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Original Article

MicroRNAs Expression Induces Apoptosis of Macrophages in Response to *Leishmania major* (MRHO/IR/75/ER): An In-Vitro and In-Vivo Study

Mostafa GHOLAMREZAEI [1], *Soheila ROUHANI [1], *Mehdi MOHEBALI [2,3], Samira MOHAMMADI-YEGANEH [4,5], Mostafa HAJI MOLLA HOSEINI [6], Ali HAGHIGHI [1], Zohreh LASJERDI [1], Faezeh HAMIDI [1], Mohammad KAZEM SHARIFI-YAZDI [7]

1. *Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
2. *Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
3. *Center for Research of Endemic Parasites of Iran (CREPI), Tehran University of Medical Sciences, Tehran, Iran*
4. *Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
5. *Department of Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences,Tehran, Iran*
6. *Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
7. *Zoonosis Research Center, Tehran University of Medical Sciences, Tehran, Iran*

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***Correspondence**
Email:
srouhani11@yahoo.com

Abstract

Background: We aimed to investigate the effect of miR-15a mimic and inhibitor of miR-155 expression on apoptosis induction in macrophages infected with Iranian strain of *Leishmania major* in-vitro and in-vivo.

Methods: RAW 264.7 cells were infected with *L. major* promastigotes (MRHO/IR/75/ER), and then were treated with miRNAs. For in-vivo experiment, BALB/c mice were inoculated with *L. major* promastigotes, and then they were treated with miRNAs. For evaluation of miRNA therapeutic effect, in-vitro and in-vivo studies were performed using quantitative Real-time PCR, Flow cytometry, lesion size measurement, and Limiting Dilution Assay (LDA). This study was performed in Shahid Beheshti University of Medical Sciences in 2019.

Results: In-vitro results of flow cytometry showed that using miR-15a mimic, miR-155 inhibitor or both of them increased apoptosis of macrophages. In in-vivo, size of lesion increased during experiment in control groups (P<0.05) while application of both miR-155 inhibitor and miR-15a mimic inhibited the increase in the size of lesions within 6 wk of experiment (P=0.85). LDA results showed that microRNA therapy could significantly decrease parasite load in mimic or inhibitor receiving groups compared to the control group (P<0.05).

Conclusion: miR-155 inhibitor and miR-15a mimic in *L. major* infected macrophages can induce apoptosis and reduce parasite burden. Therefore, miRNA-based therapy can be proposed as new treatment for cutaneous leishmaniasis.

Introduction

Leishmaniasis is a complex of diseases caused by different species of protozoan parasites belonging to genus of *Leishmania*, affecting 350 million people in more than 90 countries (1). Cutaneous, visceral, and muco-cutaneous forms are different manifestations of leishmaniasis, while cutaneous form is the most common clinical syndrome (2). Cutaneous leishmaniasis is a major health problem in Iran (3). Iran has different endemic foci for Zoonotic Cutaneous Leishmaniasis (ZCL) main reservoir hosts of which are four gerbil species (4).

Leishmania spp. are obligate intracellular pathogens of their mammalian hosts (5). Motile promastigotes are transmitted by female sand flies (6). After transmission, the promastigotes are taken up by phagocytic cells including macrophages and neutrophils (7). Macrophages are main host cells for proliferation of amastigotes (8). Pentavalent antimonials have remained a treatment choice for all clinical forms of leishmaniasis. Increasing rate of antimony resistance is becoming a serious health problem (9). Therefore, new molecular approaches are required for treatment of leishmaniasis. Nanotechnology (10) and molecular manipulation such as CRISPR/Cas9 system (11) has been introduced as novel therapeutic technologies for infectious diseases such as leishmaniasis.

Apoptosis induction in leishmanial-infected macrophages using molecular techniques can reduce parasite burden (12). Controlled program associated with distinctive morphological changes including membrane blebbing, cytoplasmic and nuclear condensation, and chromatin aggregation along with DNA breakage is triggered through Apoptosis (13). There are two main apoptotic pathways: extrinsic or death receptor pathway and intrinsic or mitochondrial pathway (14).

MicroRNAs (miRNAs), a subset of non-coding RNAs are ~22-nt long endogenously initiated short RNA molecules post-transcriptionally regulating cleavage of target mRNAs or just repressing their translation (15). MicroRNAs play significant roles in regulatory mechanisms operating in various organisms including apoptosis (16). MicroRNA-15a targets different genes including anti-apoptotic genes such as BCL-2 (17). Silencing of BCL-2 induces apoptotic pathway activated by cleaving *caspase-9*, and *caspase-3* (18). Moreover, miR-155 contributes to different signaling pathways such as apoptosis, inflammation, and immunity, which apoptosis induction by targeting *caspase-3* mRNA (19,20).

