BMC Microbiology

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# The expression profile of inflammatory microRNAs in *Leishmania major* infected human macrophages; mining the effects of *Leishmania* RNA virus

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## Abstract

**Background** Leishmaniasis is a disease caused by the *Leishmania* parasite. Recent studies suggest a critical role for double-stranded RNA virus (LRV) in the disease outcome. MicroRNAs (miRs) are small, non-coding RNA molecules that may also contribute to the outcome of infection and the pattern of disease. This study aimed to investigate the influence of *L. major* infected with LRV2 + on the expression profile of microRNAs compared to LRV2-.

**Methods** Samples were collected from cutaneous leishmaniasis (CL) patients in a leishmaniasis-endemic area of Iran. *Leishmania* species were determined using PCR (kDNA gene), and the presence of LRV2 was identified with semi-nested PCR (RdRp gene). The expression of miRs (miR-155, miR-146b, and miR-133a) was determined through quantitative real-time PCR analysis in human monocytes cell line (THP-1) infected with both LRV2 + and LRV2- isolates of *L. major* during 0, 12, 24, and 36 h post-co-infection.

**Results** The expression of miR-155 showed a significant decrease in the LRV2 + isolate compared to LRV2- at zero hours, but exhibited upregulation at 12, 24, and 36 h post-infection for both LRV2 + and LRV2- isolates compared to the control group. The expression of miR-146b was upregulated in both LRV2 + and LRV2- isolates compared to the control group at zero, 24, and 36 h post-infection. Conversely, miR-133a showed significant increases at zero and 12 h in both LRV2 + and LRV2- isolates compared to the control group, but it was downregulated in LRV2 + at 24 and 36 h compared to LRV2-.

**Conclusion** In this study, significant differences were observed in the expression profiles of miR-155, miR-146b, and miR-133a about the presence of LRV2. Our data suggest a potential determinative role for these miRs in the pathogenesis of CL.

**Keywords** *Leishmania major*, *Leishmania* RNA virus 2, miR-155, miR-146b, miR-133a, THP-1

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## Introduction

Leishmaniasis is a widespread neglected tropical disease (NTD) caused by *Leishmania* species, transmitted to mammals through the bites of female sandflies [1, 2]. According to the latest reports, leishmaniasis is responsible for 0.7-1 million new cases per year and endemic in over 98 countries [3]. Depending on the host and *Leishmania* species, the disease manifests in various forms, including cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL) [4]. The innate immune response plays a crucial role in the early defense against *Leishmania* infection. In this regard, neutrophils, dendritic cells, and macrophages are the most involved immune cells [5]. The immune responses are instrumental in limiting disease progression, and susceptibility or resistance to CL is affected by T-cell responses and the type of response, impacting ulcer development [6–8]. However, the parasite can subvert the immune responses and metabolic pathways, enabling its replication and survival within the phagolysosomal environment [9]. Recent studies indicate that, in addition to the *Leishmania* parasite species and the host's immune system response, the presence of a double-stranded RNA virus may also play a significant role in the outcome of the disease [10–12]. *Leishmania* RNA Virus (LRV) is a double-stranded RNA virus classified within the Totiviridae family. LRV1 is found in *Leishmania* species from the *Viannia* subgenus in the New World, while LRV2 is found in *Leishmania* species from the *Leishmania* subgenus in the Old World. Both LRV1 and LRV2 exhibit differences in their genome sequences and structure, reflecting their adaptation to distinct *Leishmania* hosts. LRV1 is known to exacerbate the immune response, it leads to severe inflammation and chronic MCL. LRV1 triggers a strong pro-inflammatory response through activation of Toll-like receptor 3 (TLR3). LRV2 less is known about its role in pathogenicity, but it may also influence the host's immune response in a manner specific to the associated *Leishmania* species. LRVs have been shown to facilitate immune evasion by *Leishmania* parasites and modulate the production of pro-inflammatory cytokines, ultimately contributing to the parasite's survival and the progression of the disease [13–15].

