Original Article

Synergistic Anti-Leishmanial Activities of Morphine and Imiquimod on *Leishmania infantum* (MCAN/ES/98/LIM-877)

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Abstract

Background: This study was performed to evaluate in vitro and in vivo Leishmanicidal potential of morphine (Mph), imiquimod (IQ), and their combination.

Methods: *Leishmania infantum* promastigote and amastigote assays were performed at the presence of 0.015–150µM Mph, 0.04–416µM IQ, and their combination. The inhibition effects of these drugs on promastigotes were evaluated after 24, 48, and 72h. The cytotoxic effects of the drugs were evaluated by MTT as well as flow cytometry after 72h. We explored the therapeutic effects of Mph and IQ in BALB/c mice at the end of the treatment using parasite load determination and cytokine assay. One group of mice received Mph for three weeks before infection.

Results: The results of promastigote and amastigote assays showed the cytotoxic effects of the drugs at low concentrations. The cytotoxic effects were higher on promastigotes than amastigotes ($p< 0.05$). There was a negative correlation between drug concentration and amastigote/promastigote viability. Imiquimod alone or combined with Mph showed remarkable cytotoxic effects at all concentrations ($p< 0.05$). Flow cytometry results revealed apoptosis in the parasite following exposure to the drug combinations. Accordingly, the reduction of parasite loads in the spleen and liver was observed ($p< 0.05$) with simultaneous increases in IFN-γ and IL-4. We believe that the in vivo leishmanicidal effect was mediated by Mph through IL-4 and by IQ through both IL-4 and IFN-γ.

Conclusion: Results pointed out the promising effects of Mph and IQ at low concentrations, especially when combined.

Keywords: Morphine; Imiquimod; Synergism effect; *Leishmania infantum*; Iran

Introduction

Leishmaniasis is one of the neglected tropical diseases, that caused by an obligatory intracellular parasite, the genus *Leishmania* of the family Trypanosomatidae (1). *Leishmania* parasites are transmitted by *Phlebotomus* sand flies and their infection leads to cutaneous, mucocutaneous, and visceral leishmaniasis depending on parasite species and host immunity (2). Iran is an important focus of cutaneous and visceral Leishmaniasis in the Middle East, in which the visceral form is caused by the *L. infantum*. An epidemiologic study by Mohebali, 2013 showed that the seroprevalence of VL in humans and canines in Iran are 4.7% and 12.2 % respectively (3, 4). The symptomatic form of the disease in humans, is characterized by irregular fever, anemia, hepatosplenomegaly, severe weight loss, globulinemia, and hyperglycemia. The mortality rate is 90–95% in undiagnosed and untreated cases (5). Promastigotes and immotile amastigotes are the two forms of the parasite. Amastigotes formed in host cells af-

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ter the bite of ingestion by the female sandfly containing promastigotes (6). Therefore, the parasite avoids a rapid and direct attack by the host immune system and manipulates host-parasite interactions. These results in inhibiting the host cell apoptotic machinery and ensure the parasite's extended survival in infected cells (7). Different kinds of *leishmania* spp have been reported to inhibit apoptosis in host cells (8). For example, *L. infantum* can inhibit drug-induced apoptosis in human macrophages (9). Therapeutics employed to treat leishmaniasis are limited and unsatisfactory. They include pentavalent antimony (SbV), amphotericin B (AMB) and miltefosine (MF) (10). Despite the inadequate understanding of the mechanism of these drugs, their targets are believed to differ substantially. Antimony, with a rather complex mode of action, has multiple cellular targets. MF disrupts the biosynthesis of glycolipids and glycoproteins as well as the metabolism of alkyl phospholipids. AMB exerts its toxic effects since it has a high affinity for the ergosterol of the leishmania plasma membrane (11). However, these agents have some limitations including high cost, long treatment duration, route of administration (intradermal and intramuscular injection), toxic effects on liver, heart, and kidneys as well as the lack of response to treatment in 10–15% of cases (12). Failure response to antimonial compounds has been reported from endemic areas such as India and also Iran (13).

Therapeutics that specifically kill infected macrophages may be beneficial for the treatment of leishmaniasis since the parasite resides in host macrophage or monocytes (7). Stimulation of opioid receptors in infiltrating cells may be involved in local immune response control or be a signal to produce specific cytokines or antibodies analogous to other opioid receptors. Morphine is the main alkaloid in opium and an active metabolite of heroin (Bimonte) (14). Morphine acts through opioid receptors (d, l, and j), which is related to Mph-induced macrophage apoptosis through reducing the number of murine peritoneal and rabbit alveolar macrophages (15). The Mph-induced apoptosis may be mediated by the up-regulation of Bax and p53 proteins, increased p38 MAPK phosphorylation, or TGF-β production by macrophages (16). In vivo and in vitro studies show that Mph inhibits macrophage migration, which is secondary to the apoptotic effects of Mph (17). Imiquimod, a potent toll-like receptor-7 (TLR7) agonist, exerts its effects by the modification of immune responses and stimulation of apoptosis (18). Imiquimod modulates immune responses by activating dendritic cells, macrophages, or other cell types via TLR7, pro-inflammatory cytokines including IFN-a, IFN-c, tumor necrosis factor (TNF) α/β, IL-1a, and IL-12. Treatment with IQ has an impact on the expression of various genes involved in apoptotic pathways (19).

Furthermore, IQ decreases growth and/or increases apoptosis in several human cells (20). Also, IQ has been reported to induce gene expression and protein production of opioid growth factor receptors (21). Currently, extensive research is being conducted worldwide to improve the treatment strategies of leishmaniasis. The present study was designed to investigate putative promastigote and amastigote inhibition as well as apoptotic features of Mph, IQ, or their combination on *L. infantum* and visceral leishmaniasis in BALB/C mice (22-23).