Thus, this study was carried out to investigate the effect of miR-15a mimic and miR-155 inhibitor on apoptosis induction in *L. major* infected macrophage cell line and cutaneous leishmaniasis in BALB/c mice.

Materials and Methods

This study was performed in Shahid Beheshti University of Medical Sciences, Tehran, Iran in 2019.

Parasite culture

L. major promastigotes (MRHO/IR/75/ER) (Department of Medical Parasitology and Mycology, Tehran University of Medical Sciences, Tehran, Iran), promastigotes were cultured in RPMI 1640 (Biosera, France) media supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (Gibco, USA) and 1% of penicillin (100 U/mL), and streptomycin (100 µg/mL) (Gibco, USA) and was incubated at 25 °C.

In vitro experiments

The BALB/c mice-derived macrophage cell line RAW 264.7 (Iranian Biological Resource Center, Tehran, Iran) was cultured (2×10^5 cells per well) in high-glucose Dulbecco's Modified Eagle Medium (DMEM) (Bioidea,

Iran) with 10% of FBS, and 1% of pen/strep at 37 °C in a humidified atmosphere of 5% CO_2 in 24-well cell culture plates. After 24 h, when macrophages attached media in culture wells were discarded. Macrophages in different groups were transfected with miR-15 mimic (Bioneer, South Korea), miR-155 inhibitor (Bioneer, South Korea) and both of them using Lipofectamine® 2000 (Invitrogen, USA) in serum-free media. After six hours, the media were replaced with 500 µL DMEM with 10% of FBS. The cells without any treatment and cells placed under UV light (20 min, 20 cm distance) were used as negative and positive controls, respectively. Then, plates were incubated again for 48 hours. For investigation the effect of *L. major* infection on macrophages, attached macrophages were infected with *L. major* promastigotes with the ratio of 10 promastigotes per macrophage. The cultured cells were incubated at 37 °C for 24 h in 5% CO_2 until promastigotes were phagocyte by macrophages. Then, media were discarded and replaced with serum-free media containing Lipofectamine® 2000 reagent and miR-15a mimic, miR-155 inhibitor or miR-15 mimic/miR-155 inhibitor. Negative control received no treatment. For positive control, well was placed under UV. After six hours, the media were replaced with 10%FBS containing DMEM. Plates were incubated again for 48 h.

RNA isolation, reverse transcription, and quantitative Real time-PCR analysis

The infected and uninfected macrophages were harvested 48 h after transfection by using 0.5 mM EDTA in 1X PBS and scraping. After centrifugation of the cells for 5 min at 400 × g, total RNA was extracted using miRNeasy® Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The quality and concentration of extracted RNA were determined using NanoDrop ND-1000 micro-spectrophotometer (Thermo Fisher Scientific, USA). Total RNA was used for Complementary DNA synthesis with a First Strand cDNA synthesis kit (Thermo Scientific™) following manufacturer's instructions. The miRNA-specific stem-loop primers (Zist Rouyesh, Tehran, Iran) and random hexamer primer (Yekta Tajhiz Azma, Tehran, Iran) were used for cDNA synthesis of miRNA and genes respectively. Quantitative Real-time PCR (qPCR) was performed in duplicates in ABI StepOnePlus™ instrument (Applied Biosystems, USA) using the SYBR® Green master mix Kit™ (Ampliqon, Denmark). The sequences of specific primers are presented in Table 1.

Table 1: The sequences of microRNAs and gene expression primers used for Real-time PCR assay

miRNAs / Genes	*Sequences of primers (5'-3')*
miR-15a-5p	F: AGGCATAGCAGCACATAATG
miR-155-5P	F: CACGTCCTTAATGCTAATTGTGAT
Snord-234-mmu	F: ATCTAAGTGATTTAACAAAAATTCGTCACTAC
	Reverse universal: GAGCAGGGTCCGAGGT
Caspase 3	F: TAAGAACTTCCATAAGAGCACTGG
	R: GCTATGATCTTCCTTAGAAACACTATCC
Caspase 7	F:GCTCCTCTATCATCTCCTCTATTC
	R: TCCATACCTGTCGCTTTGTC
Mmu-β2m	F: TATACTCACGCCACCCACC
	R: TCTCGATCCCAGTAGACGG

Each reaction was prepared in a final volume of 20 µL. The condition of RT-qPCR was as follow: one initial cycle at 95 °C for 15 min, and 40 cycles at 95°C for 20 sec and 60 °C for

60 seconds. Melting curve analysis was also performed at the end of cycles from 55 to 95 °C with 1 °C increment in 0.3 seconds. The expression levels of miRNAs and target genes were normalized to mmu-Snord-234 and mmu-β_2m expression (as the reference genes) respectively. Finally, the cycling threshold was calculated and data were analyzed using $2^{-\Delta\Delta Ct}$ method in REST 2009 software.

