Achievement amastigotes of *Leishmania infantum* and investigation of pathological changes in the tissues of infected golden hamsters

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Abstract *Leishmania infantum* is an agent of visceral leishmaniasis (VL). Amastigote form is a more appropriate target for investigations on vaccines, treatment, and diagnosis. This study aimed to achieve the amastigotes of *L. infantum* in the golden hamster and J774 macrophages and report the pathological changes that occur in the liver and spleen of the hamsters with VL. 4 male golden hamsters were infected with *L. infantum* promastigotes. After 5 months, the hamsters were euthanized and touch and pathology smears were prepared from the livers and spleens. Then, these tissues were homogenized and centrifuged at 100×g. Supernatants were collected and centrifuged at 2000×g and the pellets were collected. In the next part of our study, J774 macrophages were infected with *L. infantum* promastigotes. Then, the infected macrophages were ruptured. Centrifuge stages were done same the previous part. The amastigotes were observed in touch and pathology smears. A load of amastigotes in the livers was more than the spleens in both types of smears. Although the livers' structure had undergone pathological changes, the spleens were unchanged. Also, the macrophage infectivity ratio was up to 95%. Our results present a simple and accessible way of achieving a lot of pure and real amastigotes for different fields in *Leishmania*. Also, it seems that the pathological changes occurring in the spleen

and the liver of animals with VL are different and probably can be attributed to the genetic and immune process of the infected animals.

Keywords *Leishmania infantum* · Amastigote · J774 macrophage · Golden hamster · Pathological change

Introduction

Leishmania genus is an intracellular protozoan that infects the macrophage and dendritic cell lineages of their vertebrate host (Naderer and McConville 2008). *Leishmania* genus accounts for a group of diseases accompanied by a broad range of clinical manifestations together known as leishmaniasis (Goto and Lindoso 2012; Mahshid et al. 2014). *Leishmania infantum* is one of the main causes of visceral leishmaniasis (VL) (Hatam et al. 1997; Mohammadi-Ghalehbin et al. 2017). Manifestations of VL are different and vary and sometimes it could appear in a life-threatening a progressive visceral form of the disease. For instance, the coincidence of HIV with VL is a serious problem for health care conditions (Desjeux 1999).

Leishmania parasite has a digenetic life cycle with the migration between Phlebotomus and mammalian host. Amastigote and promastigote are two important forms of *Leishmania* parasites in their life cycle. Most of our knowledge about molecular biology and biochemistry of the *Leishmania* species is related to the promastigote form, because of its relatively easily cultured in vitro conditions (Pan et al. 1993). Since promastigote is present in the insect vector, it is not an appropriate target for discovering anti-leishmanial drugs, vaccines and diagnostic markers in *Leishmania* reservoirs (Fumarola et al. 2004; Mohammad et al. 2015). Therefore, having real and pure amastigote can

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be an appropriate target for designing vaccines and diagnostic approaches in *Leishmania*.

Some studies have suggested that axenic amastigote (amastigote like) can be a source of different challenges in *Leishmania* surveys (Gupta et al. 1996; Somanna et al. 2002; Habibi et al. 2008). Some researchers have shown the differences in several cellular interactions, including intracellular transportations, metabolic processes, and response to oxidative stress by comparing the axenic form of *L. infantum* amastigotes and the amastigotes obtained from the macrophage cell lines (Rochette et al. 2009). The axenic model has limitations as it does not include all stages of intracellular parasite development (Buckner and Wilson 2005). For instance, because of the axenic form inability to cross the host cell membranes or maintain stability under low pH, compounds active against these forms might be unable to reach the intracellular amastigote (Vermeersch et al. 2009). So, it seems that preparation of pure and real amastigotes (not amastigote like) is an essential step in many *Leishmania* studies.

On the other hand, previous studies mentioned that some changes can occur in the spleen and liver in animals that naturally and experimentally infected with VL. The function of the liver is resolving the *Leishmania* parasite by the creation of granulomas mediated by Kupffer cells. In contrast, the spleen is the place for the creation of cell-mediated immunity (CMI) although; ultimately the *Leishmania* parasite resists against the immune response which takes place in this area and leads to immunopathological changes. In addition, during the entire course of infection with VL, the spleen maintains the infection, but the other organs do not show this behavior (Wilson et al. 1996; Carrion et al. 2006).