MicroRNAs (miRs) are small non-coding RNA molecules that play a significant role in immune responses and are differentially expressed in various cell types, including macrophages, neutrophils, and lymphocytes [16–19]. miRs can contribute to macrophage activation, the miRs can regulate either positively or negatively cell recruitment, not only positively regulating processes like phagocytosis and apoptosis [20, 21]. The relationship between LRV and miRs during leishmaniasis is an emerging area of research. Although direct evidence linking LRV and miR expression is limited, studies suggest that both LRV and miRs play crucial roles in modulating host-parasite interactions and the immune response during *Leishmania* infections. Their combined influence on the host immune response and *Leishmania* survival is increasingly evident [18, 22]. The outcome of CL is determined by the complex interaction between *Leishmania* and host immune responses, which are regulated by a delicate balance of pro-inflammatory and anti-inflammatory signals. Recent studies showed that LRV increases the expression of inflammatory and pathogenesis factors and treatment failure [8, 10, 11, 14, 22, 23]. In the present study, based on previous research and the roles of specific microRNAs and *Leishmania* parasites, certain effective microRNAs (mir-155, mir-146b, and mir-133a) were selected [18, 24] and their expression levels were examined not only in *L. major* parasites but also with a preliminary investigation into how their expression levels respond to LRV2. To date, there have been a few reports elucidating the role of LRVs on miRs involved in the inflammatory response during infection by *Leishmania* parasites [24]. The aim of this study is to investigate any potential correlation between the expression of the specified miR-155, miR-146b, and miR-133a of *L. major* infected with LRV2 + compared to LRV2- isolates.

## Materials and methods

### Sample collection and culture

*Leishmania* isolates were obtained from CL patients referred to referral health centers of Golestan province between August 2022 and May 2022. These patients were diagnosed based on parasitological methods. Suspected CL lesions were scraped using a sterile scalpel, and the exudate materials were stained with Giemsa 10% and examined under a light microscope at X1000 magnification. The scraped materials were cultured in a two phasic NNN (Novy-MacNeal-Nicolle) medium containing RPMI-1640 medium [Gibco, Germany] supplemented with 10% fetal bovine serum (FBS) (Gibco, Germany), penicillin (100 U/mL), and streptomycin (100 µg/mL) (Sigma-Aldrich, St. Louis, USA). The culture media were incubated at 24–26 °C. After 6–8 days, the promastigotes were sub-cultured and incubated at 24–26 °C in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin for 6–7 days.

### *Leishmania* identification and LRV2 detection

DNA extraction was performed following the protocol of a commercial total DNA extraction kit (YTZ, Favorgen, Taiwan). For PCR amplification, we utilized the Forward primer (LIN4, 5' GGGGTTGGTGTAAAATAGGG 3') and Reverse primer (LIN17, 5' TTTGAACGGGATTT CTG 3') to amplify identical 680 to 720-bp fragments of the kinetoplast (kDNA) gene in *Leishmania* isolates [10].

Total RNA was extracted from $1 \times 10^{10}$ promastigotes according to the manufacturer's protocol (YTZ, Favorgen, Taiwan). The purity of the extracted RNA was assessed by a NanoDrop spectrophotometer at 260 nm (Thermo Scientific™ NanoDrop™ One Microvolume UV–Vis). Complementary DNA (cDNA) was synthesized from 250 ng of total RNA following the manufacturer's instructions (YTZ, Favorgen, Taiwan). The amplified cDNA was stored at -20 °C until it was used for semi-nested PCR. The presence of LRV2 was confirmed using semi-nested PCR methods, as previously described by Hajjaran et al. [25]. in 2016.

#### Cell culture

THP-1 cells were cultured in sterile 25 $cm^2$ culture flasks (SPL, Korea) using complete medium, containing RPMI 1640 medium (Gibco, Germany) supplemented with 10% FBS, and 1% penicillin-streptomycin. The cells were maintained at 37˚C with 5% $CO_2$ and 95% humidity. To sustain cell viability and promote optimal growth, the culture medium was refreshed every three days. The cell viability was evaluated via trypan blue staining to ensure the healthy and viability of cells in the experiments.

#### Parasites

The promastigotes of the *L. major* parasites were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% Penicillin-Streptomycin. Cultures were maintained at a temperature of 24–26 °C with a pH of 7.2 and sub-cultured every 5–6 days. After 8 days of culture, when the promastigotes reached the stationary phase, they were used in the experiments.

#### THP-1 activation and co-infection with *L. major* strains

For the infection experiments, THP-1 cells were plated in 6-well culture plates at a concentration of $5 \times 10^5$ cells/mL in complete medium. Prior to infection, the cells were treated with 50 ng/mL of 4α-phorbol 12-myristate 13-acetate (PMA) (Sigma, USA) to stimulate their differentiation into macrophage-like cells and augment their phagocytic capabilities. Subsequently, the plates were incubated for 48 h at 37 °C with 5% $CO_2$. After the differentiation period, the wells were washed twice with a culture medium to remove non-adherent cells.