Apoptosis Assay with Annexin V-PI

The Annexin V-FITC Apoptosis Detection kit (Biolegend, cat number: 640945) and BD FACS Calibur system (BD biosciences, San Jose, CA, USA) were used to detect Annexin V. Results were obtained using FlowJo software (Treestar) and base on the method applied by Flowcyt Science-Based Company, Tehran, Iran.

In vivo experiments

Thirty female BALB/c mice (6–8 wk old) (Animal Breeding Stock Facility, Pasteur Institute of Iran (IPI), Karaj, Iran) were infected with infective *L. major* using a subcutaneous injection of 2×10^6 promastigotes into the base of mice tails. Following lesion development, mice with similar lesion size were chosen to assume similar levels of infections. Chosen mice were then divided into six groups (n = 5 per group) as follows: group 1 received 25 µM of miR-15a mimic; group 2 received 25 µM of miR-155 inhibitor; group 3 received 25 µM of miR-15a mimic and 25 µM of miR-155 inhibitor. miRNAs diluted in 100 µL sterile PBS. Group 4 received 100 µL of sterile PBS (vehicle control group); group 5 included mice treated with 0.02 mg/kg of Glucantime® (positive control group), and group 6 included infected untreated mice as a negative control group. Groups 1-5 were received treatment by intralesional injection twice a week for 6 weeks. Treatment started 4 wk post-inoculation when lesions were established and appeared at injection sites. All mice in the treatment groups showed similar lesion sizes. Mice were monitored daily for 6 wk and lesion sizes were documented for each group.

Treatment evaluation

Measurement of the lesion size

Size of the lesions was measured before and after 6 wk treatment using ImageJ software (https://imagej.nih.gov/ij/). The wound regions were reported in square millimeter.

Parasite load

Limiting dilution assay (LDA) was used to calculate the parasite load spleen of *L. major*-infected mice in different groups. At end of 6th week after treatment, the mice were sacrificed by decapitation. Then, their spleens were gently crushed and suspended on RPMI 1640 with 10% FBS by using cell strainer 100 um (SPL Life Sciences, South Korea). Next, the parasite burden was measured using LDA assay on 12 serial dilutions of cells which cultured in 96-well plate (8 well for each dilution) containing RPMI 1640 with 10% FBS. After 3 to 7 d incubation at 25 °C, the number of positive wells (existence of motile parasite) and negative wells (absence of motile parasite) were determined by using invert microscope and the results were analyzed by ELIDA software (21).

Statistical analysis

Statistical analysis was done using Graph-Pad Prism 6.0 for Windows (Graph-Pad Prism, San Diego, California, USA). For in vitro and in vivo evaluation, t-test or one-way ANOVA was applied to analyze in the current study. The *P*-values less than 0.05 ($P<0.05$) were considered statistically significant.

Results

Expression of miR-15a decreased while miR-155 expression increased in RAW264.7 cells after L. major infection

Results of real-time PCR showed that infection of miR-15a mimic in the cells caused at

least 3-time overexpression of miRNA in these cells. Moreover, in the group receiving both miRNAs, miR-15a increased about 1.5 folds, but it was not statistically significant (Fig. 1). Difference in expression of miR-15a was not statistically significant in control group and Lipofectamine-receiving group (P>0.05).

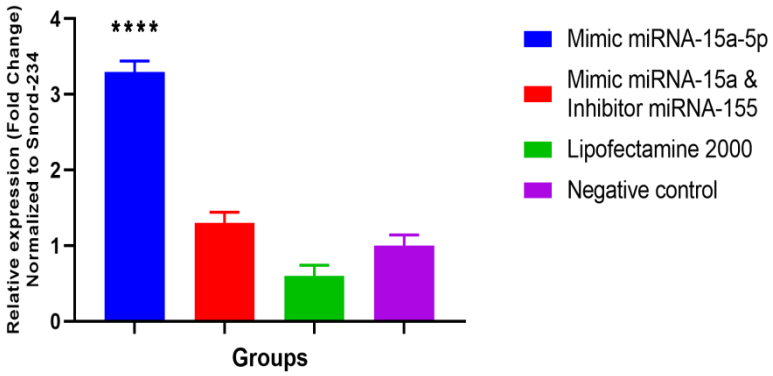

Fig. 1: Expression analysis of miR-15a-5p in *L. major* infected-RAW 264.7 macrophages after 48 h (P<0.05)

After 48 h, the result of miRNA expression analysis showed that inhibitor could successfully decline expression of miR-155 about 4 folds in the group receiving miR-155 inhibitor, however it the group receiving both mimic and inhibitor, expression of miR-155 interestingly increased (Fig. 2).