This study will establish an in vivo system for achieving the amastigotes of the *L. infantum* from infected golden hamsters with promastigotes and in vitro systems using J774 macrophage cell lines. It seems that obtaining a high number of *L. infantum* amastigotes can contribute to the development of strategies for designing effective drugs, vaccines and diagnostic methods in VL in the future. Also, we examined the pathological changes in the liver and spleen of the infected hamsters to indicate the alterations that could probably be related to VL.

Materials and methods

Experiments with hamsters were done in accordance with guidelines of the Institutional Animal Care and Committee on Ethics of Animal Experimentation from the Shiraz University of Medical Sciences. Grant Number 94-7597.

Infection of golden hamsters with *L. infantum* promastigotes and achieving amastigotes

Animals

Five male golden hamsters (*Mesocricetus auratus*) weighing 40–60 g were used in this study. They were housed in temperature-controlled accommodation, fed with standard rodent dried food, and provided with water.

Parasites

Leishmania infantum strain (MCAN/IR/07/Moheb-gh) was provided by the Department of Parasitology and Mycology, Shiraz University of Medical Sciences, Iran. Briefly, *L. infantum* promastigotes were cultured in RPMI-1640 (Shelmax Company) supplemented with 15% (v/v) heat-inactivated fetal calf serum (FCS) and 100U/ml penicillin, 100 µg/ml streptomycin at 25 °C and sub-cultured for achieving more parasites in the stationary growth phase.

Parasite inoculation in hamsters

For the immune system suppression, 1 week before injection of the parasites, 2.5 mg cyclosporine was injected to the peritoneum of each hamster. Then, four hamsters were inoculated (one hamster for negative control) with *L. infantum* promastigotes under ether anesthesia. The injection was done by both intra-peritoneal (10^8) and intracardial (2×10^7) promastigotes. The injection of cyclosporine continued for 1 week after promastigotes injection.

Preparation of touch and pathological smears from the liver and spleen of hamsters

After 5 months of promastigotes injection, the hamsters were sacrificed under ether anesthesia. The liver and spleen of the infected and uninfected hamsters (negative control) were aseptically removed. Then, six parts of each tissue (liver and spleen) were cut (2×2 mm). Then, touch smears were prepared from these tissues and stained with Giemsa. Also, these prepared tissues (2×2 mm), were used for developing the pathology blocks and smears. The pathology serial sections from all livers and spleens were prepared and stained with Hematoxylin and Eosin (H and E) (Nakanuma and Ohta 1979).

Polymerase chain reaction (PCR) for confirmation the presence of amastigotes in the tissues

DNA extraction The DNA was extracted from the spleens and livers. In brief, a small amount (2×2 mm) of each liver and spleen was mixed to 200 µL of lysis buffer

(50 mmol/L Tris–HCl (pH 7.6), 1 mmol/L EDTA, and 1% Tween 20) and 10 μ L of a proteinase K solution (containing 19 mg of the enzyme/mL) and incubated at 37 °C for 24 h. In the next step with phenol/chloroform/isoamyl alcohol, the lysate was extracted twice before the DNA was precipitated with ethanol 100%. After DNA precipitation, it was re-suspended in 100 μ L of double distilled water and stored at 4 °C (Asgari et al. 2007).

Primers and PCR reaction mixture The conserved region was selected from minicircle kinetoplast DNA because its copy number is more than 10^4 per parasite, maximizing the possibility of detection. The set of primers LINR4 (forward: 5'-GGGGTTGGTGTAATAAGGG-3'), and LIN17 (reverse: 5'-TTTGAACGGGATTTCTG-3') were used for PCR (Salotra et al. 2001, Akkafa et al. 2008). A reaction mixture containing 200 μ M (each) of deoxynucleoside triphosphate, 1.5 mM of MgCl₂, 2.5 μ L of 10 X Taq polymerase buffer, 1.5 unit of Taq DNA polymerase and 40 pmol of each primer was used in a total reaction volume of 25 μ L including 5 μ L of the DNA sample. The mixture was amplified in a programmable thermocycler (Thence Cambridge, UK) for 5 min at 4 °C (1 cycle) followed by 30 cycles at 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min followed by a final elongation at 72 °C for 5 min (1 cycle) and kept at 4 °C (Asgari et al. 2007).