Materials and Methods

In this study, all applicable international, national and institutional guidelines for the care and use of animals were followed and approved by the Medical ethics committee of Faculty of Medical Science, Tarbiat Modares University and approved under process No. 52D/3593/2015.

In vitro Experiments

Promastigote and Macrophage Culture

Promastigotes of *L. infantum* reference strain JPCM5 (MCAN/ES/98/LIM-877) provided from

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Published Online: June 30, 2021

Department of Parasitology, Kerman University of Medical Sciences (24). The promastigotes were grown in RPMI-1640 medium (Gibco, dUS) supplemented with 20% heat-inactivated FBS (Gibco, US), 100IU/mL penicillin, and 100µg/mL streptomycin in a humidified atmosphere at 24 °C. Then, 100µl of medium containing 1×10^6 promastigote cells/mL in logarithmic phase were subcultured and seeded in 96-well microplates. Mouse macrophage cells, J774 A1 (CGBR80052901, kindly offered by Professor Marcel Hommel), were cultured in RPMI-1640 medium supplemented with 10% FBS and 100 IU/mL penicillin/streptomycin at 37 °C and 5% CO_2 in a humidified atmosphere. Then, they were seeded in 12-well microplates with a density of 1×10^5 cells/well and used for the next experiments (25-26).

Drug preparation

Morphine sulfate (Temad Company, Iran) and IQ (Invivogen, Toulouse, France) was purchased. Morphine powder was dissolved in 5mL distilled water to obtain a stock solution. One mg/mL stock solution of IQ was prepared by dissolving the powder in a commercially available specific solvent. Then, the stock solutions of both drugs were diluted in RPMI to obtain the concentrations of 0.015–150µM. Glucantime (273µM) was used as control and purchased as a liquid solution (85mg/mL) from Sanofi-Aventis, France (27-28).

Study Groups and Treatment of Promastigotes, Macrophages, and Amastigote-infected macrophages

Cell treatment with drugs was performed in three groups including *L. infantum* promastigotes, J774 macrophages, and *L. infantum* promastigote-infected macrophages. In each group, treatments were performed with different concentrations of Mph, IQ, or Mph +IQ. Parallel exposures to 273µM of Glucantime or culture medium were applied as controls. The treatment groups included (i) Mph (0.015–150 µM), (ii) IQ (0.041–416µM), (iii) Glucantime (273µM), (iv) 0.015–150µM Mph + 0.041–416µM IQ.

Promastigote and Amastigote assay

Promastigote assay

The promastigotes were treated with different concentrations of Mph, IQ, and their combinations for 24, 48, and 72h. Then, the numbers of promastigotes were counted. The percentage of live promastigotes was evaluated by MTT assay after 72h. Promastigotes (1×10^6cells/mL) were exposed to studied concentrations of Mph, IQ, and their combination for up to 72h. Then, the supernatant was removed, and the cells were treated with 5mg/mL MTT ((3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma-Aldrich) solution and incubated for 4h. Afterward, dimethylsulfoxide (DMSO) was added, and the absorbance was read at 570nm. The results were expressed as the mean percent reduction of parasite number compared to the untreated controls (28).

Uninfected macrophage cells viability

We evaluated the effects of the drugs on uninfected macrophages, and the percentage of live macrophages was calculated. 1×10^5cells/mL of macrophage were cultured and treated with different concentrations of Mph, IQ, and their combinations. The viability of the cells was determined by MTT assay after 72h (28-29).

Amastigote assay

After reaching confluency, J774 A1 macrophages were seeded on 12-well microplates (Nunc) with a density of 1×10^5cells/well for 24h. Adherent macrophages were infected with the stationary phase of *L. infantum* promastigotes at a ratio of 1:10 and were allowed to infect the macrophages for 6h. The cells were washed with fresh RPMI to remove non-phagocytosed promastigotes. Infected macrophages were further incubated up to 72h in the presence of the drugs. The effects of drugs were

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calculated from the percentage of infected cells and the number of amastigotes per infected macrophage in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. Values are means of triplicate exams (29).

Evaluation of Apoptosis by flow cytometry

Promastigotes, uninfected macrophages, and infected macrophages were treated with different concentrations of Mph, IQ, and their combinations. Then, flow cytometry was performed to detect apoptotic and necrotic cells using Annexin-V FLUOS Staining Kit (Biovision, USA). 1×10^6 parasites or 1×10^5 macrophages, and infected macrophage cells were treated with the drugs and incubated at 24 °C. After 24h, they were washed with cold PBS and centrifuged at 1500g for 5min. 5µl annexin-V FITC, 500µL binding buffer and 5µL PI (propidium iodide) were added and incubated for 15 minutes at room temperature. The test was performed using CyFlow® space flow cytometry (Sysmex-Partec, USA) and data were analyzed by FloMax software (Partec, version 2.3) (30). All in vitro experiments were performed in triplicates.