According to results of the study and contrary to our expectation, *caspase-3* gene expression decreased in the group receiving miR-15a mimic. However, treatment of cells by both miR-15a mimic and miR-155 inhibitor increased expression of *caspase-3* as final effector of apoptosis (P<0.05). In other groups, no significant difference was observed (P>0.05) (Fig. 3). Consistent with results obtained from *caspase-3* expression in miR-15a receiving cells, expression of *caspase-7* also decreased and miR-155 inhibitor increased *caspase-7* expression, while the increase in the expression of *caspase-7* in *L. major* infected cells receiving both mimic and inhibitor was not statistically significant (P<0.05, Fig. 4).

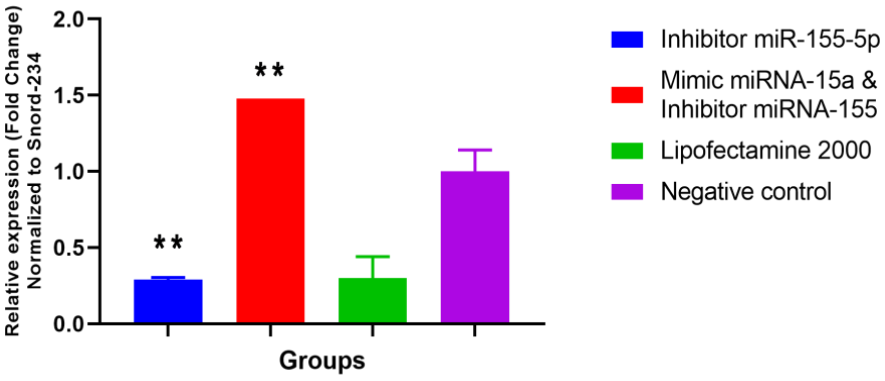

Fig. 2: Expression analysis of miR-155 in *L. major* infected-RAW 264.7 macrophages after 48 (P<0.05)

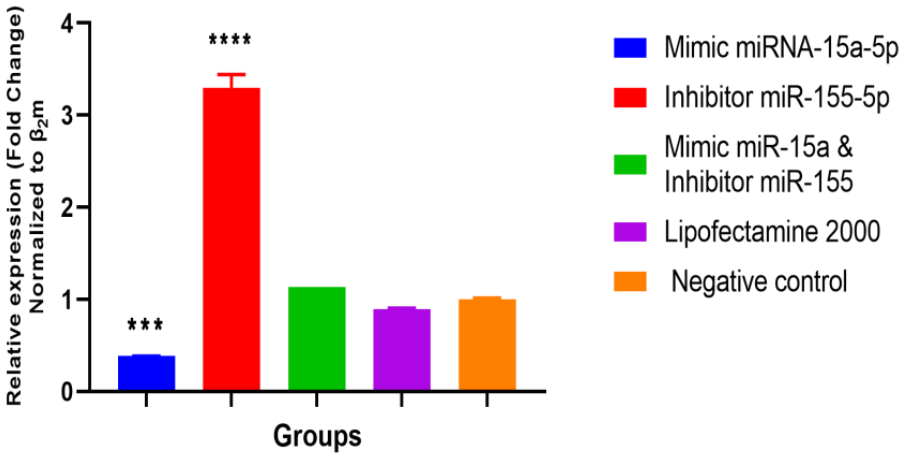

Fig. 3: Expression analysis of caspase-3 in *L. major* infected-RAW 264.7 macrophages treated with miR-15a mimic, miR-155 inhibitor and both of them after 48 h (P<0.05)

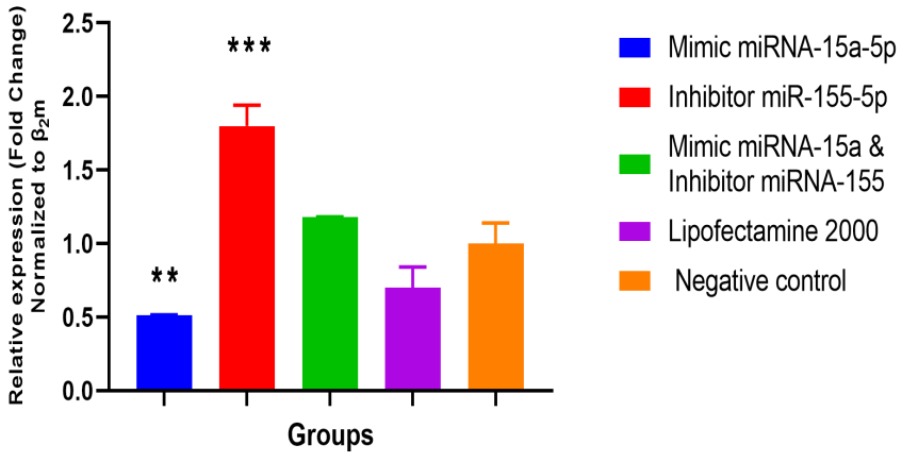

Fig. 4: Expression analysis of caspase-7 in *L. major* infected-RAW 264.7 macrophages treated with miR-15a mimic, miR-155 inhibitor and both of them after 48 h (P<0.05)