Agarose-gel electrophoresis A 10 μ L sample of the final PCR product was subjected to electrophoresis in 1.5% agarose gel. 5 μ L of loading buffer was added to the product before electrophoresis and visualized under UV light with ethidium bromide.

Isolation amastigotes from the liver and spleen of hamsters

The most parts of the livers and spleens, except those, were used for DNA extraction and pathology blocks, homogenized in Dulbecco's modified essential medium (DMEM, Sigma) containing 10% (v/v) FCS, 20 mM L-glutamine and 10 mM sodium pyruvate. The homogenates were centrifuged at $100\times g$ for 5 min at 4 °C to remove the large cell debris. Then, the supernatants were collected and centrifuged at $2000\times g$ for 10 min at 4 °C. The resulting pellets were re-suspended in DMEM containing 0.05% (w/v) saponin and incubated at room temperature for 5 min. Finally, after centrifugation ($2000\times g$ for 10 min at 4 °C), the amastigote-enriched pellets were washed twice in fresh DMEM.

For increasing the purification of amastigotes, the final pellets were solved in 550 μ L DMEM and mixed with 450 μ L Percoll 100%. Then, this mixture was added to the 1 ml Percoll 100% and centrifuged at $3500\times g$ for 30 min. After centrifugation, amastigotes formed a ring at the

interface between the 45 and 100% Percoll, while the cells and debris floated at the top near the meniscus. After separation of the amastigotes and washing with DMEM, it was re-suspended in a final volume of 2 ml DMEM and frozen at – 70 °C. The presence of pure amastigotes can be checked by smear preparation and Giemsa staining.

Infection of J774 macrophages with *L. infantum* promastigotes and achieving amastigotes

Macrophages and parasites

Semi-adherent J774 macrophage cell line was provided by Department of Immunology, Shiraz University of Medical Sciences, Iran. J774 macrophages were grown in DMEM containing 10% FCS. All media were supplemented with 100U/ml penicillin, 100 μ g/ml streptomycin. Also, *L. infantum* strain (MCAN/IR/07/Moheb-gh) was prepared and mass cultured in stationary growth phase the same as that mentioned in previous parts.

Macrophages infectivity

Briefly, after the mass cultivation of J774 macrophages in DMEM containing 10% FCS, the macrophages were pelleted at $450\times g$ for 5 min at room temperature. After diluting the pellet with DMEM containing 10% FCS and counting macrophages, 3×10^5 macrophages were added to per well (in a plate with 24 wells).

Promastigotes in the stationary growth phase were used for infecting the J774 macrophages at a final ratio of 20 promastigotes per macrophage. To this end, promastigotes were pelleted with the centrifuge at $1250\times g$ for 10 min and then resuspended in DMEM containing 10% FCS. Finally, the promastigotes (6×10^6) were added to the macrophages in each well.

For achieving high infectivity, the promastigotes should be added to the macrophages immediately, so it is an important issue that should be noticed. In the next step, the plate was incubated at 37 °C with 5% CO₂. After 24 h, unphagocytized promastigotes were removed by substitution of the supernatant with fresh DMEM containing 10% FCS. The plate was incubated for 8–10 days at 37 °C in 5% CO₂ and fresh DMEM media (450 μ L) was added to each well per day. The infectivity was checked with smear preparation and Giemsa staining.

PCR for confirmation the presence of amastigotes in the infected macrophages

The same PCR procedure was performed to confirm the presence of amastigotes in the infected macrophages.