In vivo Experiments

Animals and study groups

This experimental study was performed at the laboratories of the Parasitology Department, Faculty of Medical Sciences, Tarbiat Modares University (TMU), Tehran, IRAN from October 2016 to July 2017. Thirty female BALB/C mice, with an average weight of 18–21g and age of 5–8 weeks, were purchased from Pasteur Institute of Iran. The mice were kept in our animal facility and used for in vivo experiments. The mice were randomly divided into 5 groups with 5 mice in each group (Table 1). In all the cases, 100µl of promastigotes (2×10^7cells/mL) at the stationary phase were injected intraperitoneally into the mice. The infected mice were kept for about 18 days to allow parasite growth. Then, the treatments were performed in each group for 3 weeks. 100µl of 1500µM Mph once a week and 100µl of 624µM/mouse IMQ three times a week were injected intraperitoneally. All relevant ethical considerations in animal experiments were used before starting the study (Medical Ethics Committee of Tarbiat Modares University, No: 52D/3593/TUMS). Before treatment, two mice were sacrificed and evaluated by parasite culture to confirm the parasite growth. In the 6th group, the mice received Mph for 3 weeks and were then infected with promastigotes. In all groups, after one month, the all of mice were sacrificed. Then, their spleen and liver tissues were evaluated for parasite load by dilution method. Briefly, 30mg of spleen or liver tissues were homogenized and transferred to RPMI-1640 medium. Five dilutions of the suspensions were prepared, incubated at 26±2 °C, and followed up for two weeks. Finally, the least count of the parasite was considered as the final titer, and parasite load was calculated (26).

Extraction of spleen lymphocytes and cytokine assay

To measure IFN-γ and IL-4 levels, the mice were sacrificed at the end of the treatments, and lymphocytes were isolated from spleen tissue. The staining of lymphocytes was performed using trypan blue to obtain the percentage of live cells. Then, 1×10^6/mL lymphocytes were cultured in 12-well plates in RPMI1640 medium. Then, the lymphocytes were stimulated by 20µg/mL of SLA and incubated in the presence of 5% CO_2 at 37 °C for 72h. Finally, INF-γ and IL-4 levels in culture supernatants were measured by ELISA using U-CyTech kits (bioscience, Netherlands) according to the manufacturer's instructions. To isolate Soluble *Leishmania* Antigens (SLA), 10^8 promastigotes/mL at stationary phase was suspended in PBS and lyzed by five freeze-thaw cycles. The lysates were then centrifuged at 3000g at 4 °C for 15 minutes. The protein concentration of the supernatant was measured using Bradford assay (26).

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Published Online: June 30, 2021

Statistical analysis

Data are shown as mean ±SD from at least two independent assays. In in vitro experiments, the differences between the groups were evaluated by one-way ANOVA. In in vivo, the differences between treated and control groups were determined by an unpaired Students t-test. P< 0.05 was considered significant by SPSS version 23.

Results

The results of in vitro experiment

Cultured promastigotes were exposed to 0.015–150µM concentrations of Mph, 0.041–416µM IQ, and their combination. The promastigote inhibition was assayed after 24, 48, and 72h exposure to the drugs as shown in Fig. 1. The results showed that Glucantime, as a positive control, and Mph and IQ in high concentrations had no effect on promastigotes count (p> 0.05). In Mph and IQ group, the concentrations of a lesser extent had inhibitory effects on promastigotes in all time intervals. This effect was more obvious after 72h (p< 0.05). In the group treated with Mph +IQ, similar to Mph and IQ only, the concentrations showed the least effects on promastigotes after 72h. The observed promastigote inhibition effect can be attributed to the presence of IQ due to mostly the lack of response in the Mph group. The viability of promastigotes and uninfected macrophages was evaluated after treatment with similar concentrations of Mph, IQ, and their combination for 72h (Fig. 2). Promastigote viability results showed a significant effect on the viability for Mph in any doses (p< 0.05) except for 150 and 15µM Mph, 416 and 41.6µM IQ and their combination. The IC50 values of Mph, IQ, and combination formula on promastigotes after 72h were 0.102± 0.03, 0.235±0.01, and 0.173±0.02µM, respectively. Also, the IC90 values of Mph, IQ, and their combination on promastigotes after 72h were 0.191±0.02, 0.398±0.01, and 0.245±0.02 µM, respectively.

The results of the toxic effects of the drugs on uninfected macrophages were similar. Glucantime had the most significant effect with no effect of Mph even at high concentrations compared to the control group. In contrast, IQ and the combination of the drugs showed toxicity on macrophages at higher doses (p< 0.05). The effect of imiquimod on promastigotes in higher doses, whether alone or in combination with Mph, was not significant (p> 0.05). The IC_{50} values for Mph, IQ, and combination of these drugs were 0.8±0.01, 0.2±0.01, and 0.6± 0.02µM, respectively, after 72h on uninfected macrophages.

Fig. 3. Shows the amastigotes count after exposure to all of the studied concentrations of Mph, IQ, and their combination for 72h. In contrast to promastigote assay, glucantime showed a significant effect on amastigotes compared to the negative control (p< 0.05). Also, drugs in all doses except 150µM showed significant toxic effects both on infected macrophages or intracellular amastigotes compared to the control group (p< 0.05). The same results were observed for IQ alone or its combination with Mph especially at low doses (p< 0.05). Analysis of differences in contaminated macrophages and intracellular amastigotes showed that there were significant differences between the treatment and control groups (p= 0.01) except for high concentrations (Mph 150µM, IQ 416µM, and their same combinations).

The most significant toxicities were related to 0.04µM IQ and then 0.015µM Mph compared to other groups (p= 0.04). The IC_{50} values of Mph, IQ, and Mph+IQ on amastigotes after 72h were 18.2±0.3, 0.79±0.1, and 8.72± 0.1µM, respectively. Also, the IC_{90} values were calculated 29.7±0.1, 1.1±0.1, and 11.8±0.3µM for Mph, IQ, and Mph + IQ respectively.