Apoptosis happened in L. major infected-RAW 264.7 cells after treatment with miR-15 mimic and miR-155 inhibitor

To evaluate the effect miR-15a mimic and miR-155 inhibitor on apoptosis and necrosis of macrophage cells, 48 h after treatment of cells with miR-15a mimic or miR-155 inhibitor, the cells were collected and were stained with Annexin-V and Propidium Iodide (PI) dye. Overexpression of miR-15a or down-regulation of miR-155 caused an increase in the population of apoptotic cells (pre-apoptotic, apoptotic, and post-apoptotic/necrotic cells in Q3, Q2, and Q1 quadrants, respectively) (Fig. 5a). The effect of miR-15a mimic, miR-155 inhibitor, and both agents on macrophage apoptosis was significant compared to the control group. Apoptosis induction by UV light was used in a group as positive control (Fig. 5b).

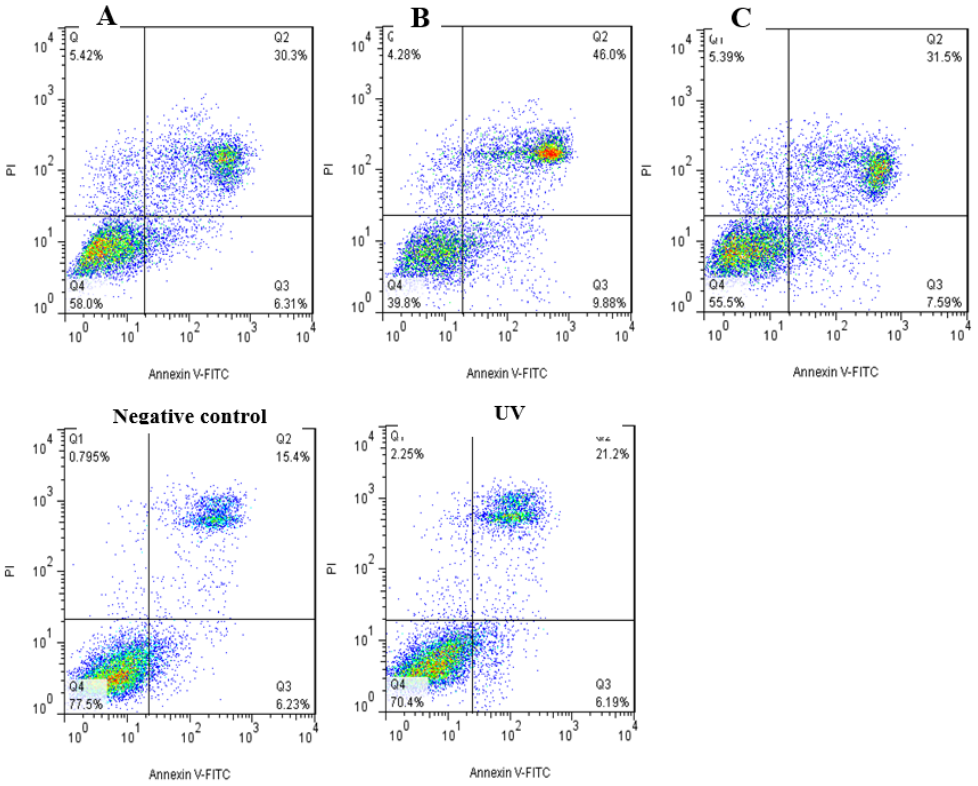

Fig. 5a: Flow cytometry analysis of the RAW 264.7 cells treated with (A) miR-15a-5p mimic, (B) miR-155-5p inhibitor and (C) miR-15a mimic and miR-155 inhibitor, under UV exposure (positive control), and untreated (negative control) 48 h after transfection ($P<0.05$)

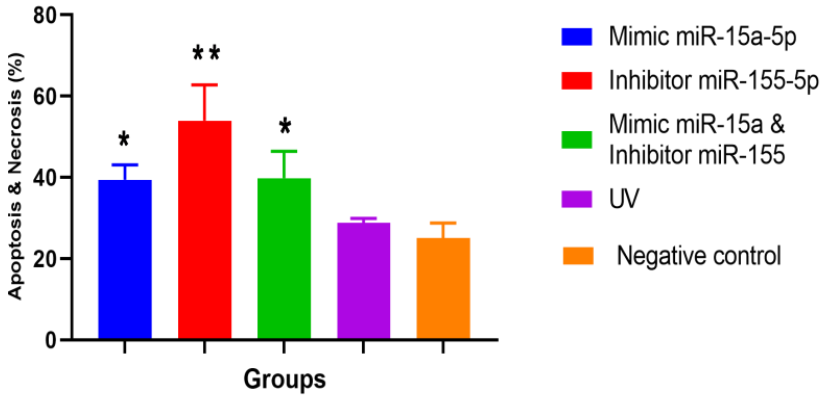

Fig. 5b: Effect of treatment with miR-15a mimic, miR-155 inhibitor, and their combination in RAW 264.7 cells compared with negative control group ($P<0.05$)

Results obtained from Annexin V-PI staining of miRNA mimic and inhibitor treatment of *L. major*-infected macrophage showed that miR-155 inhibitor had the most effect on apoptosis induction of the cells (Fig. 6a). MiR-15a mimic or its combination with miR-155 inhibitors also increased population of apoptotic cells, however their difference was not statistically significant compared to the control group ($P>0.05$, Fig. 6b).

