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Research paper

Antileishmanial effect of rapamycin as an alternative approach to control *Leishmania tropica* infection

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ABSTRACT

Cutaneous leishmaniosis (CL) is a parasitic disease in animals and human with no satisfactory treatments and vaccination. Rapamycin is a potent inhibitor of mammalian target of rapamycin (mTOR) with various applications. Here, the effect of rapamycin alone or in combination with two other drugs, namely amphotericin B (AmB) and glucantime, was investigated against *Leishmania tropica* infection. *In vitro* viability and electron microscopy evaluation of the parasites showed detrimental changes in their appearance and viability. Treatment with clinically relevant dose of rapamycin (10.2 μg/dose) is able to control the parasite load in BALB/c mice infected with *L. tropica*. Furthermore, the cytokine profiles showed significant polarization towards Th1 immune response. Surprisingly, combination therapy with either AmB or glucantime was not efficient. Rapamycin is showed an effective alternative therapy against leishmaniosis caused by *L. tropica*.

1. Introduction

Several species of the genus *Leishmania* are causative agents of leishmaniosis, which is a serious public health problem in at least 98 countries with incidence of two million new cases annually. Dogs are considered as the main reservoirs of leishmaniosis. Approximately 3500 human cases of zoonotic leishmaniosis in Brazil and 875 in Meditaranian region are reported annually. Although canine leishmaniosis (CanL) is highly prevalent in many countries, the diseases is found mainly in South America and Mediterranean region. There are three main forms of disease, including cutaneous (CL), muco-cutaneous (MCL) and visceral leishmaniosis (VL). The most prevalent form of leishmaniosis is CL, mainly caused by species such as *L. major*, *L. aethiopica* and *L. tropica* in the old world, and *L. amazonensis, L. mexicana*, and *L. guyanensis* in the new world. Leishmaniosis is a vector-borne zoonotic disease, and about 70 species of mammals, including human and dogs can act as reservoirs of the parasite. CanL, which is primarily transmitted by female *Phlebotomine* sand flies between animals and subsequently to humans, plays a major role in disseminating the diseases (Alvar et al., 2006; Desjeux, 2004; Gijón-Robles et al., 2018). Manifestation of CanL may be different from one dog to another, ranging from self-healing lesions to a progressive and lethal disease. A direct parasitological cure for CanL is not available, and clinical recurrences are frequent (Ribeiro et al., 2018). Clinical symptoms of CL caused by *L. tropica* vary from small skin lesions to disfiguring scars (Schwenkenbecher et al., 2006). CL caused by *L. tropica* is recognized as anthroponotic; however, zoonotic features have been reported (Kassahun et al., 2015). Dogs are usually considered as the main domestic reservoirs for *L. infantum*, but it has been recently reported that they also act as a carriers for *L. tropica* (Bamorovat et al., 2015; Baneth et al., 2017). There are also some reports from Greece about an increase in CanL due to *L. tropica* infection. *Phlebotomus similis* is believed to be the potential vector in this area, increasing the risk of spreading this anthroponotic infection to other regions (Ntais et al., 2013). The epidemiology of *L. tropica* infection is not fully elucidated; however, it has been reported that infection with this parasite in urban areas and highly-populated regions is more frequent (Mebrahtu et al., 1992). Control of leishmaniosis is mainly reliant on the host immune response, early diagnosis and proper treatments (Vendrame et al., 2010). Pentavalent antimonial, meglumine antimoniate (glucantime) and sodium stibogluconate are among the traditional treatments (Arevalo et al., 2007). Glucantime is often the first-line treatment for all forms of leishmaniosis, and clinicians have used it for more than 60 years (Moreira et al., 2017), although its mechanism of actions has not been clearly understood. There are some reports on its interaction with bioenergetic processes of intracellular parasites and consequently their

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depletion of adenosine triphosphate (ATP) (Demicheli et al., 2002). However, there are major concerns about drug toxicity as well as developing resistance in the parasite, making glucantime not the most desirable choice anymore (Singh and Sivakumar, 2004). AmB and less toxic liposomal AmB (Ramos et al., 1996) are other predominant treatments, but the high cost makes them unaffordable in many countries where the disease is highly endemic. Moreover AmB is not used in dogs due to the risk of developing resistance strains that could be zoonotically transmitted to humans (Mistro et al., 2016; Yardley and Croft, 1997; Solano-Gallego et al., 2009). The anti-leishmanial activity of AmB could be explained by its binding to C-24 alkylated sterols, specifically ergosterol, with various selective affinities. Higher concentrations of this drug (> 0.1 μM) can accelerate the cationic and anionic influx with the formation of aqueous pores and consequently cause cell lysis (Ramos et al., 1996; Pourshafie et al., 2004). Despite all recommended strategies to control leishmaniosis, the conventional anti-leishmanial drugs as mentioned above have low efficacy against CanL.

