Original Article

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TANAFFOS

Extracellular Vesicles from Serum of Mycobacteria Patients Accelerate Expression of Apoptosis miRNAs and Facilitate THP-1 Monocyte Cell Death

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Background: Extracellular vesicles **(**EVs) may accelerate cell death during the course of infection. Mycobacteria could invade the host's immune system and survive in the host by modulation of miRNAs. MiRNAs' differential expressions can serve as biomarkers. This study evaluates THP-1 monocyte cell death by EVs from serum of patients with mycobacteria and assesses serum-derived exosomal miRNAs to increase or decrease THP-1 monocyte cell death.

Materials and Methods: EVs were purified from serum of patients with mycobacteria and cultured with THP-1 monocyte. The cell death was determined via annexin V-FITC and PI staining. The microRNA was isolated from serum-derived EVs of the patients. Expression level of Hsa-miR-20a-5p, Hsa-miR-29a, Hsa-miR-let7e, and Hsa-miR-155 was assessed using qRT-PCR.

Results: Cell death was accelerated in 10 and 5 µg/ml concentrations of the EVs ($p<0.05$). Minimum cell death was seen in 2.5 and 1.2 µg/ml concentrations ($p<0.05$). In tuberculosis (TB) patients, expression of miR-20a-5p, miR-29a, and miR-let7e were significantly enhanced ($p\leq0.0001$), but miR-155 expression reduced. ROC analysis showed diagnostic biomarkers of miRNAs with an AUC=0.6933 for miR-20, AUC=0.6011 for miR-29a, AUC=0.7322 for miR-let7e, and AUC=0.7456 for miR-155 for active tuberculosis. Expression of miR-let7e, 20a, and 29a in *M. avium* vs. *M. tuberculosis* was overexpressed ($P\leq0.01$, $P\leq0.0001$, and $P\leq0.0001$, respectively). Also miRs let7e and 20a expression was accelerated in *M. abscessus* vs. *M. tuberculosis* ($P\leq0.0001$ and $P\leq0.002$, respectively).

Conclusion: EVs accelerates cell death and may not be ideally considered for drug delivery and vaccine developments. Circulating exosomal microRNA MiR-20, miR-let7e, and miR-155 facilitate development of potential biomarkers of pulmonary tuberculosis and non-tuberculosis.

Keywords: Extracellular vesicles (EVs); MicroRNAs; Mycobacteria; Cell death; THP-1 monocyte

INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis (Mtb)*, that has become a global health problem and a reason of morbidity among millions of people per year. The latest updates show that approximately 7.1 million people have been infected with *Mtb* in 2019 and the main reason of 1.4 million deaths every year (1). Other mycobacteria *(Non-tuberculous mycobacteria, NTM)* have been identified as a cause of acute pulmonary diseases in many geographical areas. *NTMs*

may also introduce a greater disease burden than tuberculosis. Although up to 150 species of *NTM* have been found, pulmonary infections are mainly due to *Mycobacterium avium complex (MAC)*, *Mycobacterium kansasii*, and *Mycobacterium abscessus* (2, 3). Clinical findings including radiological evidences and laboratory diagnosis such as culturing on solid media and broth are required to identify these species. Both early diagnosis of mycobacteria and differentiation of *NTM* from *Mtb* play critical roles in controlling the disease. But, due to the lack of optimal and cost-effective diagnosis techniques, determining novel biomarkers using miRNAs expression level by circulating extracellular vesicle (EVs) might be highly valuable (4).

Exosomes as extracellular vesicle (EVs) are membrane-bound extracellular vesicles of typically 30–150 nm in size that are produced from most eukaryotic cell types and formed by the fusion of multivesicular bodies (MVB) with the cell plasma membrane (5, 6). Exosomes facilitate cell-cell communication by shuttling cellular information about a disease to recipient cells in the form of basic molecules such as ribonucleic acid (RNA) cells. Pioneering reports also found RNA contents in exosomes and that genetic information could be transferred from donor to recipient cells (7). The stability of the RNAs is in maximum value and highly protected from enzymic degradation in body fluids (8). MiRNAs shuttled by exosomes regulate level of gene expression and cell function both in vivo and in vitro. MiRNAs are small 18–22 nucleotide noncoding RNAs involved in post-transcriptional regulation (9). In *Mtb*, most immune responses including cytokine production, autophagy, apoptosis, MHC class II expression and phagolysosome maturation in macrophages and dendritic cells of eukaryotic cells are regulated by miRNAs (10). Modulation and subversion of miRNAs expression facilitates these biological processes and is considered as a key mechanism implemented by bacterial pathogens to survive and persist within the host cells (11).

