



Expression of hsa Let-7a MicroRNA of Macrophages Infected by *Leishmania Major*

Nooshin Hashemi¹, Mohammadreza Sharifi², Sepideh Tolouei¹, Mitra Hashemi³,
Cyrus Hashemi⁴ and Seyed Hossein Hejazi^{5*}

¹Department of Parasitology & Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

²Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

³Deputy of Research and Technology, North Khorasan University of Medical Sciences, Bojnurd, Iran

⁴Dentist, North Khorasan, Social Security Organization, Bojnurd, Iran

⁵Skin Diseases and Leishmaniasis Research Center, Department of Parasitology & Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

*Corresponding E-mail: hejazi@med.mui.ac.ir

ABSTRACT

Leishmaniasis is a vector-borne disease caused by species of the genus Leishmania and is transmitted from host to host through the bite of an infected sandfly. MicroRNAs (miRNAs) are non-coding small RNAs with 22-nucleotide length. They are involved in some biological and cellular processes. We aimed to evaluate the expression of let-7a in human macrophages miRNA when are infected by Leishmania major. We also evaluated the impact of Leishmania major infection on the expression of let-7a at two different times, 24 and 48 hours, after infection. Blood samples were collected from ten healthy volunteers with no history of leishmaniasis. Development of macrophages from peripheral monocytes and infection with stationary phase of Leishmania major promastigotes were done through serial cultures under 5% CO₂ environment and 37°C. To measure the expression levels of let-7a real-time PCR was performed with specific related primers using the SYBR® Green master mix Kit™. The real-time PCR showed let-7a was expressed in cells infected with parasites after 24 and 48h post-infection. Comparison of let-7a miRNA expression after 24 and 48 h revealed that let-7a miRNAs were down-regulated at 48 h post-infection more than 24h after infection. The results of this study suggest that according to the main function of miRNA in repression of mRNA translation it could be possible to manipulate host cells in order to alter miRNA levels and regulate macrophage functions after establishment of intracellular parasites such as Leishmania.

Keywords: microRNA, let-7a, *Leishmania major*, macrophage

INTRODUCTION

Leishmaniasis is caused by intracellular protozoan parasites of the genus *Leishmania* that is distributed in tropical and subtropical regions of the world. Cutaneous, mucocutaneous, and visceral leishmaniasis are three main forms of the disease [1]. These neglected diseases are caused by the bites of sandfly vectors and are important for public health. Promastigotes are phagocytized by vertebrate host cells, routinely macrophages (MP), and dendritic cells and are then proliferated in parasitophorous vacuoles as amastigotes [2]. The outcome of infection depends on parasite's

ability to suppress or evade the host immune response and also the host's ability to activate macrophage killing implicated in infection [3]. Currently, chemotherapy is considered as the most original and most effective way to treat the disease. However, there is no fully effective drug available for the treatment.

MicroRNAs (miRNAs) are short, single-stranded RNA molecules containing approximately 22 nucleotides in length. Initially miRNAs were identified by studying the *Caenorhabditis elegans*, a free-living transparent nematode, which lives in temperate soil environments [4]. miRNAs affect diverse biological processes, including the cell cycle, proliferation, differentiation, growth and development, metabolism, aging, apoptosis, gene expression, and immune regulation [5, 6]. Early functions of miRNAs indicate that they regulate some kinds of physiological and pathological processes in parasites [4].

The let-7 miRNA family is functionally conserved from worms to humans [7]. Let-7 miRNA family is related to acute innate immune response, cell differentiation, and development [8]. In some studies it is reported that let-7 targets toll like receptor 4 (TLR4) and in human cholangiocytes, it is decreased when they are treated with either LPS (lipopolysaccharide) or when they are infected by a lumen dweller organism of human and some warm blooded animals, *Cryptosporidium parvum*. There is a hypothesis that repression of let-7 during infection assist innate immune responses [9]. The other organism is *Toxoplasma gondii*, an obligate intracellular parasite, which can interchange the miRNA of infected host cells [10].

Leishmania major survives in the phagolysosome compartment of host cells, which is a safe environment far from killing mechanisms of immune system. During entrance of the parasite into macrophages some interactions may occur including internal alterations such as miRNA expression to regulate macrophage function [11, 12]. Micro-RNA changes can be important for the diagnosis and treatment of parasitic diseases [13]. In recent years many cases of drug resistant leishmaniasis have been reported. An effective therapeutic approach to cure the disease with the least side effects is an emergency need for clinicians in endemic areas of leishmaniasis [14]. To achieve this goal, an appropriate interference in immune and macrophages systems is among the most important measures.

In this study, we aimed to quantify let-7a expression in 10 healthy volunteers twice after cell infection (24 and 48 hours) and then the amount of its expression at the two different times were analyzed using appropriate statistical tests.