Isolation of amastigotes from infected J774 macrophages

Firstly, 100–200 μ L of DMEM media containing 0.05% (w/v) saponin was added to each well. After turning up and down and passing the contents of each well from the needle (Gauge 27), the contents of all wells were collected in a tube and shaken firmly. Then, the tube was centrifuged at $100\times g$ for 5 min at 4 °C. In the next step, the supernatant was collected and centrifuged at $2000\times g$ for 10 min at 4 °C. The pellet was washed 2 times with DMEM without FCS at $2000\times g$ for 10 min at 4 °C. The pellet was re-suspended in a final volume of 2 ml DMEM and frozen at – 70 °C. The pure amastigotes can be checked by smear preparation and Giemsa staining. Also, for increasing the purification of amastigotes, the Percoll protocol can be used the same as previously mentioned.

Results

PCR for confirmation the presence of amastigotes

For confirmation the presence of amastigotes in the liver, spleen and in the infected macrophages, the PCR was done. By using the LIN4 and LIN17 primers, samples which produced a 720 bp band were judged to be positive for *L. infantum*. Based on the following PCR amplification and electrophoresis results (Fig. 1), in all samples, the 720 bp band was observed.

Fig. 1 PCR results: obtained pellet from the spleen (1), liver (2), infected J774 macrophages (3) and ladder 1000 bp (4)

Recovering amastigotes from golden hamsters

The first set of questions was on recovering amastigotes from the livers and spleens of golden hamsters. Our touch smears finding showed an acceptable load of amastigotes in the liver and spleen. Interestingly, a load of amastigotes in the liver touch smears was more than that of the spleen (by microscopic method and counting in different shines) (Fig. 2).

The results of pathology serial sections in the livers and spleens showed that the amastigotes existed in both tissues clearly. In the liver, the amastigotes were seen in the brown color zoon (Fig. 3). In addition, degeneration and necrosis of hepatocytes were observed. Hydropic degeneration (arrowhead) and loss of nucleus were the third phenomena in the tissue of the livers (Fig. 4). The amastigotes are seen clearly in the hepatocytes and cytoplasm are affected by hydropic degeneration (arrowhead) (Fig. 5). Finally, the lymphocytes were accumulated in the portal area (arrowhead) (Fig. 6).

Also, in the spleen tissue, amastigotes were obviously detectable (Fig. 7). Unlike the liver tissue, surprisingly no structural changes were seen in the spleen tissue (Fig. 8). The results of pathology serial section revealed that a load of amastigotes in the liver was more than that in the spleen.

In the smears prepared from the homogenized livers and spleens, the amastigotes were observed with an acceptable load (by microscopic method and counting in different shines) (Fig. 9). The isolated amastigotes were devoid of the least visible host cell debris. In this section, no comparison was done for a load of amastigotes between obtained homogenized from the spleen and the liver. As the size of these two tissues is not the same.

Achievement amastigotes from the J774 Macrophages

The second set of questions aimed to infect the J774 macrophages with the *L. infantum* promastigotes. Interestingly, the results of our experiment showed that most of the macrophages were infected and the rate of infectivity was almost 95% in all experiments (Fig. 10a). For calculating the number of amastigotes per macrophage (by microscopic method and counting in different shines), the infected macrophages were divided into groups of low and high infected macrophages, respectively. In the low infected level, the range of amastigotes was 5–10 in each macrophage. In contrast, in the high infected level, the infected macrophages had more than 10 amastigotes (Fig. 10b).

The results of amastigotes extraction from the macrophages showed that the amastigotes were clearly detectable with an acceptable load and isolated amastigotes were devoid of any visible host cell debris (Fig. 10c).

Fig. 2 Touch smears with Giemsa staining (**a** liver, **b** spleen)

Fig. 3 Liver tissue: The presence of amastigotes in the brown color zoon ($\times 40$ H and E staining)

Fig. 5 Amastigotes in the hepatocytes and hydropic degeneration in cytoplasm (arrowhead) ($\times 200$ H and E staining)

Fig. 4 Liver tissue: Hepatocellular degeneration, necrosis, hydropic degeneration (arrowhead) and loss of nucleus ($\times 400$ H and E staining)

Fig. 6 Lymphocyte accumulation in the portal area of the liver (arrowhead) ($\times 200$ H and E staining)

Fig. 7 Amastigotes in the spleen ($\times 400$ H and E staining)

Fig. 8 The unchanged structure of the spleen ($\times 40$ H and E staining)

Discussion

The first set of questions in this study sought to determine why recovering pure amastigote is important. Most studies are using cultured *Leishmania* parasites (promastigote) in vitro assays. This form of parasite seems not to be

appropriate as amastigote for in vitro experiments such as vaccine and diagnostic marker in the case of VL (Croft 2001).