Fig. 4. demonstrates the results of flow cytometry analysis. As seen, early apoptosis in promastigote cells was low in morphine treatment, 3.04% and 6.08% at 1.5 and 0.015µM, respectively. The necrosis value was 2.55%

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and 5.96% at 1.5 and 0.015µM Mph, respectively. In contrast, in IQ treatment, early apoptosis occurred more than necrosis, i.e. 4.11% and 9.18% apoptosis and 3.63%, 2.93% at 4.16µM and 0.04µM of IQ, respectively. When these two drugs were combined, the early apoptosis increased and was 13.72%. The live uninfected macrophage cells were 90.75%, 91.59%, 87.56% at 0.015µM Mph, 0.04µM IQ and 0.015µM Mph+0.04µM IQ, respectively. Apoptosis in infected macrophages was more than in other cells. Significant differences were seen in the values of apoptosis and necrosis in all groups compared with the control ($p< 0.05$).

In vivo experiments

Fig. 5. Shows that parasite load in mice was significantly reduced in all drug groups compared with infected mice treated with no drugs. The highest reduction in parasite load was observed in the groups that received 1500Mph treatment before the infection. Also, there was a significant difference between these groups in terms of reduction in parasite load ($p< 0.05$). As shown in Fig. 6, the levels of both IFN-γ and IL-4 had increased in all treated groups compared to untreated control. In mice treated with Mph, there was a significant reduction in IFN-γ. Mph seems to inhibit the parasites with a mechanism other than IFN-γ production. As seen, in the Pre-Mph group, the mice were treated before inoculation of parasites, IFN-γ higher than IL-4 level, but there are no noticeable differences between IL-4 levels compared to the control group ($p> 0.05$).

Table 1. The studied mice groups in the present study (5 mice in each group)

Mice Groups	Treatment	Prescription amounts	Route of Administration	Abbreviation
Control Groups	Uninfected-untreated	-	-	CTRL(-)
	Infected-untreated (parasite-infected treated with Glucantime	273µM	Intraperitoneal	CTRL(+)
Treatment Groups	parasite-infected treated with morphine	1500µM	Intraperitoneal	Mph
	parasite-infected treated with imiquimod	624µM	Ointment	IQ
	parasite-infected treated with morphine+ imiquimod treatment	1500+624µM	Intraperitoneal/ Ointment	Mph+ IQ
	parasite-infected after pre-treatment with Mph for 3 weeks	1500µM	Intraperitoneal	Pre-Mph

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Published Online: June 30, 2021

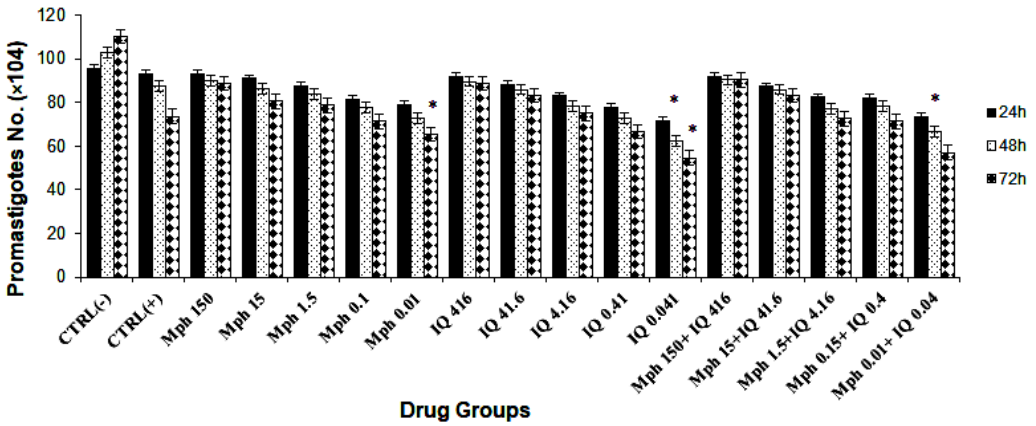

Fig. 1. Promastigotes were exposed to 0.015–150µM concentrations of Mph, (0.041–416µM) IQ, and their combination for 24, 48, and 72h. In the negative control group, no treatment was applied. Glucantime was used as a positive control. Data are mean± standard deviation of one experiment in triplicate, *p< 0.05, Tarbiat Modares University, Tehran, 2017

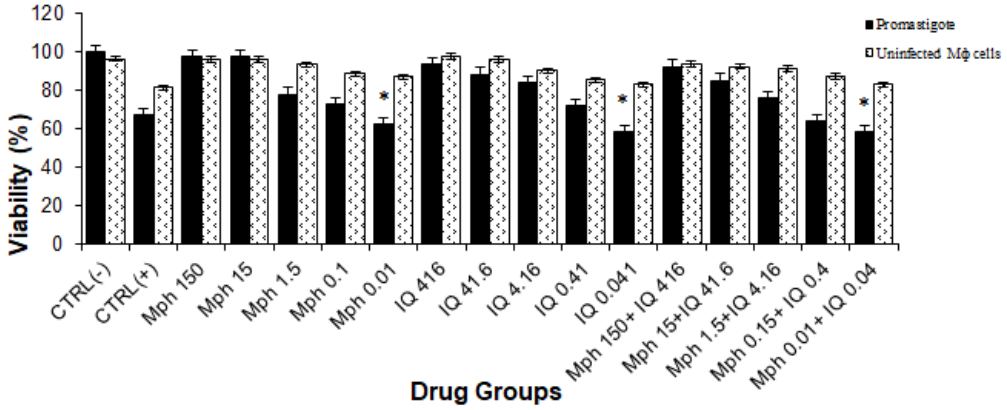

Fig. 2. Viability percentage of promastigotes and macrophages after 72h exposure to 0.015–150µM concentrations of Mph, (0.041–416µM) IQ, and their combination for 72h. After treatment, the viability was evaluated using MTT. In the control group, no treatment was applied. Glucantime was used as a positive control. Data are mean± standard deviations of one experiment in triplicate, *p< 0.05, Tarbiat Modares University, Tehran, 2017