A report by Singh and colleagues has found signature of host derived miRNAs, mRNA transcripts and mycobacteria RNAs in exosomes released from macrophages infected with *Mtb* (12). Analysis of differences in exosome content of healthy and patient's samples introduce exosomes as biomarker candidates. Exosome is recently considering as a biomarker for diagnosis of pathogenic particles(5). Exosome concentration in serum of infected mice with *BCG* or *M. tuberculosis* was significantly higher than healthy subjects, confirming the role of exosomes as a biomarker. Another report showed proteomic evaluation of highly antigenic proteins of exosomes from *Mtb*-infected and culture filtrate protein-treated macrophages (13). Approximately 29 types of *Mtb* proteins in exosomes released from CFP-treated J774 cells were found; the most detected in exosomes were only isolated from *M. tuberculosis*-infected cells confirming that exosomes released from *Mtb* antigens may be an optimal method to prepare a novel tuberculosis vaccine (14).

MiRNAs regulate cell death signaling pathways in host cells infected with *Mtb*. Recent studies report that after macrophage cell infection by mycobacterium, miRNA regulates JNK-2 signaling in human macrophage that participates in a negative regulation of apoptosis pathway. For example, upregulation of miR-20a-5p in mycobacterial infection resulted to survive Mtb within the macrophages (15). A report also shows that miR-20a-5p decreases its survival within macrophages. As a first report, the current study evaluates THP-1 monocyte cell death by exosomes released from serum of mycobacteria patients and exosomal miRNAs expression level including Hsa-miR-155, Hsa-miR-20a-5p, and Hsa-miR-29a, and Hsa-miR-let7e. Also, this study determines regulation of those exosomal apoptosis miRNAs that may affect on cell death status. Then we attempt to show accelerating or and decreasing cell death triggered by serum-derived exosome from Mtb and NTMs on monocyte cell that may change the strategies of exosomes as a developing tool for a novel vaccine or its biomarkers appliances.

MATERIALS AND METHODS

Patient characterizations

In the first step of the study, 6 patients diagnosed with *non-tuberculous mycobacteria* (NTM) species including *M. abscessus, M. Kansasi,* M. *Chelonae, M. Simiae, and M. avium* and *Mycobacterium tuberculosis* aged from 32 to 83 years were admitted at the Masih Daneshvari Hospital. Also, for microRNAs study, a total of 80 patients diagnosed with mycobacteria species including *M. abscessus, M. Kansasi, M. Chelonae, M. Simiae, and M. avium and M. tuberculosis* were recruited from Masih Daneshvari Hospital, a tuberculosis and lung disease center in Tehran, Iran, during 2018-2020. The patients were aged from 32 to 83 years old. According to the official ATS/IDSA statement, the diagnosis was based on chest radiographs and mycobacterial culture or positive bronchial washing and molecular diagnosis (16). Also, 80 healthy subjects aged from 30 to 80 were analysed for exosomal cell death miRNAs. For the patients, HIV and negative history of TB were evaluated and CRP, ESR, HIV were tested for the healthy subjects. An institutional ethical board from Masih Daneshvari Hospital, tuberculosis referral center, Tehran, Iran approved the current study (SBMU 1.REC1394.137).

Exosome isolation and characterization

Exosomes were isolated from serum samples of patients with mycobacteria using exoEasy Maxi Kit (Qiagen, Valencia, CA, USA, Cat. no. 76064) based on the manufacturer's instructions. Accordingly, buffer XBP with the final volume of 1000 µl was added to 1000 µl of the serum sample, mixed and left to warm up in room temperature. The mixture was then transferred to the exoEasy spin column and centrifuged at 500×g for 1 min followed by discarding flow-through and placing the column into the new collection tube. Only 10 ml buffer XWP was then added to the column and centrifuged 3000×g. Flow-through was discarded and 800 µl of buffer XE was added and incubated for 1 min, centrifuged at 500×g for 5 min and the flow-through was collected as exosome. Exosomes were kept at -70 °C for transmission electron microscopy (TEM) (Zeiss- EM10C-100 KV, Germany), dynamic light scattering (DLS) analysis and flow cytometry analysis.