Promastigote suspensions, with a multiplicity of infections of three parasites per cell, were prepared. The suspensions were centrifuged at 2500 rpm for 10 min, and the resulting promastigotes were added to each well. The culture plates were incubated at 37 °C and 5% $CO_2$ for parasite internalization. After 6 h of incubation, the wells were washed with culture medium to remove non-internalized parasites. Finally, 2 mL of fresh complete medium was added to each well. The plates were incubated at 37 °C with 5% $CO_2$. The cells in the 6-well plates were prepared in duplicates, and uninfected cells were as controls.

**Table 1** Primer sequences used to detect MiR by qreal-time PCR

| Gene name | Primers | Sequence 5′-3′ |
|---|---|---|
| hsa-146b-5p | Real-time | ATATAGCTACTTGGTGAGAACTGAATTCC |
| | RT | CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGCAGCCTAT |
| hsa-133a-3p | Real-time | ATTCTATAGTTATGTTTGGTCCCCTTCA |
| | RT | CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGCAGCTGGT |
| hsa-155-5p | Real-time | ACTCTCCTACTGGGTTAATGCTAATCGTGA |
| | RT | CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGAACCCCTA |
| Universal reverse | Real-time | TGGTGTCGTGGAGTCGGCAATTCAGTTG |
| U6 | Real-time | ACACTCCATCTGGGTCGTGAAGCGTTC |
| | RT | CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGAAAAATATG |

#### RNA extraction cDNA synthesis

After 6 h from the initial infection (zero h), the plates were washed, and RNA was extracted at zero, 12, 24, and 36 h post-infection using total microRNA Purification Kit (YTA, favorgen, Taiwan), following the manufacturer's instructions. The extracted RNA was then quantified and assessed for purity using a NanoDrop spectrophotometer at 260 nm (NanoDrop™, One Microvolume UV–Vis). To synthesize complementary DNA (cDNA), 250 ng of total microRNA was used in each reaction. The cDNA synthesis was performed according stem-loop protocol, as previously described [26, 27]. The resulting amplified cDNA was stored at -20 °C.

#### Quantitative real-time PCR

The expression levels of miR-155, miR-146b, and miR-133a were monitored using qreal-time PCR in a Rotor-Gene Q (Qiagen, Germany). The primers used for amplification are listed in Table 1. The real-time PCR experiments were carried out in duplicates with a final volume of 15 µL containing 5 ρM of each miR-specific forward (miR-155, miR-146b, and miR-133a) and universal reverse primer, 7.5 µL of 2X SYBR green (Ampliqon, Denmark), 5.5 µL of distilled water (D.W.), and 1 µL of cDNA. The thermal protocol for the qPCR reactions consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min.

To normalize the data, small nucleolar RNAs (RNU6) were used as reference genes. The relative expression levels of the microRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method [28]. All experiments were performed in duplicate, and the data are reported as the mean ± standard deviations (SD). Statistical comparisons were performed

between LRV2 + and LRV2- of *L. major* isolates, using a significance level of 95% and a *P*-value threshold of < 0.05.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism v.10.2 software. The statistical analysis was ANOVA (analysis of variance) for repeated measurements followed by the Tukey test for post hoc comparisons between groups. The significance level was set at $p < 0.05$.

## Results

### *Leishmania* identification and LRV2 detection

This study selected two *Leishmania* isolates from CL patients based on the study's objectives. Both isolates were identified as *L. major*. The band sizes were 560 bp for *L. major* and 720 bp for *Leishmania tropica* (*L. tropica*), respectively. One isolate was LRV2-, while the other was LRV2 + using semi-nested PCR. (Table 2) (Supl Fig. 1). These isolates were subsequently used for the analysis of miR expression using RT-qPCR methods.

### Expression level of miR-155

The expression levels of miR-155 were evaluated at different time points (zero, 12, 24, and 36 h) following co-infection of macrophages with LRV2 + and LRV2- isolates of the parasite, compared to un-infected macrophages (control group). At zero h (6 h post-infection), a significant decrease in miR-155 expression was observed in the LRV2 + strain ($p < 0.0005$), while a significant increase was observed in the LRV2- strain ($p < 0.0001$), compared to the control group. At 12 and 24 h, both LRV2 + and LRV2- strains exhibited a significant increase in miR-155 expression compared to the control group ($p < 0.0001$). At the final time point (36 h), a similar approximately significant increase in miR-155 expression was observed in both LRV2 + and LRV2- isolates (Fig. 1).