Rapamycin is a well-known inhibitor of the mammalian target of rapamycin (mTOR) and was first discovered by its antifungal effect; afterwards, due to its immunosuppressive effect, rapamycin was used clinically to prevent graft rejection in transplantation and also for its anticancer properties (Laplante and Sabatini, 2009). Its target protein, mTOR, is a highly preserved serine/threonine protein kinase that has key roles in regulation of essential mechanisms such as initiation of specific mechanisms of cell proliferation, translation and growth (Zhang et al., 2007). The catalytic subunit of this molecule is consisted of two multi-protein complexes named as mTORC1 and mTORC2. Each of these two complexes is associated with another protein: mTORC1 interacts with the regulatory associated protein of mTOR (RAPTOR), and mTORC2 interacts with the rapamycin-insensitive companion of mTOR (RICTOR) (Laplante and Sabatini, 2009). Furthermore, mTORC1 can initiate several anabolic pathways such as protein and nucleic acid synthesis. Importantly, mTORC1 suppresses the main catabolic pathways that lead to autophagy. Genetic studies on mTORC2 have indicated that this subunit plays key roles in several biological processes including metabolism and proliferation (García-Martínez and Alessi, 2008). New studies have suggested a possible anti-pathogen effect for this drug in different doses and conditions, for example against lymphocytic choriomeningitis virus (LCMV), vaccinia viruses or as a host cell mediator against tuberculosis (Araki et al., 2009; Singh and Subbian, 2018). Other studies on zebrafish model of *Mycobacterium marinum* infection indicated that mTOR can effectively boost host resistance to infection (Pagan et al., 2016). Information on the anti-leishmanial effect of this drug is still limited, although our previous study on *L. major* infection showed a strong anti-parasitic effect for rapamycin (Khadir et al., 2018). Therefore, in the present study we examined rapamycin as a mTOR inhibitor in terms of its anti-leishmanial effect against *L. tropica*. To evaluate the immune response, we assessed the Th1:Th2 cytokine bias in BALB/c mice infected with *L. tropica*. The importance of IFN-γ in Th1 response to control Leishmania infection is highlighted by studies reporting the failure of the IFN-γ knockout (KO) mice to overcome the disease (Dayakar et al., 2019). Importantly, *Leishmania*-resistant mice, such as C57BL/6, develop a T-cell response by a $CD4^+$ Th1 phenotype characterized by high levels of IFN-γ, whilst in susceptible BALB/c mice a dominant $CD4^+$ T Th2 phenotype is observed, accompanied by high levels of interleukin (IL)-4, IL-5 and IL-13 production. Therefore, the balance between Th1 and Th2 cytokine responses may be a decisive factor in determining the outcome of infection (Khadir et al., 2018; Wakil et al., 1998).

2. Materials and methods

2.1. Mice and parasites

Six to eight weeks old female BALB/c mice were obtained from the breeding supply facilities at Pasteur Institute of Iran and used for the experiments. All animal studies and protocols were approved by Institutional Animal Care and Research Advisory Committee of Pasteur Institute of Iran in compliance with the ethical code of IR.RII.REC.1894 (2015). *L. tropica* strain MOHM/IR/09/Khamesipour-Mashhad was used in this study, and its virulence was maintained by regular passages in BALB/c mice. The promastigote forms of the parasite were cultured and maintained in Schneider's Insect Medium (pH 7.4) (Gibco, Germany) at 26 °C with 10 % heat-inactivated FCS (Gibco, Germany) by adding 50 μg/L of gentamicin (Biosera, France).

2.2. Prepration of the drugs

A stock solution of rapamycin (LC laboratories, USA) was prepared by dissolving 10 mg of rapamycin powder in 1 mL DMSO and stored at −80 °C. For *in vitro* experiments, rapamycin stock solution was diluted in phosphate-buffered saline (PBS) (Sigma-Aldrich, Germany) by 1:2.5 serial dilutions. For *in vivo* studies, the stock solution was diluted in a vehicle consisted of Phosal 50 PG (95 % v/v) and Tween 80 (5 % v/v) (Sigma-Aldrich, Germany) shortly before the injection in order to obtain the required dose of 10.2 μg rapamycin in 200 mL.

A stock solution of AmB (Cipla, India) was made by dissolving 50 mg AmB powder in 10 mL distilled water. AmB stock solution was diluted in PBS before use in order to obtain the required doses. Glucantime (Sanofi. France) was diluted in distilled water and used immediately

2.3. In vitro assessment of 50 % inhibitory concentration (IC_{50}) of rapamycin

For *in vitro* experiments, wide ranges of rapamycin concentration were used to test the viability of L. *tropica* promastigotes through cell sensitivity assay. Parasites were cultured in flat-bottom sterile 96-well plates (Orange Scientific, European Union) at a density of 2×10^7 parasites/mL in presence of rapamycin with the concentration range of 0.51 μg/mL up to 50 μg/mL (serial dilution of 1:2.5 in PBS) for 48 h. Parasites treated with the same volume of PBS were used as negative control. The viability of the parasites in presence of the drug and without drug (negative control) was measured by MTT (3-(4,5-dimethylthiazol-3-yl)-2,5-diphenyltetrazolium bromide) assay (Kharaji et al., 2015). Briefly, after treating the cells with rapamycin or PBS for 48 h, 5 mg/mL of MTT component (Sigma-Aldrich, Germany) was added and the plates were incubated at 37 °C for 4 h, and then centrifuged at 800 × *g* for 5 min. The supernatant was removed and 100 μL of 99.7 % DMSO was added to each well. Optical density (OD) of the wells measured at 540 nm was used to calculate IC_{50} by curve-fitting in Microsoft Excel (Downey et al., 2008).