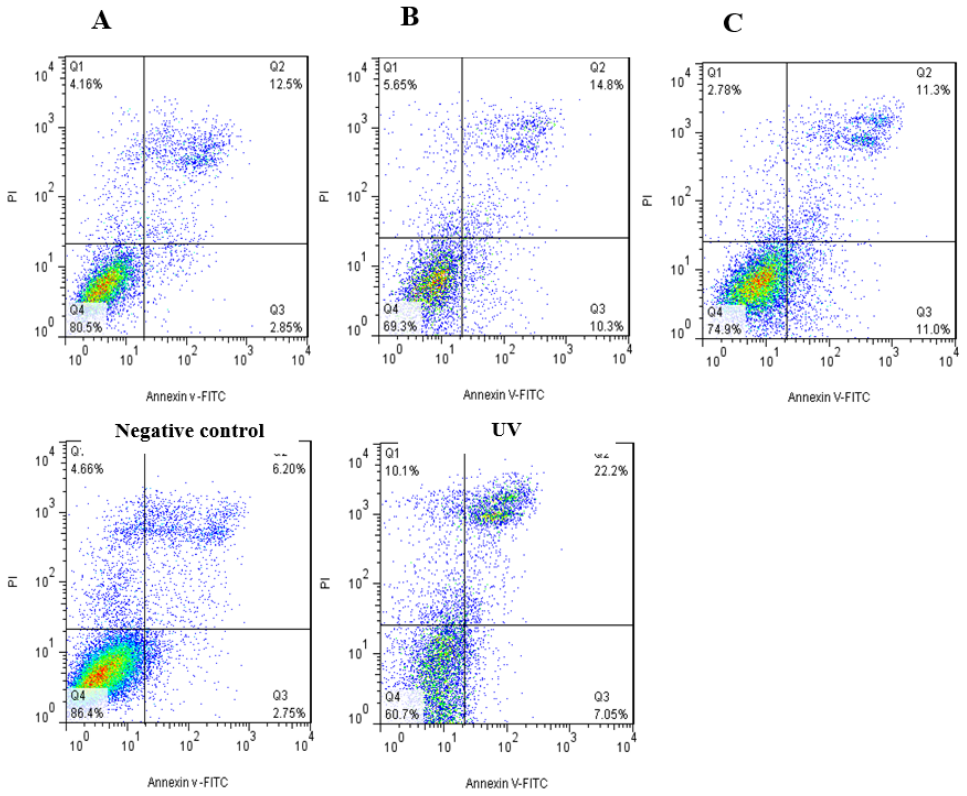

Fig. 6a: Flow cytometry analysis of the *L. major*-infected RAW 264.7 cells treated with (A) miR-15a-5p mimic, (B) miR-155-5p inhibitor and (C) miR-15a mimic and miR-155 inhibitor, under UV exposure (positive control), and untreated (negative control) 48 h after transfection ($P<0.05$)

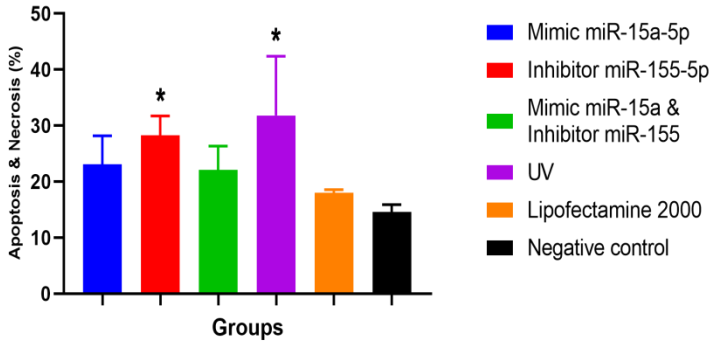

Fig. 6b: The effect of treatment with miR-15a mimic, miR-155 inhibitor, and their combination in *L.major*-infected RAW 264.7 cells ($P<0.05$)

Size of Leishmaniasis lesion remained stable after treatment with miRNA

Size of lesions increased normally in the groups receiving PBS or no intervention. Variations in lesion size (mm^2) in BALB/c mice are shown in Table 2. In treated groups including mice receiving miR-15 mimic or miR-155 inhibitor, size of lesion significantly increased within 6 wk of treatment period while the increase in the lesion size of the mice receiving both miR-15a mimic and miR-155 inhibitor was not statistically significant

(P>0.05)(Table 2). In glucantime-treated mice, as a positive control, lesion size decreased during the treatment. Furthermore, after monitoring for 6 wk, comparison of the group receiving both mimic and inhibitor with the control group (no treatment) showed a decrease in the size of lesion (Fig. 7). Figure 8 shows trend of variations in size of lesion before and after treatment of mice.