MATERIALS AND METHODS

Monocyte-derived Macrophage Generation

Human blood samples were treated with an anticoagulant heparin taken from ten healthy volunteers with no history of leishmaniasis. Blood samples were diluted with Phosphate Buffered Saline (PBS) and then added Lymphodex (Inno-Train, Germany). After Centrifugation at 750xg for 20 minutes at 20°C in a swinging-bucket rotor without brake, the interphase (Peripheral blood mononuclear (PBMCs)) cells were isolated, washed twice by PBS and subsequent incubated at 10^7 cells/ml in RPMI1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine at 37°C and at a concentration of 5% CO₂. After 8 days, monocyte-derived macrophages (MDM) were produced.

Parasites and infections

All infections were induced with *L. major* (MRHO/IR/75/ER). Promastigote parasites were cultured at 25° C without CO₂ in complete RPMI 1640 medium (20% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine). Parasites from stationary phase cultures of approximately 8 days old were collected by centrifugation and were washed three times by PBS. Macrophages were infected with metacyclic *L. major* promastigotes from stationary phase at a ratio of 10 parasites per cell.

RNA isolation

For expression of miR let-7a, RNA was extracted from control uninfected macrophages, or macrophages infected by promastigotes of stationary phase for 24 and 48 hours. RNA was extracted using miRCURY RNA Isolation Kit™ (product No: 300110; Exiqon, Denmark). RNA extraction was performed on each sample to identify the expression of miR let-7a miRNAs upon infection compared with the uninfected samples. Briefly 600 µL of lysis solution was added directly to culture plate. Then 200 µL of 100% ethanol was added to the lysate and mixed by vortexing for 10 seconds. In the final step, lysate was transferred to a microcentrifuge tube and centrifuged for 1 min on 14000g. The

RNA was washed using washer solution and was eluted with the elution buffer. RNAs were quantified by a Nanodrop (WPA Biowave II, USA).

Reverse transcriptase microRNA real time polymerase chain reaction

cDNA was synthesized with a Universal cDNA Synthesis Kit™ according to the manufacturer's instructions (Exiqon, Denmark). For Real-Time PCR was used SYBR® Green master mix Kit™ (Exiqon, Denmark) and specific miR let-7a primers. Synthetic RNA spike-in templates and its primer (Exiqon, Denmark) were used for RT-PCR internal control. For RT-PCR experiments was used ABI Step One Plus device (ABI, USA) and the result were calculated with $\Delta\Delta C_t$ method.

Statistical analysis

All the experiments were carried out in triplicate. The statistical analysis was done using SPSS software (IBM), version 20 Kolmogorov-Smirnov and paired *t* tests were used as appropriated.

RESULTS

Macrophage differentiation

To identify let-7a for which expression is altered upon *L. major* infection of human macrophages, blood samples were collected from 10 healthy donors. Transformation of the monocytes to macrophages take place after 8-day culture (Figure 1).



Figure 1: Differentiated macrophages from monocytes in 8-day culture

Parasites and infections

Giemsa staining of coverslips containing attached cultured macrophages that had been put at the bottom of flasks before cell culture and direct microscopy revealed 70% of the cells were infected by promastigotes of *L. major* (Figure 2).

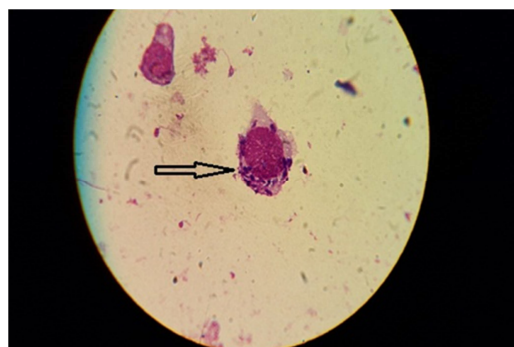


Figure 2: Photograph of MDM cells infected by metacyclic *L. major*

miRNA expression during *L. major* infection

Let-7a expression was significantly altered in infected compared with uninfected control macrophages. Figure 3 indicates that the mean up- or down-regulation of let-7a expression is different for each time point.

48 hours after infection of MP cells, mean let-7a expression was more down-regulated compared with the mean let-7a expression at 24 h post-infection (figure 3). However, in all two time points, the mean expression of let-7a was considerably lower in the tests group compared with the control group ($P < 0.001$). Paired t test showed that there was statistically no significant difference between the two groups between 24 and 48 hours ($P < 0.001$). Kolmogorov-Smirnov test was used to assess the data normality. The collected data followed a normal distribution.