### Expression level of miR-146b

The results obtained for miR-146b at 0 h showed a significant increase in expression in the LRV2- and LRV2 + isolates compared to the control group ($p < 0.05$). At 12 h post-infection, the expression level of miR-146b significantly decreased in the LRV2 + isolate compared to the control group ($p < 0.0001$). Conversely, there was no significant change in expression observed in the LRV2- isolate compared to the control group. At 24 h, both the LRV2 + and LRV2- isolates exhibited an increase in miR-146b expression compared to the control group, although the significance level was not specified. At the final time point (36 h), a significant decrease in miR-146b expression was observed in both the LRV2 + and LRV2- isolates compared to the control group ($p < 0.0001$) (Fig. 2).

**Table 2** Characteristics of isolates from CL patients

| Isolate code | Source of isolate | Nomenclature codes | Leishmania species | LRV2 status |
|---|---|---|---|---|
| LRV2- | Clinical | MHOM/IR/21/Leish.z.1 | *L. major* | LRV2- |
| LRV2+ | Clinical | MHOM/IR/21/Leish.z.2 | *L. major* | LRV2+ (ACC number: OR493488) |

### Expression level of miR-133a

The analysis of miR-133a expression at various time points yielded noteworthy results. At zero h post-infection, both the LRV2 + isolate ($p < 0.05$) and the LRV2- isolate ($p < 0.0001$) exhibited a significant increase in miR-133a expression when compared to the control group. Similarly, at 12 h post-infection, both the LRV2 + and LRV2- isolates demonstrated a significant increase in miR-133a expression compared to the control group. However, at the mid-time point of 24 h post-infection, both the LRV2 + and LRV2- isolates displayed a significant and nearly equal decrease in miR-133a expression in comparison to the control group. Notably, the expression level continued to show a significant decrease in both the LRV2 + and LRV2- isolates when compared to the control group (Fig. 3).

## Discussion

Macrophages play a dual role in cutaneous leishmaniasis (CL), acting as both host cells and primary effectors against the parasites. Macrophages create a favor environment for *Leishmania* survival, but paradoxically, they can also eliminate the parasites through various mechanisms such as phagocytosis and the production of reactive oxygen species [29]. *Leishmania* has developed strategies to evade the host immune response, including the modulation of miR expression [16]. However, few studies have investigated the role of LRV in the expression of miRs [24]. Based on previous research and the significant importance of microRNAs, mir-155, mir-146b, and mir-133a were selected. Their expression levels were examined, particularly in relation to the presence of LRV2+ [18, 30–32].

It was suggested that miR-155 plays a significant role in controlling infections, including parasitic infections like *Leishmania*, as well as those caused by DNA viruses, where it can modulate the host immune response and potentially facilitate viral replication [33, 34]. In a study conducted by Eren et al. [24], the impact of LRV1 infection on the expression of miR-155 in macrophages within *L. guyanensis* was evaluated that the results suggested an effectively role of LRV1 on TLR3/miR-155/Akt signaling axis to enhance macrophage survival and facilitate the persistence of intracellular *Leishmania* parasites. Other studies have reported an upregulation of miR-155