2.4. Analysis of cytotoxicity of rapamycin

Cytotoxicity measurement for rapamycin was done on THP-1 cells (human monocytic cell line derived from an acute monocytic leukemia patient; ATCC TIB-202). The cells were seeded in flat-bottom 96-well culture plates (Orange Scientific, European Union) and treated with various concentrations of rapamycin. To promote adhering the cells to the culture plate, 50 ng/mL of phorbol 12-myristate 13-acetate (PMA) was added to the culture media. The cells were then incubated for 24 h in complete RPMI medium at 37 °C with 5 % CO_2, followed by adding rapamycin at a final concentration of 0.14 μg/mL up to 25 μg/mL (1:2.5 serial dilutions) and incubation for 48 h. The supernatant was removed and 5 mg/mL MTT reagent in serum-free RPMI was added to each well and incubated for 4 h at 37 °C. CC_{50} of the drug on uninfected THP-1 cells was determined using MTT assay as described in Section 2.3 above.

2.5. Evaluation of effective concentration of rapamycin (EC_{50}) on amastigote form of the L. tropica

Viability of intracellular parasites was measured using parasite rescue and transformation assay. THP-1 cells with 50 ng/mL PMA in complete RPMI-1640 medium with 10 % FCS and 50 μg/L gentamicin were seeded (5×10^5 cells/well) in a flat-bottom plate and incubated at 37 °C for 24 h with 5 % CO_2. To infect the cells, stationary-phase *L. topica* promastigotes were added at a promastigotes to cell ratio of 10:1. Wells were washed with PBS 24 h later in order to remove the non-phagocytized parasites. THP-1 cells were then treated with different concentrations of rapamycin, ranging from 0.51 μg/mL up to 50 μg/mL (1:2.5 serial dilutions) for 48 h. Infected THP-1 cells without treatment were used as negative control (Kharaji et al., 2016). Afterwards, the infected THP-1 cells were washed with serum-free RPMI-1640 medium, and lysed by adding 0.05 % sodium dodecyl sulfate (SDS) in serum-free RPMI-1640 (20 μl/well). To allow the rescued live amastigotes to transform into the promastigote form, Schneider culture medium supplemented with 10 % heat-inactivated FCS and 50 μg/L of gentamicin was added to each well (180 μl/well), after shaking the plate for 30 s. The plates were then incubated for 72 h at 26 °C in 10 % Schneider medium. Finally, the efficacy of rapamycin-treated cells to remove the intracellular *L. tropica* parasites was evaluated compared to the untreated control, and the EC_{50} (50 % effective concentration) was determined using MTT assay as described in section 2.3 above.

2.6. Cell morphology analysis by Scanning Electron Microscopy (SEM)

Based on the results of viability MTT assay, 2×10^7 parasite/mL of *L. tropica* promastigotes were treated with three different concentrations of rapamycin, covering concentrations around IC_{50} of the drug (5 μg/mL, 10 μg/mL and 20 μg/mL), or left untreated as control group. To prepare a sample of fixed promastigotes suitable for SEM, the cells were centrifuged at 1800 × *g* for 10 min, the supernatant was removed, and 2.5 % EM-grade glutaraldehyde (Sigma-Aldrich, Germany) in PBS was added to the cells as primary fixative at room temperature for 2 h. Then, the cells were spread on coverslips (Moreno and de Meirelles, 1998). To remove the excess buffer salts, the coverslips were washed in double distilled water (ddH_2O). The coverslips were treated with increasing percentages of ethanol (30 %, 50 %, 70 %, and 90 % (v/v), in ddH_2O for dehydration, completed by washing three times in 100 % ethanol (De Souza, 2007). Coverslips were air-dried and mounted on SEM silver stubs (Ted Pella, USA). The sliver stubs were left overnight at room temperature. Finally, the fixed samples were gold-coated with sputter coater (Agar scientific, UK) and prepared for use in SEM (Zeiss DSM-960A, Germany) (Chang, 1979).

2.7. Ultra-structure analysis by Transmission Electron Microscopy (TEM)

The effect of rapamycin on the ultrastructure of the THP-1 cells infected with L. *tropica* was examined using TEM. The infected THP-1 cells were treated with EC_{50} concentration of rapamycin (7.6 μg/mL). Non-infected THP-1 cells only treated with PMA and also infected THP-1 cells not treated with rapamycin were used as control, For a better observation of the efficacy of rapamycin on the intracellular parasites, the cells were collected at two different time points, 24 h and 48 h. the cells were then detached on ice blocks and centrifuged at 600 × *g* for 7 min. The fixation method consisted of two different stages; primary fixation using glutaraldehyde, and secondary fixation with osmium tetroxide (Sigma-Aldrich, Germany). The cells were spun at 1800 × *g* for 10 min, the supernatant was discarded, and the cells were fixed with 2.5 % glutaraldehyde diluted in 0.1 M cacodylate buffer, pH 7.2 at 4 °C for 1 h. Then the cells were washed two times with 0.1 M cacodylate buffer and treated with 1 % osmium tetroxide for 1 h to complete the fixation. Series of acetone were used to dehydrate the cells on electric turn-table. To enhance the rate of Epon penetration, an equal amount of propylene oxide and Epon resin were added to the samples and left for 24 h at room temperature; fresh 100 % Epon was added to samples for another 24 h. The blocks were then polymerized in fresh Epon resin (Hatam et al., 2013).

Semi-thin sections of 1 − 2 μm in thickness were prepared using glass knife and ultramicrotome (Reichert, USA) for light microscopy observation. The sections were then stained with methylene blue. Sections of 50 − 70 μm in thickness were then prepared, stretched using chloroform vapor to reduce the artifacts, placed on 200-mesh copper grids with carbon stabilized formvar support films (Agar scientific, UK) and then stained with immersion in saturated uranyl acetate for 10 min. The grids were then washed in running distilled water, stained for 5 min in lead citrate, and finally washed well with running distilled water and left to air-dry. The sections were screened using a Philips EM-10C transmission electron microscope at voltages of 60–100 Kv.