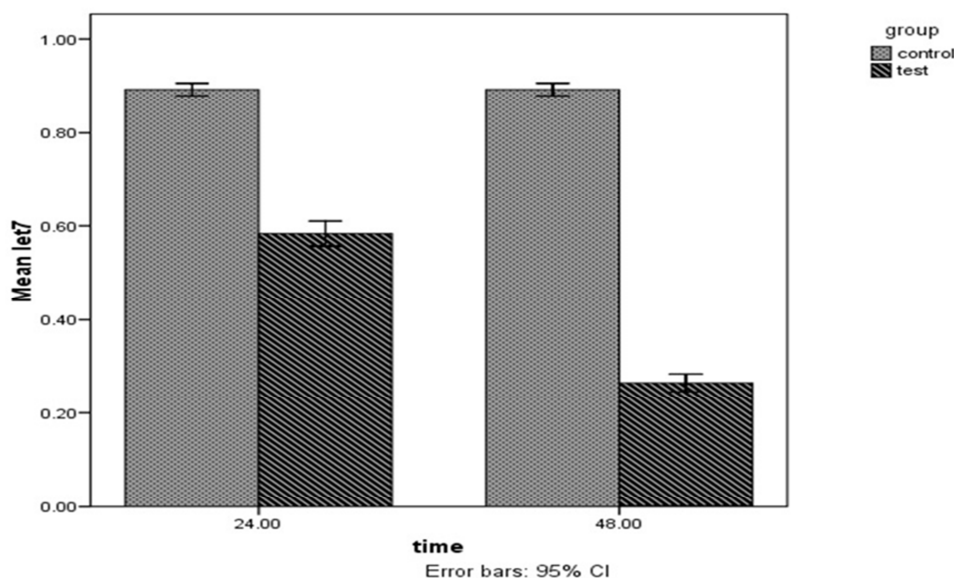


Figure 3: Assessment of the Let-7a level by real time PCR, 24 and 48 hours after transfection compared with the control group (uninfected MDM). The $\Delta\Delta Ct$ method was used for data analysis. In all two time points, the mean expression of let-7a was considerably lower in the tests group compared with the control group ($P < 0.001$)

DISCUSSION

To understand the pathogenesis of parasitic diseases at molecular level, identification of related miRNAs is one of the main strategies. miRNAs are among factors that have recently been recognized and have a very important role in biological processes, immunity regulation, and host-pathogen interactions [13, 15]. We studied the changes at the level of let-7a expression in human monocyte derived macrophages infected by *Leishmania major*. Fluctuations of miRNA 24 and 48 hours after infection with metacyclic promastigotes were studied to distinguish early parasite-host cell responses. Our hypothesis was that parasites capable of maintaining an infection in the host would induce let-7a within the macrophages they inhabit.

In this study data showed that infected macrophages with *L. major* resulted in diminished expression of let-7a which is time dependent and this reduction is more noticeable with time progress (Comparison between 24 and 48 hours). The mean let-7a expression 48 h after infection was down-regulated and the mean let-7a expression in uninfected cells (control) was higher than the infected cells.

Changes in macrophage expression miRNA during infection with *Leishmania* can have impression on parasite survival and dissemination within the host. Changes in expression patterns provide insight into the host-parasite interaction. Previous studies have reported that important changes in macrophage gene expression can be caused after infection with *Leishmania* [16].

Approximately 1300 miRNA are recognized in parasites and their number is still increasing. Having considered the life cycles of parasites with several developmental stages in vertebrate and invertebrate hosts and a unique collection

of genes expressed at different developmental stages, it is particularly important to elucidate the roles of miRNAs in the growth and development of parasites and their abilities to regulate infection of mammalian hosts[4].

Geraci and colleagues showed that cells infected by *L. major* downregulate let-7a and only dendritic cells and MPs infected by *L. donovani* upregulate those miRNAs [17]. In our study, RT-PCR confirmed that let-7a expression 48 h after infection was more down-regulated than let-7 expression after 24 h. Targeting transcripts of genes by let-7 family is responsible for the induction of inflammation as feedback inhibitors during bacterial infections[18]. Because of this process, cells infected by *L. major* present inflammatory markers such as IL-12 at maximum levels [19, 20]. So it can be concluded that micro-RNA let-7a expression may be related to the mechanisms of inflammation.

Lemaire and co-workers reported that the activity of macrophages infected by *L. major* was regulated by changes in miRNA levels. Let-7a, miR9, miR132, miR-146a, miR-155, and miR-187 are among microRNAs that were up-regulated 3 and 6 hours after infection with *L. major*[11]. In our study, Let-7a down-regulated 24 and 48 hours after infection with *L. major*. Research on miRNA may lead to discover new biomarkers related to disease progression. Such studies may reveal the roles of miRNAs in pathogenesis and may help in the discovery of new therapeutic targets.

Erik Bruce Wendlandt reported in logarithmic phase of promastigote multiplication five miRNAs of the eight (miR-200b, let-7b, miR-339-5p, miR-423-5p, miR-744) significantly altered in infected macrophages whereas only three miRNAs (miR-195, miR-324-3p, miR-708) were identified in MDMs infected with metacyclic promastigotes[1]. Significant changes were seen in selected macrophage transcript abundance when they infect with the protozoan parasite *Leishmania major*. It can be interpreted that these alterations are defense mechanisms which induced by the macrophages as an antimicrobial pathway, or changes could represent a route of evasion of intracellular organisms from internal killing mechanisms.

Finally, it is suggested that more studies on candidate miRNA in cell infections of *Leishmania* model be performed to ensure the design of proper control strategy for this important parasitic disease.

CONCLUSION

According to the main function of miRNA in repression of mRNA translation, it could be possible to manipulate host cells in order to alter miRNA levels and to regulate macrophage functions after establishment of intracellular parasites such as *Leishmania*.

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