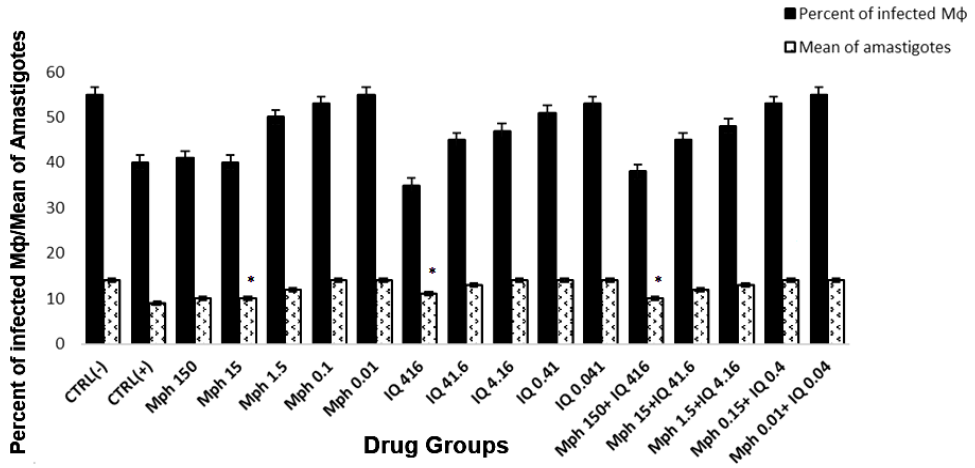

Fig. 3. Amastigotes were exposed to 0.015–150µM concentrations of Mph, (0.041–41µM) IQ, and their combination for 72h. In the control group, no treatment was applied. Glucantime was used as a positive control. Data are mean± SD, *p< 0.05, Tarbiat Modares University, Tehran, 2017

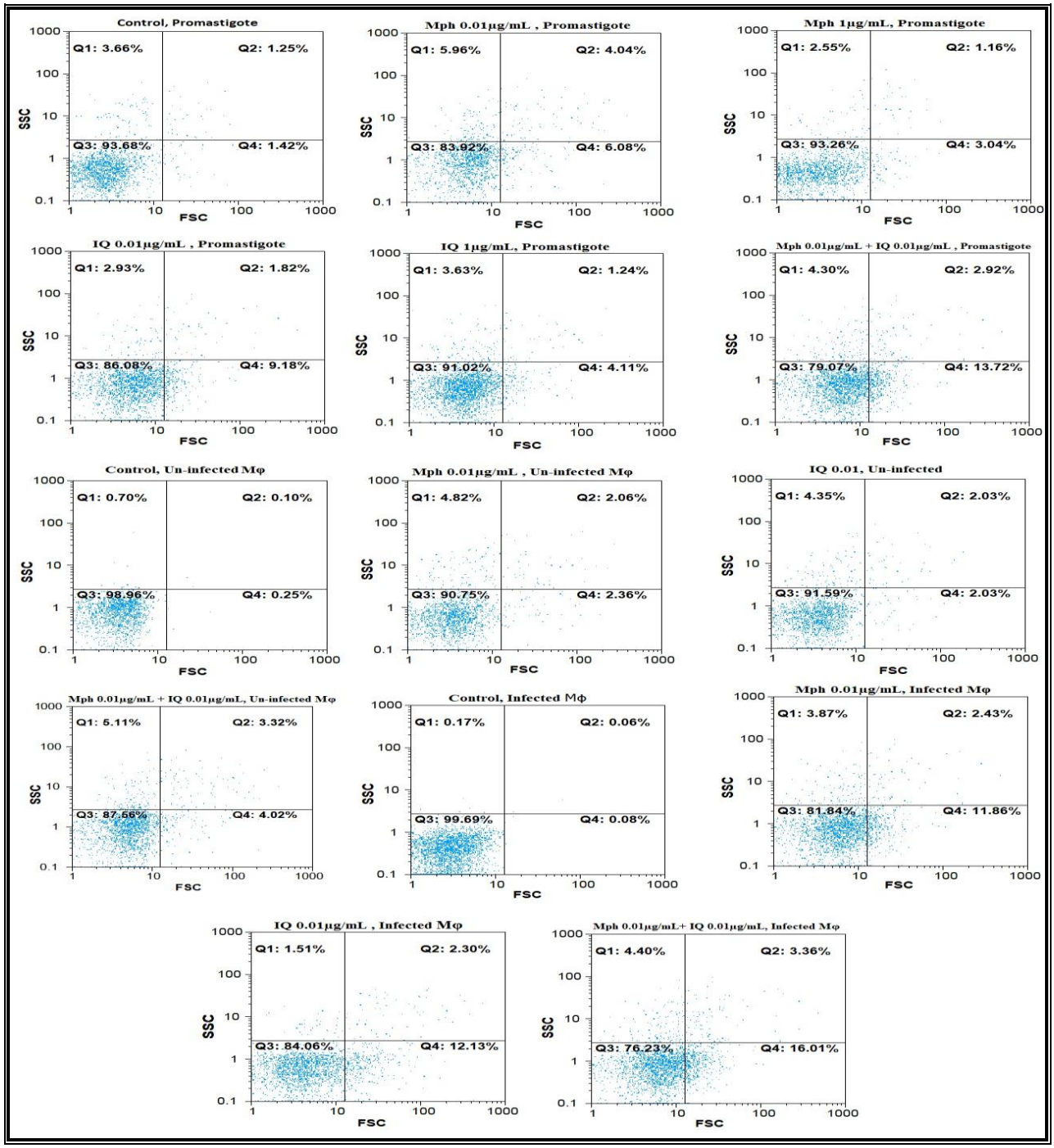

Fig. 4. The results of flow cytometry analysis of promastigotes, un-infected, and infected macrophages. Early and late apoptosis as well as necrosis has been shown after 72h, Tarbiat Modares University, Tehran, 2017

