles of the cortex was remarkable (Figure 6).

3.4.4. Day 45

In infected mice with passage 1 of the live attenuated parasite, the reduction of inflammation in different layers and the presence of parasite amastigotes in macrophages in the dermis layer of the skin, liver with severe tissue inflammation and granulomatous reactions were seen, there was a proliferation of Kupffer cells and an intense infiltration of inflammatory cells in the portal. The spleen showed a reduction in the size of the white pulp as well as scattered necrosis and fibrosis in this area. The lymph nodes were moderately hyperemic and scattered foci of necrosis were seen in the cortex (Figure 7-arrows).

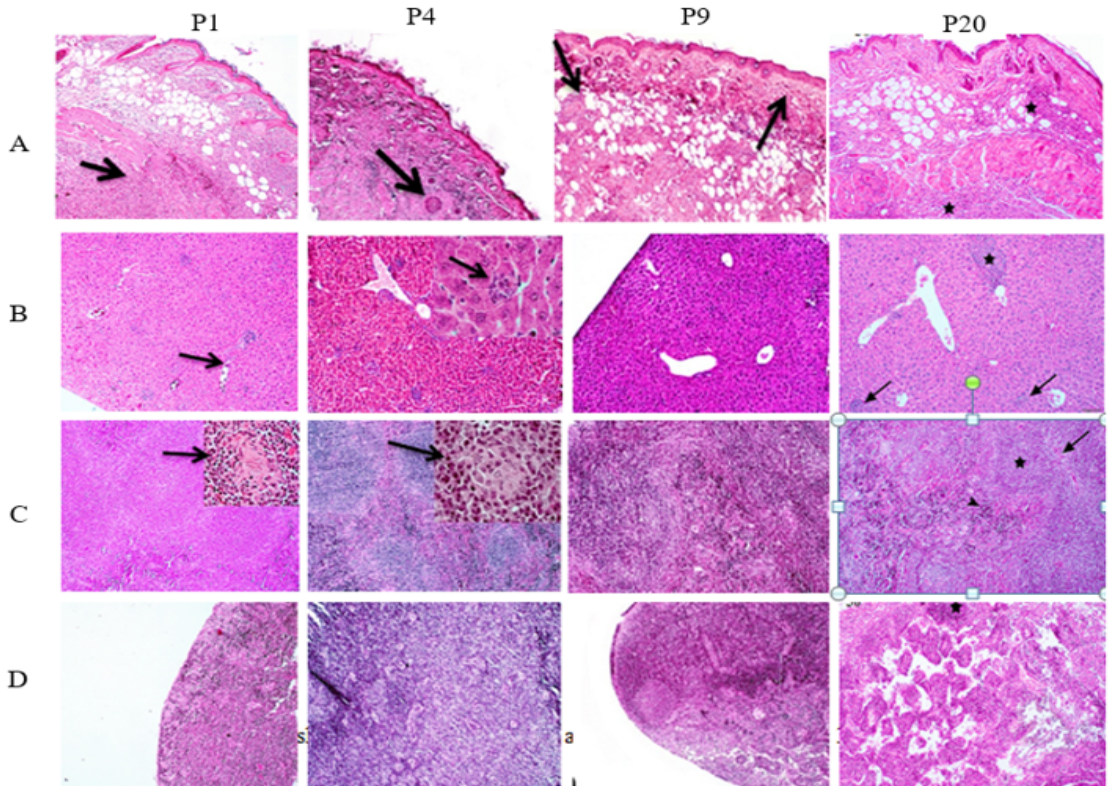


Figure 6. Histopathological changes of skin tissues, liver, spleen and lymph nodes on day 30

A) Skin, B) Liver, C) Spleen, D) Lymph node from mice infected with live attenuated parasite

P: Passages of live attenuated *L. major*.

In infected mice with passage 4 of the live attenuated parasite, inflammation was observed in the dermis and hypodermis along with attempts to repair and the presence of amastigote parasites in skin macrophages. There was moderate inflammation of the liver tissue along with an increase in Kupffer cells and scattered infiltration of inflammatory cells in the lobules of the organ, in the spleen, reduction in the size of the white pulp and reducing hyperemia, necrosis and fibrosis scattered in the spleen, moderately hyperemic lymph nodes and the presence of necrosis and fibrosis foci in the lymph nodes (Figure 7-arrows).