THP-1 monocyte cell culture with mycobacteria and control exosomes

The human acute monocytic leukemia cell line THP-1 (ATCC, catalog number: ATCC® TIB-202™, USA) was grown in RPMI 1640 (Gibco; Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 25 mM HEPES (Gibco), 100 units/ml penicillin (Sigma, Munich, Germany), 100 µg/ml streptomycin (Sigma, USA), and incubated for 24 h at 37 °C under 5% CO2. In order to classify dose-dependent of cell death with THP-1, and based on our bicinchoninic acid assay findings (Pierce BCA Protein Assay (µg/ml) - Thermo Fisher Scientific. no. 23225, USA), each well was separately coated with 200,000 cells/ml inoculated with 10, 5, 2.5, and 1.2 µg/ml exosomes, and exosome-depleted FBS (Gibco™ A25904DG, USA) to the final volume of 200 µl. Each exosome concentration was repeated over three times. Each experiment was composed of lipopolysaccharides (LPS, 100 ng/ml) and unstained controls. The cells were incubated at 37 °C under 5% CO2 for 24 h. The cells were then centrifuged at 40 x g for 5 min and the pellet was used for cell death assay via annexin V-FITC (Invitrogen, 0.25 µg/mL) and Propidium iodide (PI) (Invitrogen- thermo fisher scientific, Lot: 1989095, USA) by a flow cytometry (FACS Calibour, USA). Pellets were precipitated with annexin V for 15 min with incubation in a dark place. Finally, 10,000 events were monitored and cell death was analysed.

Exosomal RNA extraction and cDNA synthesis

RNA was extracted from serum-derived exosomes of the patients with mycobacteria using FavorPrep miRNA kit (Cat.:FAMIK001, Iran) according to the manufacturer's instructions. First, 200 µl Lysis Buffer was added into the tube containing 1x10 of the exosome, shaked and incubated at room temperature for 10 minutes. Only 20 µl

2M NaOAc, pH 5.2 was added, inoculated with 180 µl ddH2O saturated phenol, mixed with 40 µl chloroform and vortexed for 2 minutes. The mixture was centrifuged at 12,000 rpm for 3 minutes and the upper phase was transferred in a clean tube. Ethanol to 35% volume was added and transferred to the RNA column and left for 1 minute, centrifuged at 12000 rpm for 30 seconds and thus the filtrate was collected. Ethanol to 70% volume was added and mixed. The mixture was then transferred to another RNA column in the collection tube, incubated for 1 minute and centrifuging at 12,000 rpm for 30 seconds. miRNA was bound to the membrane, washed with 200 µl wash buffer and then incubated for 1 minute. Centrifuging at 12,000 rpm for 1 minute was repeated to reduce the residue liquid. RNA column was replaced with a clean tube. Only 50 µl release buffer was added and incubated for 3 minutes. miRNA was collected by centrifuging at 12,000 rpm for 3 minutes. miRNA was kept at -70°C for further analysis. For cDNA synthesis, 20 ng of the miRNA was reverse transcribed by the Yekta Tajhiz cDNA Synthesis Kit (Cat No: YT4500, Iran) according to the manufacturer's instructions. Loop primers for each apoptosis miRNAs were generated and a one step of thermal cycler was programmed at 42°C for 60 minutes, at 70°C for 5 minutes.

RT-PCR and miRNA quantification

Real-time PCR was performed using 2x qPCRBIO SYBR® Green Master Mix kit (Biosystems) for Hsa-miR-155, Hsa-miR-20a-5p, Hsa-miR-29a, and Hsa-miR-let7e according to the manufacturer's instructions. The cDNA product was diluted 10x and added to the real-time PCR reactions. A two-step real-time PCR was done using an initial denaturation step at 95°C for 2 min, a total of 50 amplification cycles including a denaturation step at 95°C for 10 s, and an annealing step at 30°C for 63 s. Expression levels of all the miRNAs were normalized according to the expression level of U6 as an endogenous control by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Experiments were statistically analysed for significant difference ($p<0.05$) using multiple t tests and analysis of one-way variance (ANOVA). Graphs were generated by GraphPad Prism (v.8) software. The difference in gene expression was computed using Genex 6 software. The association between apoptosis miRNAs and accelerating serum cell death that could introduce biomarkers was determined using the sensitivity, specificity, and area under the receiver operating characteristic (ROC) curve. The area under the curve (AUC) was resolved with a 95% confidence interval (CI) and ROC analysis was done using SPSS (v.21). Relative expression levels were computed by the $2^{-\Delta\Delta Ct}$ method.