2.8. Treatment of the mice infected with the parasite

L. *tropica* parasites were cultured at 26 °C in Schneider's Insect Medium (pH 7.4) with 10 % heat-inactivated FCS and 50 μg/L gentamicin. The metacyclic promastigotes were prepared using ficoll 400 gradient centrifugation method (Späth and Beverley, 2001). The mice were randomly divided into six groups (n = 5 per group), consisted of control cohort (vehicle 0.2 % DMSO in diluent) as shown in Table 1. The mice were infected in the left footpad with 1×10^7 metacyclic promastigotes in total volume of 50 μL. Treatment started at six weeks post-infection with 10.2 μg/dose of rapamycin and 8 mg/kg AmB (Abdossamadi et al., 2017a,b; Araki et al., 2009) injected intraperitoneally (i.p.) and 200 mg/kg glucantime injected subcutaneously (s.c.), once a day for 10 consecutive days. Combination therapy started on the same day with single shot therapy of AmB and glucantime, respectively. The treatments were continued with rapamycin for 10 days (Fig.S1). Control groups received vehicle (0.2 % DMSO in Phosal 50 PG (95 %) and tween 80 (5 %) for 10 days as well. All the mice in each group were euthanized at the end of the treatment period (day 52 post-infection). The inguinal lymph nodes and spleens were isolated aseptically.

2.9. Quantification of the parasite load in lymph nodes

Parasite burden in the infected lymph nodes was determined by quantitative real time PCR (qPCR). Genomic DNA was prepared from

Table 1
Treatment regimens of *Leishmania tropica* infected mice. BALB/c mice were randomly divided into 6 groups (n = 5) and treated with the drugs, concentrations and using the route of administration as indicated.

Treatment	Dose	Route of injection
Vehicle	0.2% DMSO in diluent in total volume of 200 μL	*i.p*
Rapamycin	10.2 μg/dose in total volume of 200 μL	*i.p*
AmB	8 mg/kg in total volume of 200 μL	*i.p*
Glucantime	200 mg/kg in total volume of 200 μL	*s.c*
Rapamycin + AmB	10.2 μg/dose, 8 mg/kg in total volume of 200 μL	*i.p*
Rapamycin + Glucantime	10.2 μg/dose, 200 mg/kg in total volume of 200 μL	*i.p, s.c*

each lymph node with GF-1 Tissue DNA Extraction Kit (Vivantis, Malaysia) following the manufacturer's protocol, and DNA concentration was measured by a Nanodrop spectrophotometer (ND-1000, Thermo Fisher Scientific, USA). Each reaction contained 100 ng of the genomic DNA, 500 nM of L. *tropica* kDNA primers kDNA1 F (5′-GGGT AGGGGCGTTCTGC-3′) and kDNA1R (5′-TACACCAACCCCCAGTT TGC-3′) and 10 µL of 2X high ROX SYBR Green, PCR master mix (Ampliqon, Denmark) in a total volume of 20 µL. Reactions on serial dilutions of L. *tropica* gDNA corresponding to 10^2 up to 10^7 parasites were included in order to derive a standard curve. The reaction was performed in 40 cycles followed by melt curve analysis in a 7500 Real-Time PCR system (Applied Biosystems, USA) according to the manufacturer's instructions.

2.10. Production of the parasite antigens

A total number of 1×10^8 stationary-phase promastigotes in one mL PBS were subjected to repeat cycles of freezing in liquid nitrogen and thawing in 37 °C water bath until the body of the parasites were completely lysed, as evaluated by light microscopy. Protein concentration was measured using Pierce BCA Protein kit (Thermo Fisher Scientific, USA).

2.11. Evaluation of cytokines by ELISA

Single cell suspensions of spleen cells were prepared using the following method. First ammonium-chloride-potassium (ACK) (Sigma-Aldrich, Germany) lysis buffer was used for erythrocytes lysis, afterwards the cells were washed and then re-suspended in complete DMEM medium (Gibco, Germany) supplemented with 10 % FCS and cultured at 5×10^6 cells/mL in a flat-bottom polystyrene 48-well plate (Orange Scientific, European Union). The cells were stimulated with 15 µg/mL of *L. tropica* frozen and thawed antigens. Concanavalin A (Con A) at concentration of 5 µg/mL was used as the positive control. IFN-γ and IL-4 cytokines in culture media were measured by ELISA kit (R&D, USA) at 24 h and 72 h post-stimulation.

2.12. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software Inc., USA). Student's *t*-test and two-way ANOVA were used to evaluate the difference between the groups. A *P* values of less than 0.05 was considered as significant.