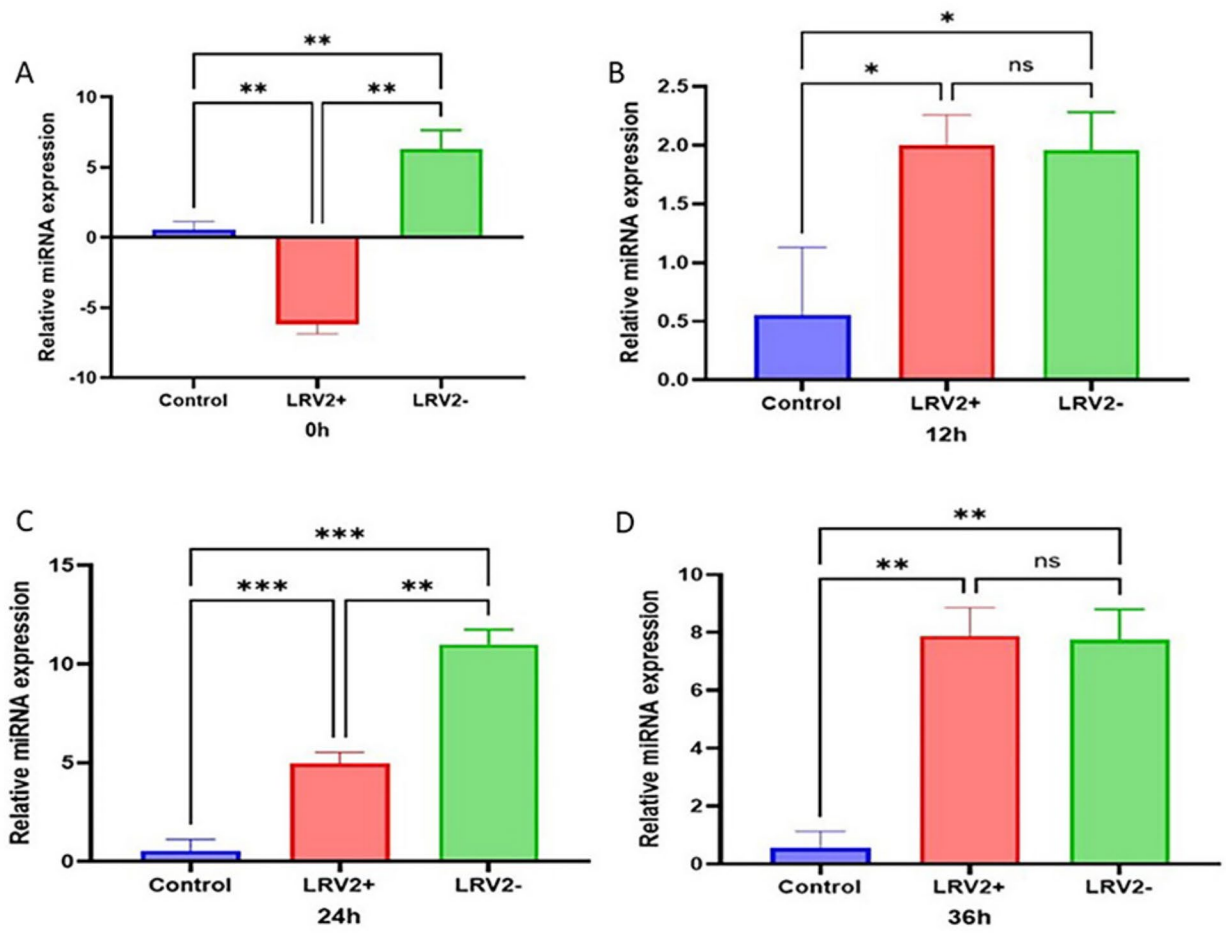


**Fig. 1** miR expression of miR-155 in LRV2 + and LRV2- isolates compared to control [(un-infected macrophage) at different times (**A**: Zero, **B**: 12, **C**: 24, and **D**: 36 h) of co-infection. THP-1 cells were plated in 6-well culture plates at a concentration of $5 \times 10^5$ cells/mL in complete medium, stimulated with PMA for macrophage differentiation, and infected with *Leishmania* Data analysis was done using two-way ANOVA for repeated measurements followed by the Tukey test. Bars represent mean ± SD. * Symbol represents the meaningful difference between groups. (ns: not significant, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$)

expression during *L. major* infection, providing valuable insights into the complex interplay between miR-155 and *Leishmania* infection, shedding light on the intricate mechanisms employed by the parasites to establish and maintain infection within the host [35–37]. Elevated levels of miR-155 can influence *Leishmania* infection outcomes by promoting Th1-type immune responses, crucial for parasite clearance, though its overexpression may also cause excessive inflammation and chronic disease. In a study, miR-155-deficient mice exhibited reduced Th2 responses and enhanced Th1 immunity, resolving *L. major* infection faster [41–43]. This highlights miR-155's role in susceptibility to CL by promoting Th2 responses and suppressing protective Th1 immunity. Interestingly, miR-155's role differs in VL caused by *L. donovani*, where it supports host immunity without being critical for infection resolution [5, 44, 45]. Immune mechanisms differ between *L. major* and *L. donovani*, as Th2 cytokines (IL-4, IL-13) worsen CL but protect in VL, while IL-17 enhances immunity to *L. major* but increases susceptibility to *L. donovani*. miR-155 also promotes Th1 responses and IFN-γ production while modulating Th2 cytokines IL-5 and IL-13, underscoring its dual role in immune regulation [46–49]. All of these studies support our results indicating changes in the expression levels of miR-155 during infection with *L. major* isolates, particularly with the presence or absence of LRV2. During the early hours, we noted a reduction in miR-155 expression in the LRV2 + isolate, while the LRV2- isolate exhibited an increase compared to the control group. However, as the infection progressed, both the LRV2 + and LRV2- isolates displayed a significant upregulation of miR-155 in the middle and final hours. Notably, there is a scarcity of previous reports examining the association between miR-155 expression, LRV + status, and *L. major* infection [24]. Therefore, based on our findings, we put forth