RESULTS

Characterization of serum exosomes

Exosome was isolated from serum of patients with *Mycobacterium tuberculosis (MTB)*, *non-tuberculous mycobacteria (NTM)* species and healthy controls and thus analysed by transmission electron microscopy (TEM) and characterized for size distribution by dynamic light scattering (DLS). Serum-derived exosomes are found as spherical particles with an average of 50-150 nm in size. The release of exosomes was expressed as high levels of the marker protein CD81 as determined by flow cytometry (Figure 1).

THP-1 cells death by serum exosome of mycobacteria and controls

Analysis of cell death induced by exosomes from serum samples of tuberculosis and *non-tuberculous mycobacteria* patients on THP-1 monocyte cells was performed. The cell death of THP-1 monocyte cells was evaluated by different concentrations of exosome released from *Mtb* and *NTM* patient's serum samples by annexin V-FITC and Propidium Iodide (PI) staining. Most cell death of THP-1 monocyte cells were determined in 10 and 5 µg/ml concentrations of the exosomes ($p<0.05$). However, minimum cell death was found in 2.5 and 1.2 µg/ml concentrations ($p<0.05$) (Figures 2 and 3). The apoptosis of

monocyte cell by the exosomes released from control cells was analysed. The cells were viable in different concentrations of healthy exosomes.

Relative expression level of cell death induced by exosomal miRNAs

The expression level of apoptosis miRs including miR-20a, miR-29a, and miR-let7e enhanced, but miR-155 showed a decline in patients with mycobacteria compared to normal subjects. The relative expression of nuclear RNA U6, as an endogenous control, was assessed. Each experiment was done in duplication. The quantitative expression of miRNAs compared to level of normalized U6 was consistent in healthy controls considering as a reference for TB and *NTM* samples. Overexpression of miR-20a (2.61 − fold increase, p≤0.03), (8.75− fold increase, p≤0.003), (2.40–fold increase, p≤0.7), (5.9 − fold increase, p≤0.03), (7.37−fold increase, p≤0.01), (7.72 − fold increase, p ≤ 0.008) was statistically significant in *M. tuberculosis, M. avium, M. simiae, M. chelonae, M. abscessus* respectively, compared to healthy controls. But, it was not significantly found for *M. kansasi* (2.4-fold increase, p ≤ 0.7). For miR-29a, overexpression was statistically significant in *M. avium* (upregulated 6.82-fold increase, p ≤ 0.01), *M. kansasi* (upregulated 7.08-fold increase, p ≤ 0.01), and *M. chelonae* (upregulated 9.38-fold increase, p ≤ 0.002); however, significant differences was not found in the expression of miR-29a in *M. tuberculosis* (upregulated 1.93-fold increase, p≤0.3) and *M. simiae* (upregulated 2.28-fold increase, p≤0.7) compared to healthy subjects. A significant upregulation was statistically seen in miR-let7e expression of *M. tuberculosis* (2.36-fold increase, p≤0.01), *M. avium* (5.68-fold increase, p≤0.005), *M. kansasi* (4.85-fold increase, p ≤ 0.01), and *M. simiae* (5.05-fold increase, p≤0.01), whereas no statistical significance upregulation of miR-let7e was seen in *M. chelonae* (2.98-fold increase, p≤0.21) in comparison to healthy subjects. In contrast, only the expression level of miR-155 was downregulated compared to healthy subjects. A significant downregulation was found in expression level of miR-155 from *M. tuberculosis* (-2.05-fold decrease, p ≤ 0.006) and *M. kansasi* (-3.14-fold decrease, p≤0.03), but no significant differences were determined in *M. avium* (downregulated -1.49 fold decrease, p≤0.9), *M. simiae* (down regulated -1.51 fold decrease, p≤0.9), *M. chelonae* (down regulated -1.28 fold decrease, p≤0.9), and *M. abscessus* (down regulated -1.68 fold decrease, p≤0.7) compared to healthy controls. The expression level of miRNAs of combination of all mycobacteria species was statistically significant (Figure 4, 5 and 6).