3. Results

3.1. Rapamycin reduces the viability and changes the morphology of L. tropica parasite in vitro

In vitro efficacy of rapamycin on *L. tropica* promastigotes, intracellular amastigotes and on THP-1 cells was presented in Fig. 1A–C. The IC_{50} of rapamycin for *L. tropica* was 8.2 µg/mL (Fig. 1A), EC_{50} of the drug for intracellular parasites in the host cells was 7.6 µg/mL (Fig. 1B), and the CC_{50} (50 % cytotoxic concentration) of the drug on THP-1 cells was 48 µg/mL (Fig. 1C). The effect of rapamycin on the morphology of the parasites as evaluated using SEM is presented in Fig. 1D. Ultrastructural changes in morphology and size of the parasites were observed in a rapamycin dose-dependent manner. Parasites treated with high doses seemed to be smaller compared to the non-treated cells, based on direct observation. Furthermore, the treated parasites were disintegrated and round-shaped. Treatment with a high dose of rapamycin caused severe changes in appearance of L. *tropica* parasites; notably, the cells were surrounded with cellular debris, that implies cell rupture and cell death. However, the medium and high doses of rapamycin had almost the same effect on the cells in terms of morphology and physical disintegration

As shown in Fig. 1E, no sign of cell injury including vacuole formation is evident in non-treated THP-1 cells. However, in the infected THP-1 cells (Fig. 1F). Intracellular *L. tropica* amastigotes were observed in the cytoplasm, the nuclei became dense and pyknotic and the cell had several vacuoles (Fig. 1F–G). A representative SEM image of an infected THP-1 cell treated with EC_{50} of rapamycin (7.6 µg/mL) for 24 h is presented in Fig. 1G. The frequency of formation of abnormal amastigotes within the rapamycin-treated THP-1 cells was higher in comparison with the control group (infected cells without any treatment). Fig. 1H shows a representative TEM image of infected THP-1 cells treated with EC_{50} of rapamycin for 48 h. Absence of amastigotes in the infected THP-1 cells after treatment with EC_{50} dose of rapamycin was evident. Importantly, presence of vacuoles along with dense nuclei in those cells could be a sign that the cells were experienced celluar stress and transforming back to their normal conditions. Accordingly, rapamycin was effective in a range of doses, based on IC_{50} and EC_{50}.

3.2. Rapamycin decreases the parasite load in lymph nodes of the L. tropica-infected mice

Parasite burden in the lymph node of the *L. tropica*-infected BALB/c mice treated with drugs are shown in Fig. 2, parasite load in the mice treated with 10.2 µg/dose of rapamycin was significantly lower compared to the vehicle-treated group ($p = 0.001$). These results indicate that parasite propagation in infected lymph nodes of the rapamycin-treated group was inhibited with a therapeutic dose of the drug (Fig. 2). Besides, the parasite burden in the infected lymph nodes of BALB/c mice treated with AmB and glucantime was also significantly lower in comparison with the mice treated with vehicle ($p = 0.003$). However, combining rapamycin with AmB or glucantime did not lead to a synergistic therapeutic effect, at least in terms of parasite load in the lymph nodes. Altogether, treatment with rapamycin had a strong effect on reduction of the parasite load.

3.3. Rapamycin treatment can induce significant ratio of IFN-γ/IL-4 production in response to L. tropica antigen

The Th1 *versus* Th2-type cytokine responses subsequent to the monotherapy with rapamycin, AmB and glucantime or the combination therapy of rapamycin with AmB or rapamycin with glucantime are shown in Fig. 3. The supernatant of F/T (freeze and thaw) antigen-stimulated splenocytes from the mice treated with rapamycin revealed higher level of IFN-γ production in comparison to the vehicle- group (Fig. 3A, $p = 0.006$). Notably, treatments with AmB and glucantime also increased the production of IFN-γ when compared to the vehicle group ($p = 0.030$), to the extent that there was no significant difference between the three monotherapy groups ($p = 0.033$). Surprisingly, the level of IFN-γ production was significantly higher in the combination modalities including rapamycin with AmB and also rapamycin with glucantime ($p = 0.026$). Although there were some variations in IL-4 production within other treated groups, animals in the rapamycin monotherapy group showed the lowest IL-4 level with minimal variation, virtually identical to the vehicle group (Fig. 3B). Furthermore, the ratio of IFN-γ/IL-4 was almost equal within the groups treated with rapamycin, AmB and glucantime, and significantly higher than the vehicle-treated group ($p = 0.013$). Interestingly, this ratio was higher in the combination therapy groups than in the monotherapy groups, although the differences were not significant ($p = 0.880$) (Fig. 3C).

4. Discussion

Rapamycin is a potent mTOR inhibitor with a broad range of applications, such as use as an immunosuppressant or an anti-pathogen compound. The mTOR signaling pathway modulates diverse cellular mechanisms in mammalian cells which are associated with the host immune response to pathogens (Zhang et al., 2017). An important