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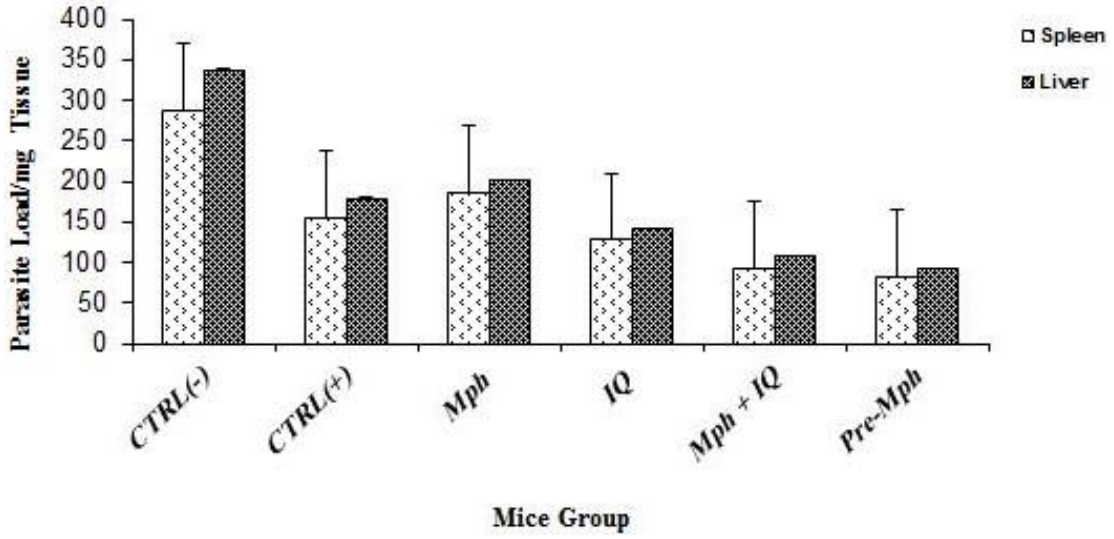

Fig. 5. The mice were infected by *Leishmania infantum* and then treated with studied drugs. At the end of treatment (one week later), the mice were sacrificed, and the parasite loads were evaluated in their liver and spleen. Data were presented as Mean±SD of experiments in 6 mice groups: CTRL (-): infected with no treatment, CTRL (+): parasite-infected with 273µM Glucantime treatment, Mph: parasite-infected with 1500µM Mph treatment, IQ: parasite-infected with 624µM mouse IQ treatment, Mph + IQ: parasite-infected with 1500µM Mph + 273µM IQ treatment, Pre-Mph: parasite-infected after pre-treatment with 1500µM Mph for 3 weeks, Tarbiat Modares University, Tehran, 2017

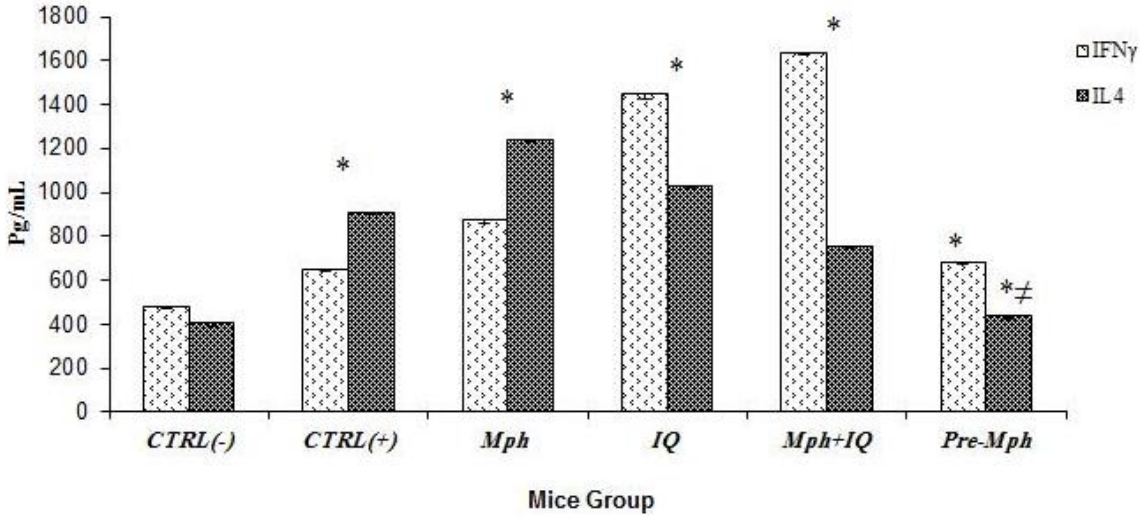

Fig. 6. The mice were exposed to *Leishmania infantum* and then treated with studied drugs. Afterward, the mice were sacrificed, and the cytokine levels were evaluated in their spleen using ELISA. Data are presented as Mean±SD deviation of experiments in 6 mice groups: CTRL (-): infected with no treatment, CTRL (+): parasite-infected with 273µM Glucantime treatment, Mph: parasite-infected with 1500µM Mph treatment, IQ: parasite-infected with 624µM mouse IQ treatment, Mph + IQ: parasite-infected with 1500µM Mph +273µM IQ treatment, Pre-Mph: parasite-infected after pre-treatment with 1500 µM Mph for 3 weeks, *p< 0.05, Tarbiat Modares University, Tehran, 2017