Diagnostic potential of serum apoptosis miRNAs from *Mycobacterium tuberculosis* and healthy individuals

Due to inadequate sample sizes of *non-tuberculous mycobacteria* species, biomarker analysis of miR-20a, miR-29a, and miR-let7e and miR-155 was only evaluated for *Mycobacterium tuberculosis (Mtb)* in comparison to controls. Upregulation of each miRNA alone may differentiate *Mtb* from controls with AUC= 0.73 (95% CI: 0.6041 to 0.8603, p < 0.002) for miR-let7e with a cut-off value Youden's Index 0.47 (sensitivity 0.80, 95% CI: 0.6 to 0.9, specificity 0.67 95% CI: 0.48 to 0.80), AUC= 0.69 (95% CI 0.55 to 0.8, p < 0.01) for miR-20a with a cut-off value of the largest Youden's index 0.37 (sensitivity 0.60, 95% CI: 0.42 to 0.75, specificity 0.77, 95% CI: 0.59 to 0.88), for miR-29a AUC=0.6 (95% CI: 0.45 to 0.74, p < 0.1) with a cut-off value of Youden's index 0.33 (sensitivity 0.37, 95% CI: 0.21 to 0.54, specificity 0.97, 95% CI: 0.83 to 0.99), and a significant difference of downregulated miR-155 was seen compared to healthy subjects AUC=0.7 (95% CI: 0.61 to 0.87, p < 0.001) with a cut-off value of Youden's index 0.47 (sensitivity 0.8, 95% CI: 0.62 to 0.9, specificity 0.6, 95% CI: 0.48 to 0.8). The AUC was improved by combinations of all miRNAs. The combination of miR-20a, miR-29a, and miR-let7e and miR-155 represents an AUC= 0.86 (95% CI: 0.77–0.95, p < 0.0001) with a sensitivity 0.86 (95% CI:0.70 to 0.94, specificity 0.76, 95% CI:0.59 to 0.88 and showing a cut-off value with Youden's index 0.63 in comparison to control individuals (Figure 7).

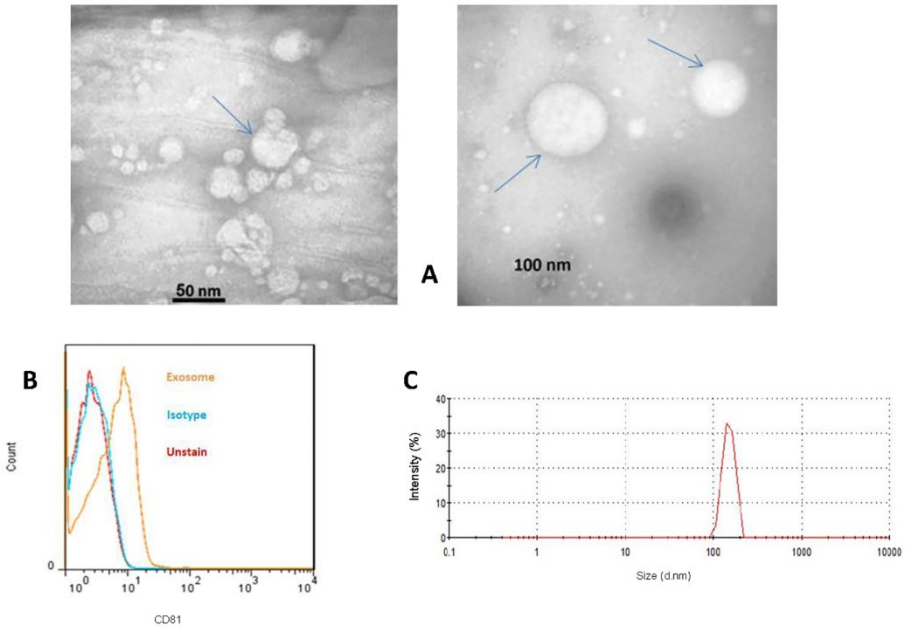

Figure 1. Exosome characterization by transmission electron microscopy (TEM), Dynamic light scattering (DLS), and flow cytometry analysis. Exosomes were isolated by exoEasy Maxi Kit (Qiagen, USA) **a)** TEM showing serum exosomes are spherical particles with an average of 50-100 nm in size. **B)** Detection of exosomal CD81 surface marker using flow cytometry. MFI (mean fluorescence intensity) represents the expression of CD81 on the surface of exosomes. **C)** Dynamic light scattering shows the size distribution of the exosomes as 147.6 ± 3.4 nm