In previous studies, some antigenic targets on amastigotes of *Leishmania* species were introduced as effective candidates for vaccines in leishmaniasis. For instance, the P-8 proteoglycolipid complex (P-8 PGLC), an amastigote antigen of *L. pifanoi*, has been shown to protect the mouse after challenge with the parasite (Carrillo et al. 2007). In another investigation, the authors examined the protection and diagnostic effects of LiHyp1 and LiHyp6 proteins originating from amastigote of *L. infantum* for canine visceral leishmaniasis (CVL) (Martins et al. 2015).

Accordingly, based on the mentioned information, having pure and real amastigotes (not amastigote like) will increase the accuracy and precision of the studies, especially *Leishmania* proteomics approaches which are conducted for discovering new vaccines and diagnostic markers. Although in the first impression, the preparation of real and pure amastigote seems to have a simple protocol, according to our experiences and few previous studies in this regard, it is not a simple task (Menezes et al. 2013).

Recovering amastigotes from golden hamsters

In some studies, the animal models have been used for different *Leishmania* researches. Outbred mice are not very suitable for in vivo studies which are in the field of *Leishmania* due to self-curing infections. Then, having a good model is very essential. For VL, the best animal model is golden hamster (*Mesocricetus auratus*) because it is highly susceptible to this disease. Nevertheless, few studies have been released in regard to the experimental infection of hamsters with *L. infantum* for achieving amastigotes in vivo. Therefore, we presented a method for infection of golden hamsters with *L. infantum* promastigotes.

Fig. 9 Prepared smears from homogenized tissues with Giemsa staining: The presence of amastigotes in the arrowhead (a liver, b spleen)

Fig. 10 **a** The infectivity percentage of J774 macrophages (up to 95%), **b** an infected macrophage within more than 10 amastigotes, **c** extracted pure amastigotes from J774 macrophages

Our data showed that the golden hamster is a susceptible model for collecting the amastigotes *in vivo* similar to some previous studies (das Does Moreira et al. 2016). Some experimental studies could not infect the hamster with *L. infantum* promastigotes. It can be attributed to the genetic factors and resistance of this animal against the VL that we solved it through injection of immunosuppression drug (Stanley and Engwerda 2007).

Infection of hamsters with *L. infantum* almost mimics the clinical and pathological aspects of human VL (das Does Moreira et al. 2016). Our study results illustrated that a load of amastigotes in the liver (in touch and pathology smears) is more than the spleen and the essential pathological structural changes were seen in the livers, but the structure of the spleen is unchanged. In regard to the control group which was treated with cyclosporine, we found that the injection of this drug did not have any effect on the pathological feature of the liver and spleen. Also, in spite of suppression of the animal's immune system, all animals stay alive until the end of the experiment.

According to the earlier studies, the liver and spleen of the infected hamster with *L. infantum* promastigote showed a big change and a load of parasitemia in both tissues was

high (das Does Moreira et al. 2016). In a study on dogs which were naturally infected with VL (Manochehri et al. 2012), histopathological changes in the spleen including severe infiltration of the plasma cells, the presence of megakaryocytes indicating extramedullary hematopoiesis, inflammatory granulomatous reaction, atrophy of the lymphoid follicles and loss of germinal centers, lymphoid follicles hypertrophy were observed. In this study, amastigotes in a high load were seen just in one case. However, no changes were seen in the livers.

Another study was performed in 2008 on the stray dogs (Santana et al. 2008). They observed amastigote in 10% of the spleens. In infected spleens, they observed a higher frequency of perisplenitis, granuloma, structural disorganization and atrophy of the lymphoid follicles. This study also reported changes in the white pulp of the spleen which are related to naturally acquired visceral leishmaniasis. In this study, the livers were not examined.