Discussion

Finding new treatments for leishmaniasis has been the target of ongoing efforts for decades. Current medications for the treatment of visceral leishmaniasis are pentavalent antimo-

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ny, pentamidine, miltefosine, and amphotericin B. However, problems such as the emergence of resistance and severe toxic effects are limiting these drugs' usefulness (31). Thus, several new drug candidates have been proposed by many researchers (32). In this study, we used Mph and IQ to evaluate their potential toxicity on *L. infantum* promastigotes as well as uninfected and infected macrophages. The IC_{50} values of Mph, IQ, and combination formula on promastigotes after 72h were 0.102± 0.03, 0.235±0.01, and 0.173±0.02µM, respectively. Also, the IC90 values of Mph, IQ, and their combination on promastigotes after 72h were 0.191±0.02, 0.398±0.01, and 0.245±0.02 µM, respectively. These values for uninfected macrophages were 0.8±0.01, 0.2±0.01 and 0.6± 0.02µM, respectively. These data showed that Mph, IQ, and their combination could be considered as new antileishmanial drug candidates at a minimum concentration of drugs. Jabari et al. showed noticeable results of these drugs on *Leishmania. major* (*L. major*) in in vitro conditions (28). In our present study, the drugs showed anti-leishmanial effects in the lowest concentration compared to the control group ($p< 0.05$). On the other hand, simultaneous usage of Mph and IQ showed promising results against promastigotes compared with the control ($p< 0.05$). Glucantime, as the positive control, showed a poor effect on promastigotes. Many studies have pointed that glucantime has low anti-leishmanial activities on promastigotes of *Leishmania* spp (34). The IC_{50} of Mph, IQ and their combination was found to be 18.2± 0.3, 0.79±0.1, and 8.72±0.1µM, respectively, on *L. infantum* amastigotes. Also, the IC90 values were calculated 29.7±0.1, 1.1±0.1, and 11.8±0.3µM for Mph, IQ, and Mph +IQ, respectively on amastigotes. The low toxicity of these drugs on J774 macrophages indicated the high effect of these drugs on intracellular *Leishmania* parasites. These results were consistent with results obtained by others on *L. major* amastigotes (27, 33). In the present study, glucantime was more toxic to uninfected and infected macrophages than Mph and IQ ($p< 0. 05$).

Flow cytometry results indicated a lower value of early and late apoptosis in Mph or IQ alone than their combinations. The results of early and late apoptosis were consistent with other studies that were performed on *L. major* (28). The difference between our study and others is perhaps related to drug doses and incubation time. The in vivo experiments on BALB/ c mice confirmed the in vitro results. The parasite burden of spleen and liver tissues in test groups decreased significantly compared to the control group ($p< 0.05$). The positive synergistic effect of Mph +IQ showed more efficacies in controlling the parasite multiplication rate compared to Mph or IQ alone. On the other hand, when Mph was administered before inoculation of parasites, the growth rate of parasites in BALB/c mice was inhibited, and the parasite rate was at a minimum compared to the control and other test groups. The parasite count decreased to 48%, 63%, 67%, and 80% in Mph, IQ, Mph +IQ, and pre- Mph groups, respectively. The results of cytokine assay showed that IL-4 was produced more than IFN-γ in Mph group. In other groups, IFN-γ was produced more than IL-4. The role of Mph as an immunomodulator in protection against leishmaniasis by producing cytokines of CD4 + helper T-cells has been shown in many studies (14). Other similar studies on *L. major* have shown contradictory results (34). In another study, the augmented effect of Mph at low doses was approved in murine visceral leishmaniasis (35). Many studies have shown that these drugs affect the parasite by involving immune system receptors. This is true especially for Mph, which may induce the activation of opioid receptors as an immunomodulator (36). Due to the selective pressure of the host defense system, pathogens have evolved different mechanisms helping them to antagonize apoptotic death of the invaded host cells such as macrophages. This not only gives more time for parasite replication (37) but also accelerates the ingestion of apoptotic infected macrophages