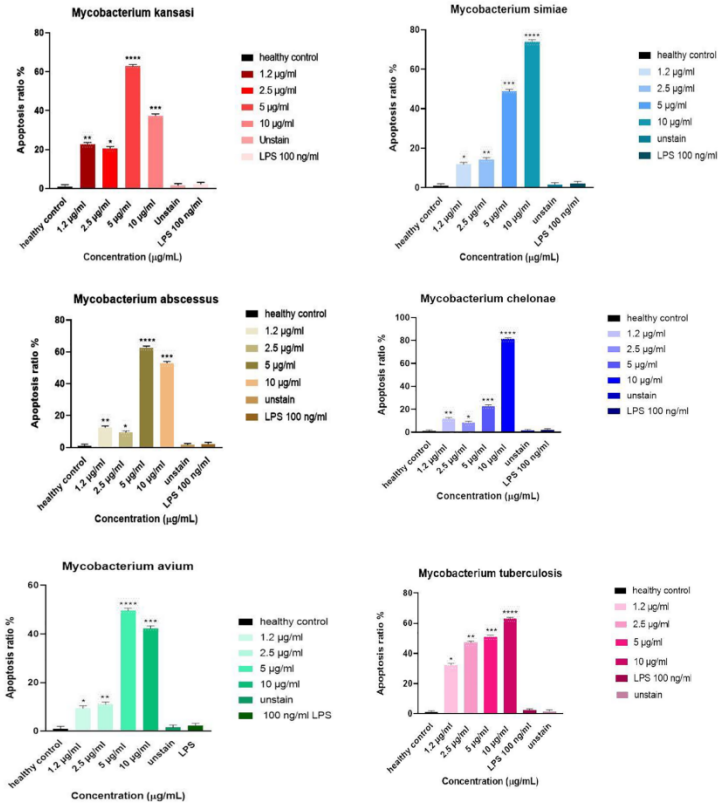

Figure 2. Apoptosis analysis of different concentration of mycobacteria exosomes on THP-1 monocyte cells. Graphs show most apoptosis of THP-1 cells by increasing concentrations of exosomes directly derived from mycobacteria species. Lowest concentrations of mycobacteria exosomes induce minimum exosomal apoptosis on THP-1. Apoptosis was increased in highest mycobacteria exosome concentrations 10 and 5 µg/ml, and it was decreased in 2.5 and 1.2 µg/ml of the exosomes concentrations. **** ≤0.000001, *** ≤0.00001, **≤0.0001, *≤0.001