Table 2: Variation of lesion size (mm^2) of BALB/c mice, before and after treatment

Groups	*Before treatment*	*After 6 weeks treatment*	*P-value (before/after)*	*P-value (compare to negative control)*
miR-15a mimic	12.74 ± 1.18	15.675 ± 2.19	0.0366	0.2174
miR-155 inhibitor	12.66 ± 0.93	16.24 ± 3.3	0.0497	0.3274
miR-155 inhibitor & mimic miR-15a	10.68 ± 2.02	10.35 ± 3.2	0.8577	0.0003
PBS (vehicle)	10.8 ± 2.3	19.78 ± 2.6	0.0013	0.7629
Glucantime®	10.1 ± 1.83	5.64 ± 1.11	0.0016	<0.0001
Control Negative	10.4 2± 1.97	17.88 ± 2.4	0.0007	

Values are represented as mean ± SD, Positive control (Glucantime®): the mice were treated twice a week with 300 µl (5 mg/ml) intralesional injection.

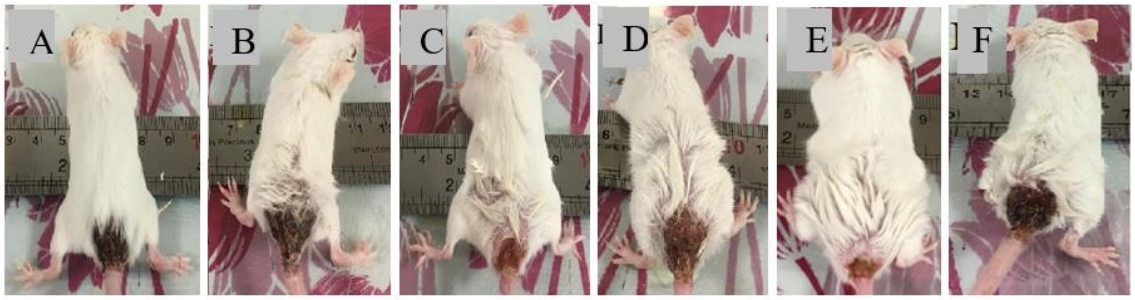

Fig. 7: Lesions of BALB/c mice infected with *L. major*. Photographs were taken on the last days of the treatment: A) miR-15 mimic; B) miR-155 inhibitor; C) miR-15 mimic & miR-155 inhibitor; D) PBS, E) Glucantime®; F) Negative control (untreated)

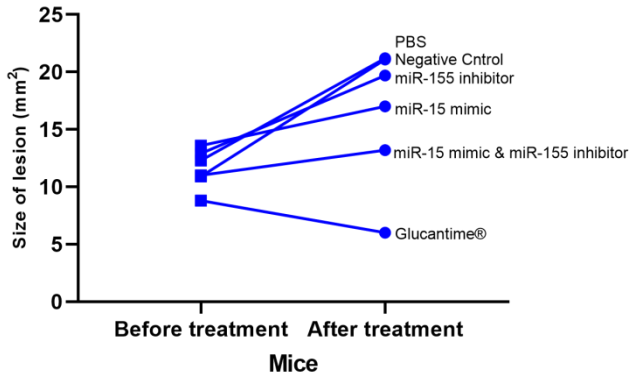

Fig. 8: Trend of the size of lesion before and after treatment in mice

LDA showed a decrease in the number of parasite in spleen of leishmanial-infected Mice treated with miRNA

Motility of the promastigote was investigated by inverted microscope and was statistically calculated by ELIDA software. Load of parasite declined in all treatment groups receiving miRNAs compared to the controls (P<0.05) (Fig. 9).