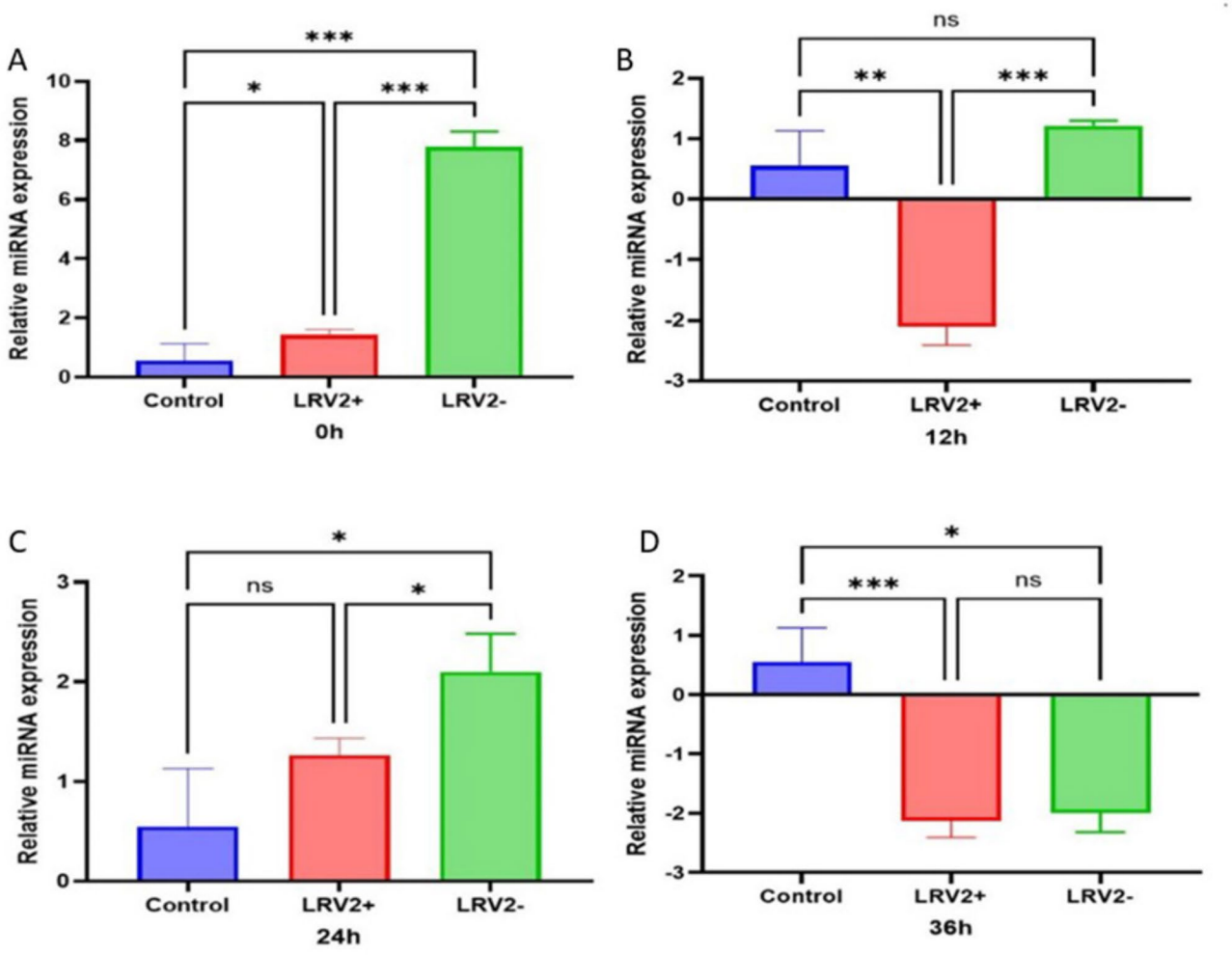


**Fig. 2** miR expression of miR-146b in LRV2 + and LRV2- isolates compared to control (un-infected macrophage) at different times (**A**: Zero, **B**: 12, **C**: 24, and **D**: 36 h) of co-infection. THP-1 cells were plated in 6-well culture plates at a concentration of $5 \times 10^5$ cells/mL in complete medium, stimulated with PMA for macrophage differentiation, and infected with *Leishmania* Data analysis was done using two-way ANOVA for repeated measurements followed by the Tukey test. Bars represent mean ± SD. * Symbol represents the meaningful difference between groups. (ns: not significant, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$)

the hypothesis that miR-155 levels are elevated during *L. major* infection, shedding light on a novel aspect of the interplay between miR-155 and LRV2 + isolate in the context of *Leishmania* infection.