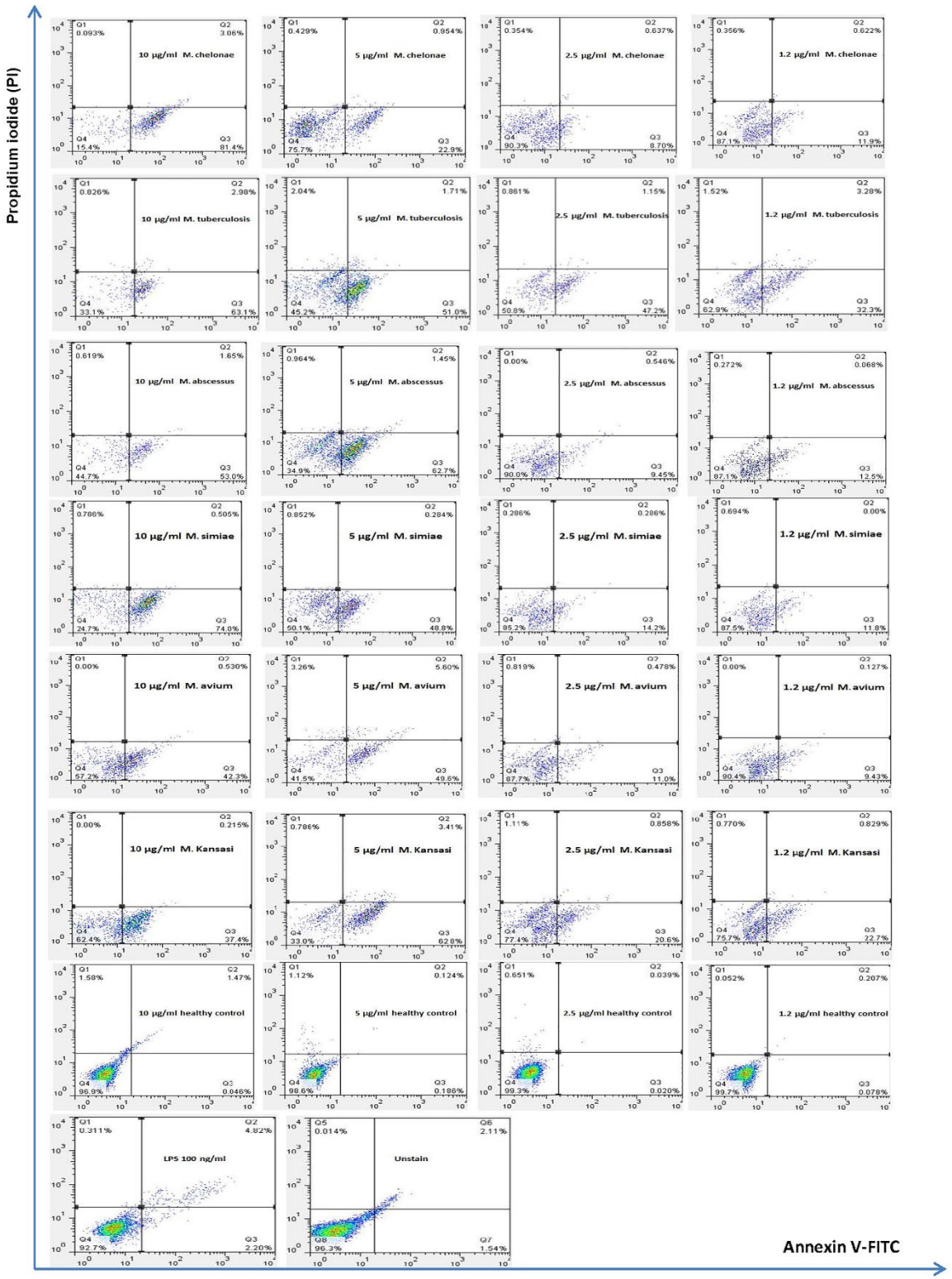

Figure 3. Flow cytometry data of annexin V FITC-PI for exosomal apoptosis of *M. avium, M. abscessus, M. kansasi, M. chelonae, M. simiae, M. tuberculosis* with THP-1 monocyte cells. THP-1 monocyte cells treated with different concentrations of mycobacteria exosome (10 µg/ml, 5 µg/ml, 2.5 µg/ml and 1.2 µg/ml), unstain cell treated alone, LPS –treated control, and exosome from healthy controls, showing % of apoptotic cells (annexin-V positive cells). In most mycobacteria species, THP-1 exosomal apoptosis was increased by enhancing mycobacteria exosome multiplicities. P. value < 0.05 was obtained from each experiment comparable with apoptosis of the healthy controls.