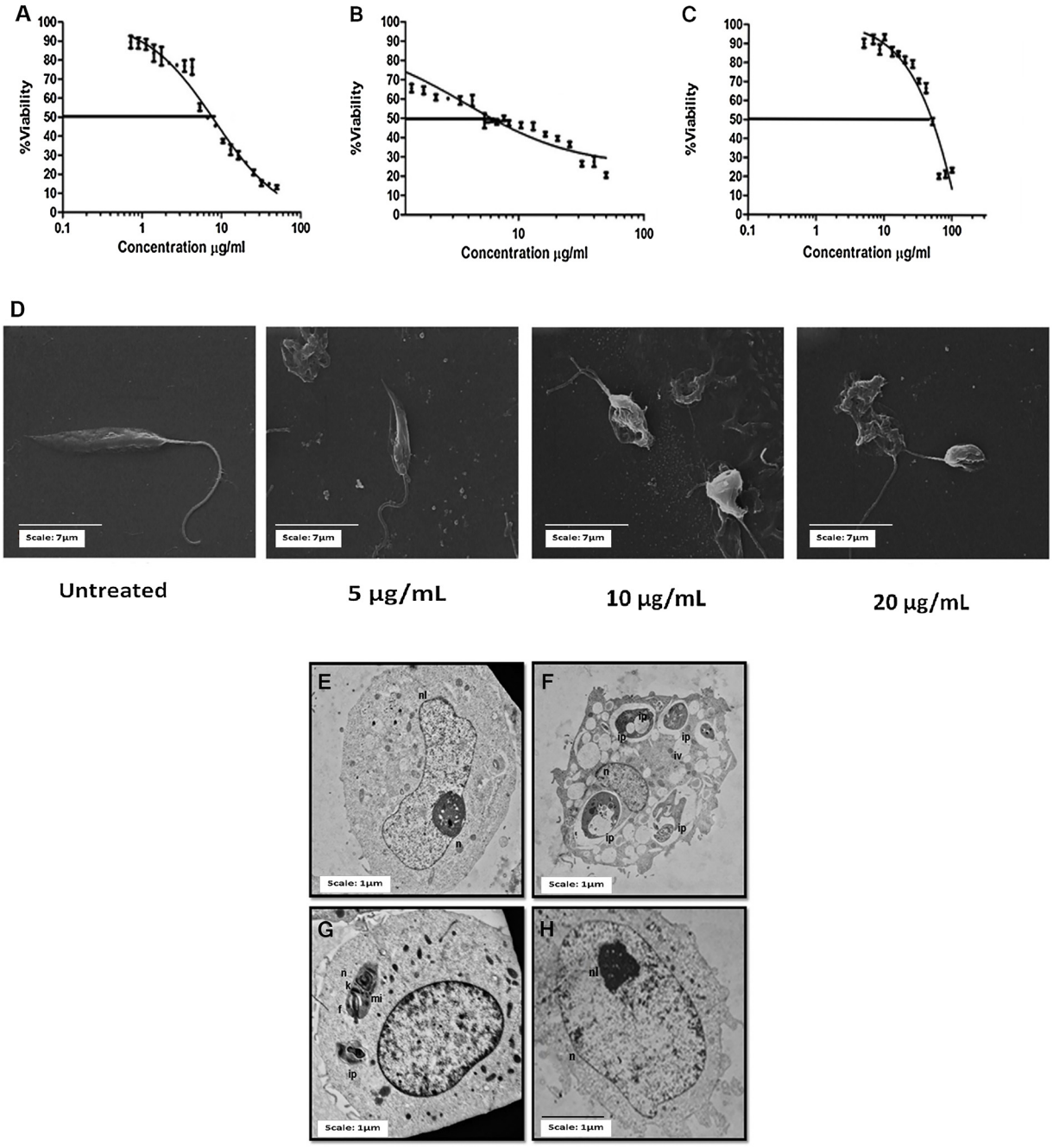

Fig. 1. Effect of rapamycin on morphology and viability of *Leishmania tropica in vitro* and in the host cells.
Viability of cultured *L. tropica* promastigotes as measured in a range of rapamycin concentrations. The IC_{50} was 8.2 µg/mL (A). Viability of *L. tropica* amastigotes in THP-1 host cells as measured in a range of rapamycin concentrations. The EC_{50} of rapamycin was 7.6 µg/mL (B). Cytotoxicity of rapamycin on THP-1 cells as measured on a concentration range. CC_{50} was 48 µg/mL (C). Parasites treated with low, intermediate and high concentrations of rapamycin (5, 10 and 20 µg/mL, respectively) as observed by SEM (D). Normal THP-1 cells (E) and the THP-1 cells infected with *L. tropica* parasites and left untreated (F) or treated with EC_{50} of rapamycin (7.6 × µg/mL) for 24 h (G) or 48 h (H) as observed by TEM.
n: nucleus; nl: nucleolus; fp: flagella pocket; ip: intracellular parasite; mi: mitochondrion; g: golgi apparatus; k: kinetoplast; iv: intracellular vacuole; c: carbohydrate droplet; ld: lipid droplet; ed: electron dense structure.

finding suggest that mTOR inhibition may regulate the host defense against invading pathogens (Harrison et al., 2009). Moreover, some other studies have emphasized on the efficacy of rapamycin as a mTOR inhibitor to boost immune responses against viral pathogens (Araki et al., 2009). In particular, an important study by Singh and Subbian (2018) focused on host-pathogen interactions in presence of rapamycin for intracellular pathogens such as tuberculosis. The potential anti-pathogen effect of the drug despite its known immunosuppressive properties raised intriguing questions regarding how it could be applied for the intracellular parasites such as *Leishmania*.

Leishmaniosis is a parasitic disease with serious problems and obstacles in term of treatment and vaccination. TOR (target of rapamycin) from *Leishmania* parasite is a key molecule in mechanisms related to autophagy (Duszenko et al., 2011; Liu and Bassham, 2010). Especially, TOR1 and TOR2 proteins are essential in growth and virulence of the parasite (Madeira Da Silva and Beverley, 2010; Zhang et al., 2014). Therefore, it could by hypothesized that inhibiting these particular molecules with rapamycin may inhibit parasite growth and