In infected mice with passage 9 of the live attenuated parasite, the decrease of inflammation in different layers and the presence of parasite amastigotes in macrophages in the dermis layer of the skin was remarkable, the liver had severe tissue inflammation and granulomatous reactions, there was a proliferation of Kupffer cells and severe infiltration of inflammatory cells in the portal area. Reduction in the size of the white pulp and scattered necrosis and fibrosis in the spleen, lymph nodes with moderate hyperemia and scattered foci of necrosis in the cortex were seen (Figure 7). In infected mice with passage 20 of the live attenuated parasite, the reduction of inflammation in different layers and the presence of parasite amastigotes in macrophages were observed in the dermis layer of the skin (Figure 7-asterisk), the livers had granulomatous reactions and the proliferation of Kupffer cells was detectable throughout the liver (Figure 7-arrowheads), the spleen was severe structural irregularities with destruction and necrosis of white and red pulp (Figure 7-arrow), Lymph nodes became hypertrophied due to the recruitment of lymphocytes.

4. Discussion

The occurrence of leishmaniasis depends on the virulence of the *Leishmania* species, the genetics and the immune response of the host cell. Prevention of the disease is very difficult due to the different geographical distribution of the related vectors and reservoirs [12]. Leishmaniasis is still considered one of the most common infections. There are several reasons for this, notably disease recurrence, bacterial wound infections, side effects, drug resistance, the high cost of treatment and

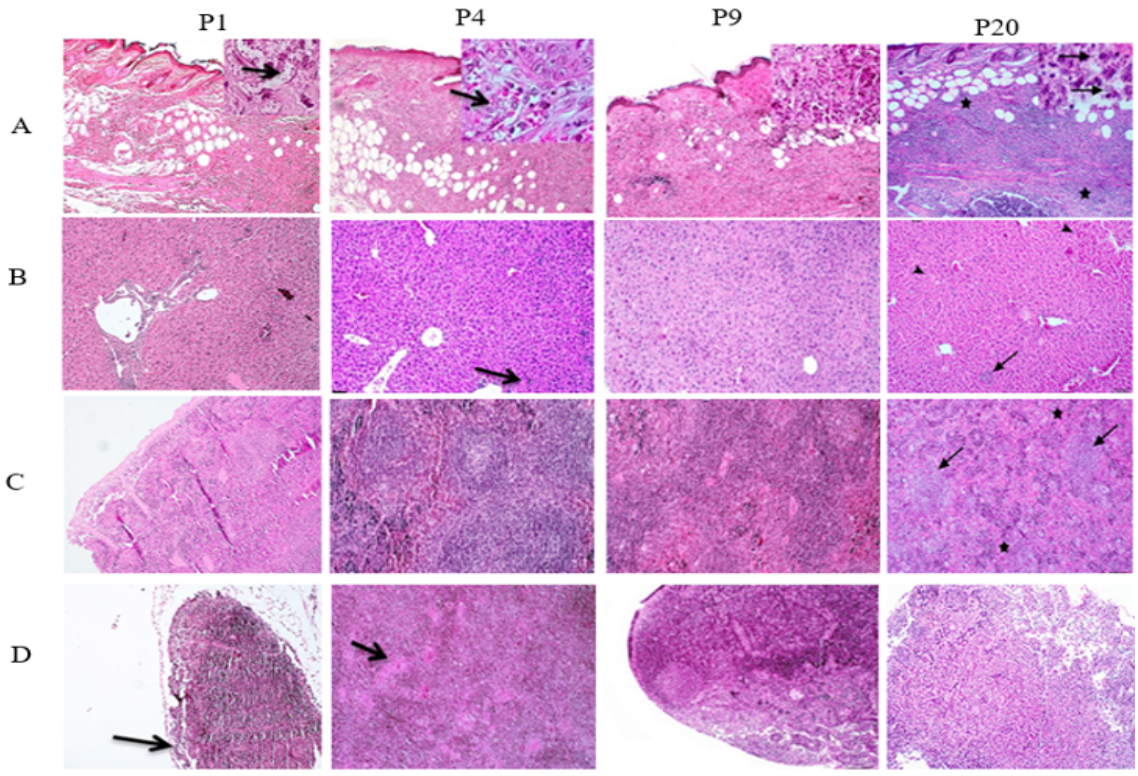


Figure 7. Histopathological changes of skin tissues, liver, spleen and lymph nodes on day 45