Most studies on miR-146 have focused on its role in inflammation, identifying this miR as one that is notably induced in response to cytokines and pathogenic components in macrophages and dendritic cells [38, 39]. Our study revealed that miR-146b exhibited upregulation patterns in both LRV2 + and LRV2 + during different time points of infection. This regulation might indicate a parasite evasion mechanism from the immune system. Supporting our findings, Mendoca et al. [18], reported a significant upregulation of miR-146b expression in patients with CL, which was associated with inflammasome expression. Lemaire et al. [36], observed an up regulation of miR-146 in the early hours (3 h) after infection in human macrophages, while another study reported a down-regulation after 24 h of infection in human dendritic cells [36, 40]. These studies are supported our results suggesting elevated levels of mir-146 at the first time points, while it was downregulated at 36 h. Increased miR-146 expression has also been observed in infections caused by other protozoa than *Leishmania* such as *Trypanosoma cruzi*, *Toxoplasma gondii*, *Cryptosporidium*, and *Plasmodium* [41–44]. Based on these findings, we hypothesize that *L. major* parasites may alter the expression of miR-146b in infected macrophages during the initial and middle hours after infection, potentially indicating a high division rate of the parasites.

miR-133 is widely recognized as one of the most extensively studied myo-miRs, predominantly expressed in muscle tissue, and it plays a crucial role in the functioning and development of both skeletal and cardiac muscles