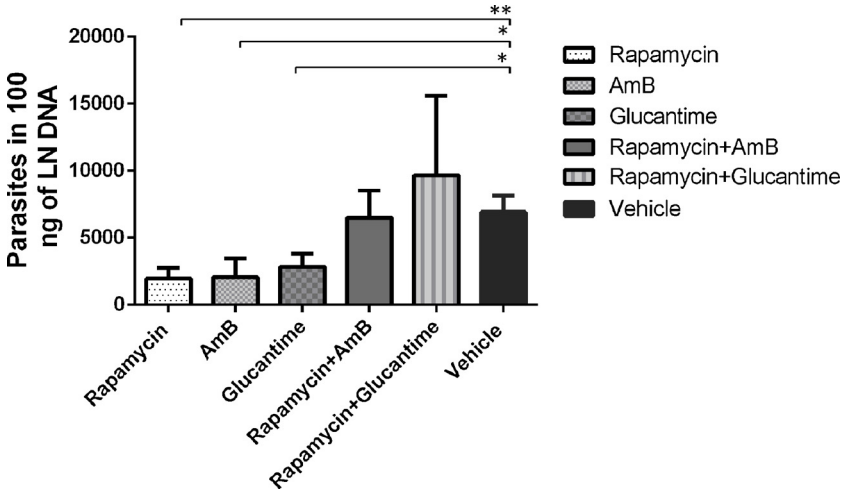

Fig. 2. Quantification of the parasite load in lymph nodes of BALB/c mice infected with *L. tropica* and treated with different modalities.
The parasite load in infected popliteal lymph nodes of infected footpads was determined by qPCR. Absolute copy numbers of the *Leishmania* kDNA in lymph node of mice treated with vehicle, 10.2 μg/dose of rapamycin, 8 mg/kg of Amb, 200 mg/kg of Gluantime or combination therapy of 8 mg/kg of Amb (single shot) followed by 10.2 μg/dose of rapamycin or 200 mg/kg glucantime (single shot) followed by10.2 μg/dose of rapamycin is indicated. The error bars represent the standard error of the mean (SEM). The experiments were repeated two times.

proliferation and reduce its viability. In this study, we aimed to evaluate rapamycin as a monotherapy or combination therapy with AmB and glucantime against *L. tropica* infection in BALB/c mice. The efficacy of these treatment regimens was evaluated by parasite load and cytokine production after stimulation with *leishmania* antigens. Our recent findings proved that rapamycin has a remarkable therapeutic effect against *L. major* parasite (Khadir et al., 2018)., which corroborates with the results of other studies on this topic. One of the first studies on rapamycin in the field of leishmaniosis was an *in vitro* study that indicated the effect of rapamycin on IL-12/IL-10 axis in *L. donovani* infection (Cheekatla et al., 2012). Furthermore, another study showed various effects of different mTOR inhibitors on *Leishmania* and trypanosomes and depicted a range of therapeutic doses that may be comparable to that of the current anti-leishmainal agents (Diaz-Gonzalez et al., 2011).

Our recent study regarding the effect of mTOR inhibitors on *L. major* infected animals yielded some interesting results. Analogs and dual mTOR/PI3K inhibitors enabled effective control of the disease (Khadir et al., 2018). Here, consistent with our previous study, we tested various concentrations of rapamycin *in vitro* against *L. tropica* parasites. The inhibitory effect of the rapamycin on extra- and intracellular *L. tropica* parasite was observed with μM concentrations of the drug. By inspecting the actual morphology along with viability, we observed differences between the treated and untreated extracellular and intracellular parasites using SEM and TEM. The results confirmed the parasiticidal effect of rapamycin. The ultrastructural data by SEM suggested that rapamycin changed the morphology of the parasite in a dose-dependent manner. On the other hand, the TEM ultrastructural