A) Skin, B) Liver, C) Spleen, D) Lymph node from mice infected with live attenuated parasite

P: Passages of live attenuated *L. major*.

the resistance of vectors to insecticides, and the opportunistic spread of the disease in immunocompromised people, especially in developing countries. The researchers found that subcultures of *Leishmania* parasites over a prolonged period reduce the infectivity of metacyclic promastigotes [13]. This is an important point in the development of future vaccines against leishmaniasis by considering the immune response. Parasites with high virulence are able to infect more macrophages and cause wounds by spreading amastigotes [14]; therefore, knowledgen of the mechanisms of immune response in from different dimensions and the nature of the immune system response to the parasite leads to the development of vaccines based on attenuated parasites. Glennie mentioned that central memory and skin-resident memory T cells are long-lived play an important role in vaccine research [15]. Continuous axenic cultivation of *Leishmania infantum* in-vitro reduces the virulence of the parasite and differentiates into amastigote forms, in addition these parasites gradually lose their pathogenicity after several passages in culture [16].

In this study different passages of the live attenuated parasite in the stationary phase (1, 4, 9 & 20) were used to investigate immunogenic responses in order to achieve virulence of the parasite capable of eliciting an immune response but lacking pathogenicity. The histopathological effects caused by this parasite at the skin, liver, spleen and lymph nodes have been studied in detail. As the liver, spleen and lymph nodes can be targets of the *Leishmania* parasite, so the presence of Leishman bodies and pathological effects have been reported in these tissues; Lymphocyte proliferation in splenic tissue has been evaluated as an immunological responses (cellular and humoral immunity), and macrophage phagocytosis has also been measured as the main host immune response against *Leishmania* [17]. After the injection of different passages of the live attenuated parasite (1, 4, 9 and 20), the time of appearance of the wound was almost the same but with the reduction of virulence, the size of the wound decreased significantly, so that the largest size was associated with passage 1 of live attenuated and the smallest size with the injection of passage 20 of live attenuated. The liver, spleen and lymph nodes can be targets of the *Leishmania* parasite, so the presence

of Leishman bodies and pathological effects have been reported in these tissues. Although macrophages as the main host cells for *Leishmania* parasite and play a role in the survival or control of infection, neutrophils and dendritic cells also have an important function in contact with the *Leishmania* parasite [18]. Researchers showed that Langerhans cells have an regulatory role and are responsible for suppressing the inflammatory response against infection with *L. major*. Dendritic cells of dermis (DCs) stimulate the effective immune response of T cells against *L. major*, as dendritic cells of dermis are placed in the outer paracortex and after their migration to the lymph node cause the activation and proliferation of T effector lymphocytes with the Th1 phenotype, the Th1 cells migrate to the site of infection and stimulate the macrophages to kill the parasite but after their migration to the lymph node, the Langerhans cells activate a type of T lymphocyte that has the property of suppressing the immune system [19]. Immunization with live attenuated parasites obtained from the subculture of a leishmaniasis wound parasite to a naive individual, known as Leishmanization was performed to protect against natural infection. Currently, a live attenuated vaccine for *L. major* used to humans clinical trials [20]. In-vitro studies have also used other selective methods to reduce the virulence of the parasite such as the gentamicin, which can also be used to reduce the virulence of the parasite, attenuated parasite with gentamicin were infected but could not survive in the macrophages of BALB/C mice, IgG1 levels were lower in BALB/C mice immunized with gentamicin attenuated *L. major* than in mice infected with *L. major*, reflecting Th1 activation [21]. Despite extensive studies on live vaccines against leishmaniasis, this research has been conducted in laboratories and tested in rodent models that are better suited for efficient results in human clinical trials [22].