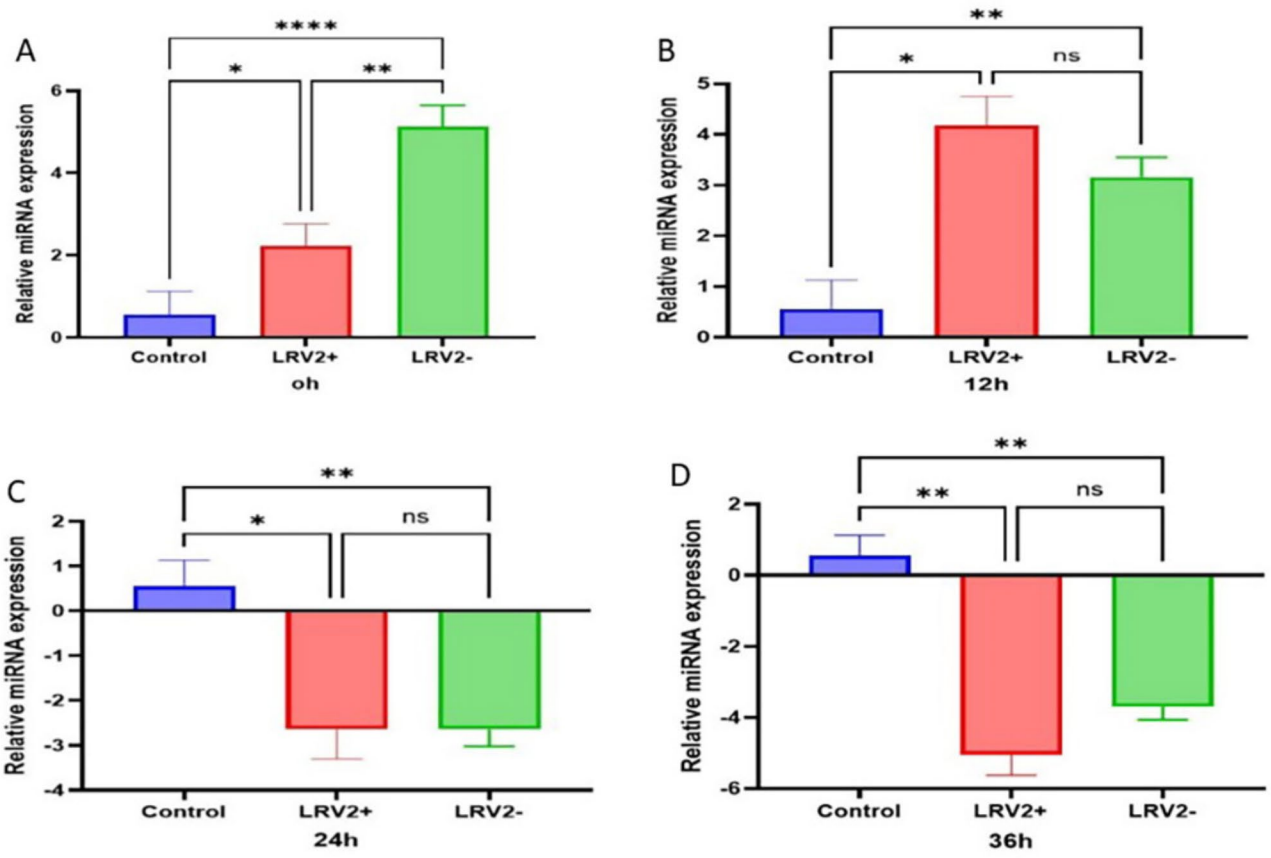


**Fig. 3** miR expression of miR-133a in LRV2 + and LRV2- isolates compared to control (un-infected macrophage) at different times (**A**: Zero, **B**: 12, **C**: 24, and **D**: 36 h) of co-infection. THP-1 cells were plated in 6-well culture plates at a concentration of $5 \times 10^5$ cells/mL in complete medium, stimulated with PMA for macrophage differentiation, and infected with *Leishmania* Data analysis was done using two-way ANOVA for repeated measurements followed by the Tukey test. Bars represent mean ± SD. * Symbol represents the meaningful difference between groups. (ns: not significant, [*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$)

[45]. Our results revealed an initial increase in miR-133a expression in both the LRV2 + and LRV2- isolates at zero and 12 h post-infection. However, at 24 h post-infection, there was a significant decrease in miR-133a expression in both isolates compared to the control group, which continued to be significant compared to the control group. In a study conducted by Mendonca et al. [18], they reported a significant increase in miR-133a expression in patients with CL. The role of miR-133a in regulating the UCP2 gene has important implications for the activation of inflammasomes, which are involved in the inflammatory response [46, 47]. These findings strongly suggest that miR-133a actively promotes the activation of inflammasomes. Understanding the intricate interplay between miR-133a, UCP2, and the regulation of inflammasome activation represents a complex and important aspect of modulating inflammatory responses and this understanding could potentially lead to the development of targeted therapies for inflammatory disorders.

A study examined the role of miR-133a in inflammasome activation and IL-1β production. miR-133a-1, initially characterized in mice, was investigated for its involvement in activating the inflammasome (NLRP3) and promoting IL-1β production. This microRNA is known to target mitochondrial uncoupling protein 2 (UCP2) [48]. UCP2 is an integral membrane protein and a member of the larger UCP family. It plays a role in ATP synthesis and the regulation of reactive oxygen species (ROS) production [49]. Previous research demonstrated that UCP2 negatively regulates ROS levels and induces an anti-inflammatory response. Additionally, UCP2 is a crucichondrial function in phagocytosis, helping to modulate immune responses [50].

Our results confirmed previous studies and the central role of mir-133a in regulation of inflammasome and production of IL-1β. Accordingly, *Leishmania* parasites, regardless the presence of LRV, seem to manipulate production of IL-1β at the beginning of the infection. These findings suggest a regulation of miR-133a expression during the course of infection and indicate its potential role in the immune response to leishmaniasis.

## Conclusion

Our study, along with previous investigations, emphasizes the significance of differentially expressed miR targets in critical pathways during *Leishmania* infection. However, in this study, we did not observe a significant difference in the expression levels of mir-155, mir-146b, and mir-133a between LRV2 + and LRV2- isolates. Therefore, the hypothesis that changes in the expression of specific microRNAs may play a role in the immune-genesis or pathogenesis of leishmaniasis requires further and more comprehensive investigation.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03901-z.

Supplementary Material 1

**Acknowledgements**

This research reported in this publication was supported by the Elite Research Grant Committee under award number (IR.NIMAD.REC.1399.233) from the National Institute for Medical Development (NIMAD), Tehran, Iran.

**Author contributions**

HH and MM designed the study. ZM contributed to manuscript writing, and HM participated in the revising of the manuscript. HMR and FG contributed to Real-Time PCR performing, prepared figures and analysis. All authors read and confirmed the final version of the manuscript.

**Funding**

This study was supported by the National Institute for Medical Development (NIMAD), Tehran, Iran.

**Data availability**

Generated data including figures and tables were not submitted elsewhere and are included in the article.

## Declarations

**Ethics approval and consent to participate**

This study was conducted in accordance with the ethical standards of the Declaration of Helsinki. This in vitro research was approved under award number (IR.NIMAD.REC.1399.233) from the National Institute for Medical Development (NIMAD), Tehran, Iran. Written informed consent was obtained from the patients. All methods were carried out in accordance with relevant regulations and guidelines.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no competing interests.

Received: 30 July 2024 / Accepted: 18 March 2025
Published online: 02 April 2025

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