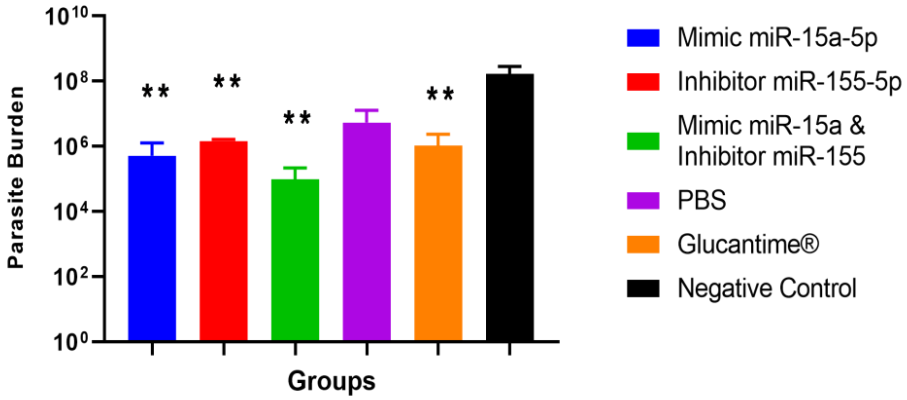

Fig. 9: Parasite load in spleen of mice in different groups after 5 days

Discussion

Concerning lack of a decisive and safe treatment for Zoonotic Cutaneous Leishmaniasis (ZCL) (22), in this study, a new approach was applied for cutaneous leishmaniasis treatment. Inhibition of apoptosis by intracellular pathogens has been described as a mechanism of securing long-term survival of their host cells (23).

MicroRNA therapy is potential manipulation of host's responses through parasite miRNAs, given that miRNA machinery is particularly interesting to seek alternative therapeutic approaches against parasites (24). miRNAs regulate a variety of developmental and physiological processes in parasites (25). Identification and characterization of microRNA (miRNAs) in *Leishmania* and their possible biological action hopefully would facilitate discovery of potential drug targets against leishmaniasis (26).

Inhibition of host cell apoptosis by obligate intracellular bacteria, protozoa, and viruses is a common process leading to proliferation and evasion of organisms from killing mechanisms. *Leishmania* infection inhibits apoptosis of macrophage (27). Infected cells neutralize invasive pathogens via initiating their death, called as apoptosis. This interaction is influenced by two different internal and external pathways (28). Already, Miltefosine® induces apoptosis-like death in *Leishmania donovani* promastigotes (29). Miltefosine® is also effective on *L. major* (30). Gastrointestinal side effects, almost involving vomiting, and diarrhea were frequent. This drug may cause destructive effects on kidney and liver (31).

In the present study, miR-15a mimic, miR-155 inhibitor, and their combined treatment were applied by miR-155 inhibitor, miR-15a mimic and both of them, which increased apoptosis in normal and infected macrophages in RAW 264.7 cell line. Resende et al reported miR-15a down-regulated expression along with high B-cell lymphoma-2 **(**Bcl-2) mRNA expression in Odontogenic Keratocyst (OKC) samples. They attempted to delete *MIR15A* genes causing down-regulation of miR-15a expression, which resulted in overexpression

of Bcl-2. However, this was not true for OKC (32). In our study, in agreement with results of another study, using miR-15a mimic did not cause an increase in the expression of *caspase-3*. However, these treatments decreased load of parasite in spleen of BALB/c mice. Targeting anti apoptotic miR-21 was effective for treatment of pancreatic cancer in mice (33).

Herein, miR-155 as an anti-apoptotic miRNA was also inhibited and an increase was observed in the number of apoptotic cells, as well as an increase in *caspase-3/7* expression and a decrease in parasite load in spleen of the mice. Lasjerdi et al showed miR-24-3p as anti-apoptotic miRNA, upregulated after *L. major* infection in macrophages (34). In this study, miRNAs was used to elucidate whether miR-15a mimic and/or miR-155 inhibitor can induce apoptosis in *L. major*-infected cells and reduce parasite burden.

Meanwhile there is not enough evidence about miRNA therapy on parasitic disease. Apoptosis and necrosis was evaluated after transfection of Locked Nucleic Acid (LNA) inhibitor of let-7a in human macrophages upon infection with *L. major*, and showed that it could induce apoptosis and necrosis in macrophages infected with *L. major* (35). Our study results also showed that treated cells after 48 h of miR-155 inhibition and/or use of mimic miR-15a displayed apoptotic condition, however, miR-155 inhibition was significantly more effective in apoptosis induction. Moreover, results of real-time PCR showed that using miRNA-155 inhibitor and miR-15a mimic caused down-regulation and up-regulation of miRNAs expression, respectively. However, their combination was not effective, and compensatory pathways hided their effects. Efforts have been made in treatment of many diseases and cancers using miRNA.

In this study, using miR-155 inhibitor and miR-15a mimic in RAW 264.7 cells increased apoptosis and necrosis. Thus, it may be an effective therapeutic protocol for treatment of leishmaniasis. Furthermore, we are performing further investigations on safety and side effects of miRNA therapy in *L. major*-infected mice to overcome toxicity of conventional drugs.

Conclusion

The use of miRNA-155 inhibitor and miRNA-15a mimic can be considered as potential novel treatment for leishmaniasis. It is recommended to perform more evaluations with larger sample size, higher doses and longer treatment periods *in -vivo* model.

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Conflicts of interest

The authors declared no conflict of interest.

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