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by uninfected ones. This event provides a way for amastigote spreading and subsequent infection (38). Promastigotes of *L. infantum* and their surface lipophosphoglycan have been shown to prevent the apoptosis of macrophages, and this allows the survival of intracellular parasites. To unravel the mechanisms behind the resistance of *L. infantum* to apoptosis, some experiments have been performed. These experiments indicate that *L. infantum* inhibits apoptosis via the regulation of the IAP (apoptosis inhibitor) family of proteins including cIAP1 and cIAP2. IAP modulation will result in the apoptosis resistance of human macrophages after *L. infantum* infection. In addition, infection with *L. infantum* changes the expression of apoptosis-related proteins including Bcl-2, BAX, caspase-3, caspase-8, and caspase-9 (7). This shows that promastigotes employ multiple signals to confer more anti-apoptotic effects human macrophages. Although, PI3K is not directly involved in Bcl-2 regulation, a considerable level of PI3K phosphorylation occurs in *L. infantum*-infected cells. In this regard, MAPKs and PI3K are probably engaged during *Leishmania* infections and take part in the apoptosis or survival of host cells (39). Opioids, including Mph, have immunoregulatory effects and do this through interactions with their receptor on immune cells. They are thus classified as cytokine families. The molecular basis underlying this effect is the modulation of cytokines and altered expression patterns of some cytokine receptors (40). Several researchers have demonstrated the protective role of Mph against leishmaniasis. In a study, low doses of Mph were injected subcutaneously to *L. donovani*-infected BALB/c mice or hamsters. The results showed significant suppression or even sterile clearing of the infection. In contrast, high doses exacerbated the infection (41). In another study, Poonawala et al. showed that Mph improved the healing of ischemic wounds through stimulating nitric oxide (NO) via opioid receptors (42). The exact mechanisms of the protection role of Mph against leishmaniasis are not completely clear. Macrophages also have TLR7, which one of its potent ligands is imidazoquinoline compound IQ (18). It has some roles in the modulation of the immune system through the activation of macrophages or other cells via TLR7 (19). In the present study, Mph and to a lesser extent IQ as well as their combination showed significant toxicity on promastigotes both in vitro and in vivo. In vitro experiments showed that drugs, at high doses, had no effect on promastigotes. The results of Mph were similar to in vivo experiments. Mph was not able to induce apoptosis in parasites, but prophylaxis with Mph before infection made the mice resistant to the parasite, an effect that was far more effective than IQ in *in vivo*. IQ was able to reduce parasite load *in vivo*, but its effect became stronger when it was synergistically used with Mph. Researchers have shown that IQ treatment stimulated genes and protein expression of the opioid growth factor receptor (21). The synergistic effect observed in this study may be attributed to this phenomenon. According to the results of many other studies, IQ is effective on *Leishmania* (43), viruses, and tumor cells (44). Imiquimod is used as a drug in warts, basal cell carcinoma, and Kaposi carcinoma, chronic hepatitis type C, intraepithelial carcinoma, melanoma, lung sarcoma, and breast cancer (45). A clinical trial study conducted by Firooz et al. on efficacy and safety of IQ combined with glucantime for cutaneous leishmaniasis of *L. tropica* expressing no beneficial effect of combining treatment with 5% IQ cream with meglumine antimoniate in patients (46). Another study by Mohebali et al. (47), showed safety, but low efficacy (40.40%) of Alum-ALM mixed with BCG and imiquimod on Canine visceral leishmaniasis. Several studies in Iran and other countries showed contradictory results of imiquimod treatment on CL lesions. The genus of *Leishmania*, treatment duration, type application of IQ (topical or subcutaneous injection), and clinical manifestation of lesions were reported responsible for cure rate in

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Published Online: June 30, 2021

IQ treatment (46-47).

The mechanism of action of IQ is unknown, but it is an agonist of TLR7 and affects immune responses, induce TNFa, IL-1a, and IL-12 production, and stimulates apoptosis pathways (48). Various cytokines are produced during leishmaniasis such as tumor necrosis factor (TNF) and interferon (IFN), which enhance macrophage activation and other inflammatory responses (49). Macrophages are the main cells against *Leishmania* and have prominent roles in immune response such as phagocytosis. Therefore, to better understand the mechanisms involved in killing *leishmania*, the spleen lymphocytes were exposed to the parasite. The results showed that in Mph group, increased the level of IL-4 strongly. The results for IQ or IQ +Mph -treated mice were contrasted, showing the equal increase of IL-4 and IFN-γ. This may imply that the apoptosis mechanism is different in the two treatments. Studies have demonstrated that IQ enhances IFN-γ production both in human and murine cutaneous leishmaniasis. IFN-γ kills the parasite and causes protective immunity. Also, IL-4 enhances programmed apoptosis in stimulated human monocytes (50). Susceptibility to infection is associated with the activation of Th2 or Th1 cells and secretion of IL-4, IL-5, IL-6, IL-10, IL- 12, IFN-γ, and lymphotoxins. This could help animals to kill parasites and control infections such as leishmaniasis (51). IFN-γ is important to the immune defense against intracellular pathogens. In leishmaniasis, IFN-γ promotes Th1 differentiation and macrophage activity. IFN-γ signaling in macrophages results in the activation of host defense mechanisms. Additionally, IFN-γ induces genes such as nitric oxide synthase, the most important molecule responsible for killing *leishmania* parasites by macrophages (52). Macrophages that are activated by cytokines can produce large amounts of nitric oxide, one of its functions is a defense against intracellular pathogens particularly *Leishmania*. Nitric oxide has been demonstrated to kill *leishmania* parasites by inducing amastigotes apoptosis (53). IFN-γ is important since it has immune-stimulatory, immune regulatory, and immune-modulatory effects. In a study on the effects of artemisinin on VL, the authors demonstrated the ability of lymphocytes of infected mice to produce INF-γ during treatment with artemisinin (54). Our study revealed a considerable IFN-γ response in the cultured splenocyte of test groups.

There are several reports on different aspects of leishmaniasis in the country including the resistant status of vectors to different WHO recommended insecticides, reservoirs, reservoir control, vector control, ecology, novel approaches, training, and epidemiology (55-90). These reports will provide a guideline for disease control.

Conclusion

According to the results of the present study, Mph and IQ alone or in combination with Mph at low concentrations could inhibit the multiplication of the *L. infantum* promastigote and amastigote. The drugs eliminated the parasite growth and the development of murine visceral leishmaniasis. Based on current and future in vitro and in vivo studies, Mph and IQ alone or in combination may be considered as a new therapeutic agent for the treatment of visceral leishmaniasis. The authors declare that they have no competing of interests.

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