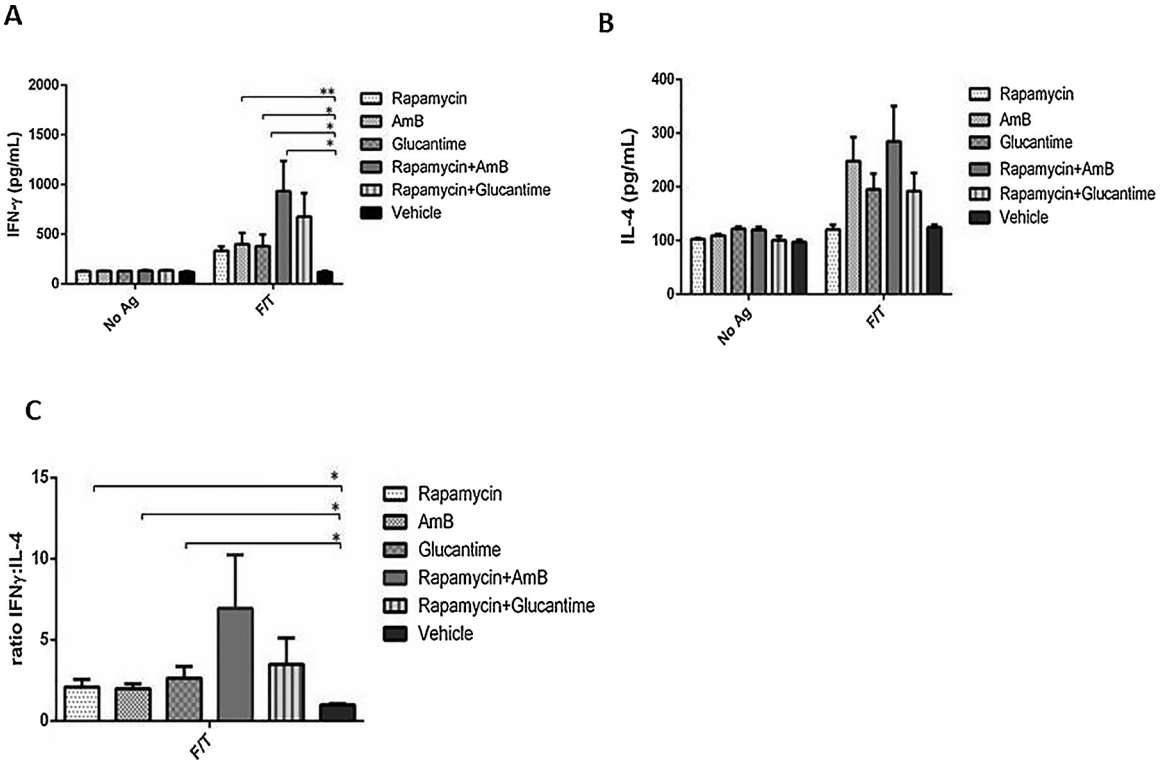

Fig. 3. The *L. tropica* infected mice treated with rapamycin showed Th1 cytokine responses.
IFN-γ (A) and IL-4 (B) secreted by the splenocytes stimulated with *L. tropica* antigens (F/T, frozen and thawed) were measured. The IFN-γ/IL-4 ratios are also presented (C). Error bars indicate the standard error of the mean (SEM), and the data was pooled from two independent experiments. ANOVA test was used to evaluate the statistical significance of the differences between the groups. *: $p < 0.05$; **: $p < 0.001$.

results suggested that only in one effective concentration the intracellular parasite was almost wiped out in the infected host cell. Similarly, some studies applied the same method of observation with electron microscopy after testing the efficacy of a new compound on *Leishmania* and *Trypanosome* parasites (Britta et al., 2014).

Besides all findings from our *in vitro* study, we decided to use the clinically relevant doses of the drug *in vivo*. This enabled us to compare our new findings with the same concentrations of rapamycin against other pathogens (Araki et al., 2009; Foster and Toschi, 2009). Our results suggested that the parasite load was effectively reduced in the group treated with rapamycin as well as in the groups treated with AmB and glucantime. On the other hand, combination therapies using rapamycin together with either AmB or glucantime did not decrease the parasite burden in the infected mice. Importantly, IFN-γ and IL-4 production in the rapamycin-treated group was similar to that of the mice treated with AmB and glucantime. The ratio of IFN-γ to IL-4 in the rapamycin-treated group was higher than in the vehicle control group and very close to the ratio observed in the groups treated with AmB or glucantime. These results indicated a remarkable bias towards Th1 responses in rapamycin, AmB and glucantime groups. Besides, decreased parasite burden in the rapamycin-treated group was consistently associated with high level of IFN-γ production, which is related to cellular immune responses. Importantly, rapamycin was able to mimic the action of front line anti-leishmanial drugs such as AmB and glucantime in decreasing the parasite load and inducing cytokine production. Numerous studies have used the levels of IFN-γ and IL-4 and their ratio as a measure of the efficacy of a drug against *Leishmania* (Abdossamadi et al., 2017a,b; Heidari-Kharaji et al., 2016; Hossain et al., 2017). Our previous study indicated that the concentration of rapamycin required for killing the parasite directly *in vitro* (IC_{50} and EC_{50}) was different from the concentration that its immunomodulatory effects are detectable. In fact, our present results confirm the previous observations that rapamycin acts as an immune regulator in controlling the infection. Furthermore, our new findings suggest that *L. tropica* parasite is more sensitive to rapamycin compared with *L. major*, based on IC_{50} and EC_{50} values.

Another aim of this study was to compare the effect of combination therapy on *L. tropica*, using rapamycin as an immunomodulator with two well-known anti-leishmanial drugs AmB and glucantime. There are various studies on combination therapy approaches against leishmaniosis (Bryceson, 2001; Meheus et al., 2010; Sundar and Chatterjee, 2006). Surprisingly, our data indicated that the two combination therapies tested in the present study were not able to control parasite propagation significantly, as no parasite load reduction was observed in the infected lymph nodes. Although the level of IFN-γ production in the two combination therapy groups was significantly higher than in the vehicle group, there was no significant difference in IL-4 production and even IFN-γ to IL-4 ratio between said groups. Certainly, further investigation is needed in order to find the mechanism underlying these unexpected results. A recent study) suggested that treatment with mTOR inhibitors promotes resistance to AmB (Bojsen et al., 2016). Although it is a matter of speculation, the idea may support the reduced efficacy of AmB in killing the parasites when administered together with rapamycin. Furthermore, it might be that the cells survived from AmB treatment may show resistance to the Ras-pathway once again with mTORC1 inhibition. This hypothesis may explain how combination therapy with AmB is not successful at least in reducing the parasite load. It is necessary to mention that the results for combination therapy with glucantime were not encouraging either. Nevertheless, considering that the mechanisms of action of these drugs are not yet fully understood, there is abundant room for further research in this context. Moreover, we hypothesize that control of *Leishmania* infection with the tested combination of the drugs failed because of their potentially conflicting mechanisms, especially Ras-pathway. Therefore, further studies using rapamycin and other anti-lesihmanial drugs without such interactions will be very informative and highly recommended.

5. Conclusion

- Different concentrations of rapamycin could change the morphology of *L. tropica*.
- Systemic treatment with rapamycin is able to control the parasite propagation in *L. tropica* infected BALB/c mice.
- mTOR may be an attractive target for development of novel and potent anti *Leishmania* agents.
- Rapamycin is as potent as AmB and glucantime in controlling the parasite propagation and immune activation against *L. tropica*.
- Rapamycin combination therapy with either AmB or glucantime were not satisfactory and needed further investigations for possible interactions and their antagonistic activities

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.108976.

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