Our results revealed an increase in phagocytosis in the injection of passages 20 and 4 on days 15 and 30, and passage 4 in 45 also had a significant increase compared to the other inoculations, but virulence 1, caused an increase in lymphocyte proliferation, this result shows an increase in the proliferation of TH2 lymphocytes due to the activity of Langerhans dendritic cells, but with lower virulence, dermis dendritic cells likely have increased TH1 lymphocytes and activated macrophages. The size of the wound in the infection with high virulence of the parasite increased significantly compared with the infection with lower virulence (Figure 1), and the wound healing time was also shorter in the infection with passage 20 of attenuated parasite and there was no mortality; this is consistent with the results of histopathologic examination of skin and spleen tissue for detection of parasites [23], 15 days after parasite injection, the skin tissue around the wound in three passages (1, 4 and 9) of the attenuated parasite showed severe inflammation with infiltration of inflammatory cells and microgranulomas in the liver of all three passages of parasite, but the degree of inflammation in the skin of passage 20 of the attenuated parasite was less. At day 30 after parasite injection the morphology of the skin, spleen, liver and lymphoid tissue showed no significant difference between the three passages of the parasite, but the degree of inflammation and lymphocytic infiltration was lower in passage 20 infection (Figures 5, 6, and 7). A decrease in inflammation was observed in spleen tissue infected with passages 9 and 20 (Figure 6) of live attenuated with no intracellular *Leishmania* amastigotes and the parasite burden of Leishman bodies was not observed in the liver and spleen tissues at passage 20 of live attenuated, but it was seen in other virulences, destruction of lymph node capsule and follicular hyperplasia with germinal centers were visible at 4 passages in three times. The data obtained are similar to the results of Oryan et al., who studied the systemic effects of inoculation of *L. major* parasites with high virulence, and they confirm our findings regarding the histopathologic changes in the spleen, liver and skin [17], also, *L. mexicana* infection was found to destroy lymphoid tissue, particulary the lymph node parenchyma due to the presence of infected macrophages as well as the red pulp of the spleen and the portal of the liver [24]. The use of killed and live forms of *L. major* has shown differences in the maintenance of immune response against live form of *leishmania* [25]. Hence, further study on the pathogenicity and immunogenicity of the attenuated strain of *L. major* parasite can be important for vaccine development.

Investigation of *Leishmania* infection by injection of 4 different virulences of the live attenuated parasite showed that *Leishmania* parasites in lower passages of attenuated parasite such as 1 and 4 in this study had no significant differences in wound size, phagocytosis and proliferation of splenocytes, but even in some cases the inflammation and infection was more severe in the passage 4 of attenuated parasite, the histopathologic results also confirmed this result. Passage 9 of the attenuated parasite also showed inflammation and structural changes in the lymphoid tissue along with less intensity of inflammation and minor structural changes in the lymphoid tissue, but the destructive effects of the parasite were observed when using of passage 1 of attenuated parasite. The pathological results in the skin, confirmed by the decrease in wound size. The significant increase in phagocytosis in the middle of the infection period as well as in some of the pathologic results of the splenic

lymphoid organ and lymph node at passage 20 of attenuated parasite overlapped with the results of passage 9 of attenuated parasite; therefore, the increase in parasite passage with a the decrease in virulence may be important for studies related to the development of vaccines against live attenuated strains.

Acknowledgements

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Compliance with ethical guidelines

This study was approved by the Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (Code: IR.AJUMS.ABHC.REC.1398.074).

Data availability

Data for this finding are available on request from the corresponding author.

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Authors' contributions

Conceptualization, study design: Hossein Rezvan; Experiments and data interpretation: Hossein Rezvan, Sahar Hamoonnavard; Statistical analysis: Sahar Hamoonnavard; Administrative, technical, and material support: Habib Habibpour, Ehsan Mohseni, Mohammad Ghasemi and Rezvan Dalvand; Writing the original draft: Hossein Rezvan and Sahar Hamoonnavard; Review and editing: Hossein Rezvan, Mohammad Hossein Feiz Haddad, and Sahar Hamoonnavard; Data acquisition and supervision: Hossein Rezvan and Mohammad Hossein Feiz Haddad.

Conflict of interest

The authors declared no conflict of interest.

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