One study performed in naturally infected dogs showed that the dogs which signs of infection have a higher frequency of parasitism compared with the asymptomatic group. Inflammatory changes were severe in the symptomatic group and were related with parasitism. In spite of

other studies, they observed histopathological changes in their liver. These changes were evaluated by measuring the biochemical alterations according to the progression of clinical forms of CVL. They indicated that there was a relationship between clinical symptoms and frequency of hepatic parasitism (Giunchetti et al. 2008). In contrast, in a study which naturally infected dogs with *L. infantum*, no sign of change was observed in the liver of dogs (Natami et al. 2000).

Taken together by reviewing the previous investigations, it seems that the pathological changes that can occur in the spleen and liver of animals with VL are different in natural and experimental infections. We suggest that these different patterns can be attributed to the amount of inoculated promastigotes, the way of injection for producing infectivity, the genetic aspects of animals and the immunological process that can especially occur in the spleen. Furthermore, the unchanged structure of the liver or spleen in some studies can be related to the duration of the infection. It means that probably these organs were not infected simultaneously. Generally, it seems that the main reason for different patterns of VL in the liver and spleen is not clear, so further investigation is required in the future.

Achievement amastigotes from the J774 Macrophages

For preparing the pure amastigotes of *L. infantum* in vitro, a study (Maia et al. 2007) used different macrophages. They examined the susceptibility of human peripheral blood-derived macrophages, macrophages derived from the mouse bone marrow, mouse peritoneal macrophages and those differentiated from the cell lines U-937 and DH82. Their results indicated that infectivity of both human peripheral blood macrophages and DH82 cells was less than others. It was revealed that U-937 and the cells prepared from the mouse bone marrow had the most infectivity with *L. infantum* promastigotes.

According to our obtained in vitro data and comparison with the mentioned studies, we found out that the J774 macrophage could be the first choice for preparation of amastigote due to its rate of growth. Furthermore, the infectivity rate of these cells with *Leishmania* promastigote was also high. For this reason, we suggest that this cell line can be the first selection for getting *L. infantum* amastigotes in comparison with other cell lines used in other studies.

Generally, it should be mentioned that for preparing lots of amastigotes, using animal models (hamster) and J774 macrophages are better than using methods that producing amastigote like. It's obvious that getting amastigotes by means of in vivo methods using the animal model gives us the same amastigotes in all aspects which are in the

reservoirs of *Leishmania*. In contrast, using in vivo method is not a simple duty due to a long time is needed to make animal infect with the parasite. Furthermore, some studies fail to infect the hamster is another disadvantage of in vivo study. In spite of in vivo method which is too long, using J774 macrophages help us to have a large number of amastigotes in a short time, whatever it is a tedious and liberos duty. In addition, it needs an expert laboratory scientist. Taken together, obtained amastigotes from both mentioned sources are reliable for different fields in *Leishmania* such as vaccines, diagnostic tests, and drug assays.

Conclusion

Based on our data, we propose a method for preparing real and pure amastigotes for different purposes, such as potential anti-leishmanial drugs, proteomics surveys, vaccines, isoenzyme surveys and diagnostic tests in VL. With this protocol, the purity of the extracted antigens from amastigote will be increased, and useless antigens that can probably originate from other extra cells will be omitted. Ultimately, using real and pure amastigotes (not amastigote like) will enhance the accuracy and precision of the future experiments. On the other hand, keeping the structures of amastigotes intact is on demand in some approaches. In both parts of our study by choosing appropriate methods, the amastigotes were extracted with unchanged structures. Also, our finding and comparison with previous studies showed that experimental surveys in vivo cannot be an appropriate way for the investigation into VL pathological changes in the liver and spleen because the results in this regard vary. It is recommended that we should rely on the results of human experiments in this regard.

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Authors' contribution Sajad Rashidi: Data collection and manuscript writing, Kurosh Kalantar: Project development, manuscript editing, and data analysis, Gholamreza Hatam: Project development, manuscript editing.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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