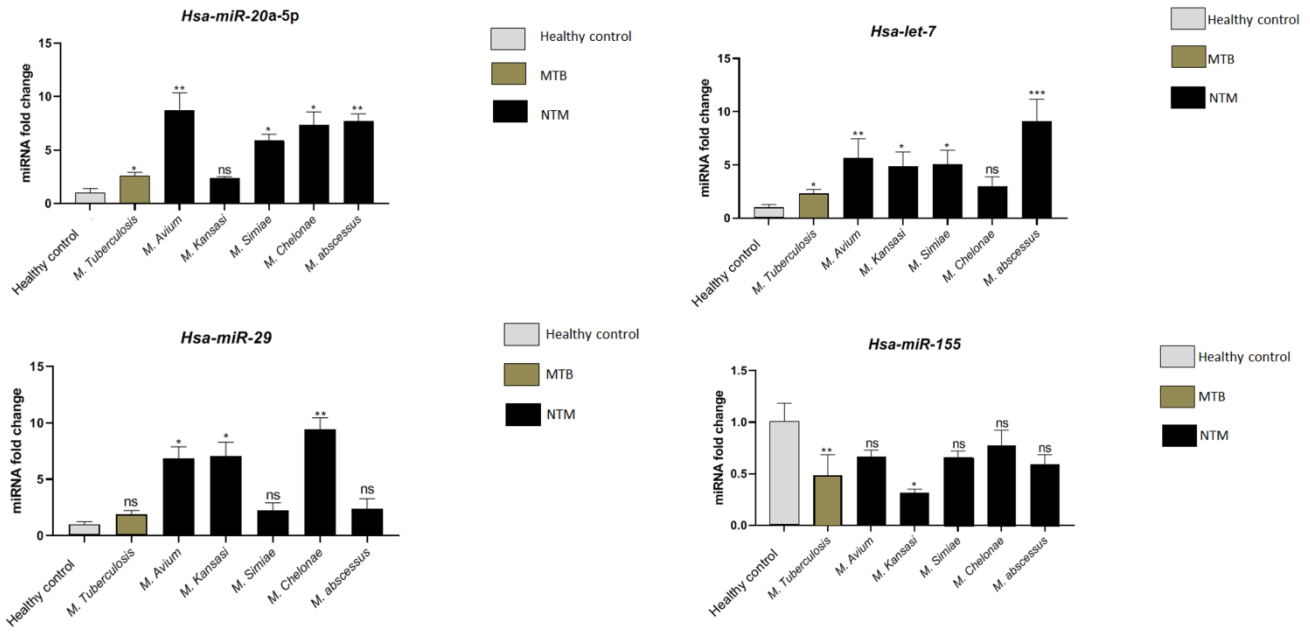

Figure 4. The relative expression of exosomal miR-20a-5p, miR-29a, miR-let7e, and miR-155 in M. tuberculosis and *non-tuberculous mycobacteria* patients compared to healthy controls. Real-time PCR of exosomal miR-20a-5p, miR-29a, miR-let7e indicated upregulation in the patients compared to healthy controls (*p < 0.05, **p <0.001, and *** p < 0.0001 respectively). But, miR-155 determined downregulation in comparison with healthy controls. Upregulation of exosomal miR-20a-5p, miR-29a, miR-let7e was found, however, miR-155 downregulated. Data represent mean ± SEM from 55 mycobacteria patients and 30 control subjects.

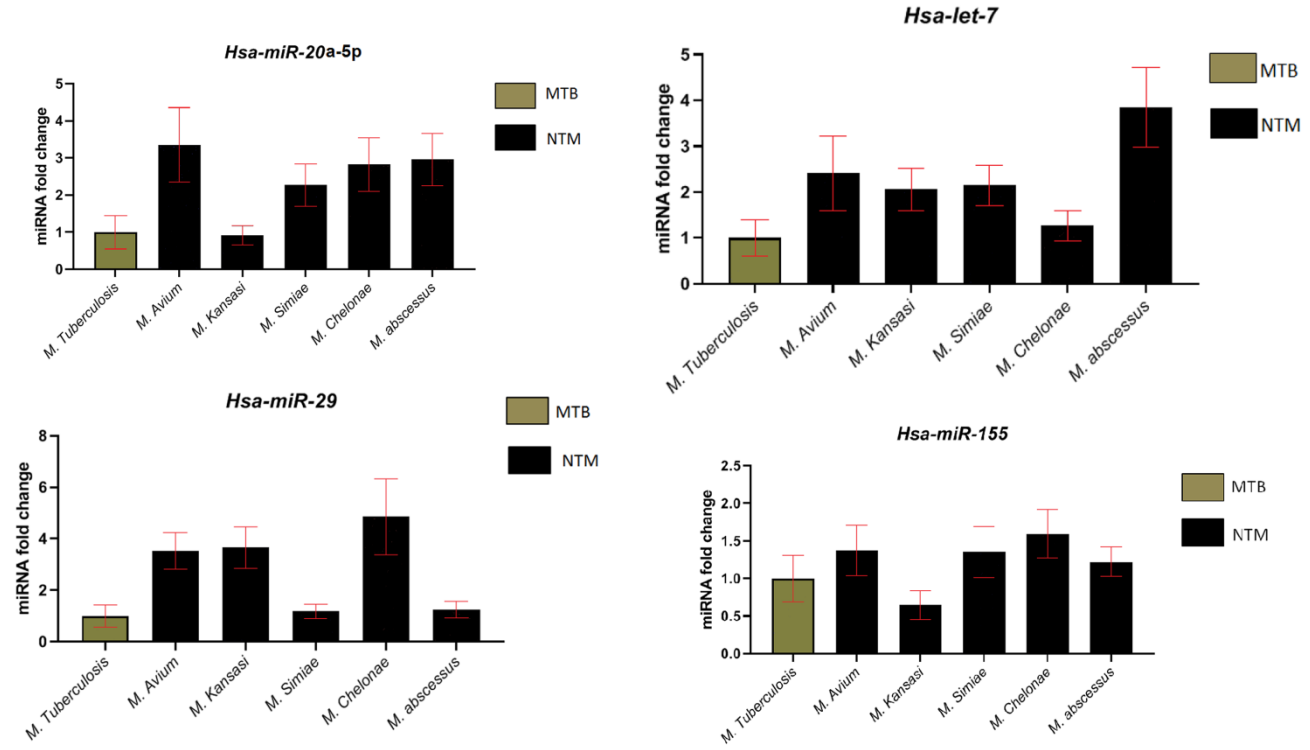

Figure 5. The relative expression of exosomal miR-20a-5p, miR-29a, miR-let7e and miR-155 in *Mycobacterium tuberculosis* patients compared to *non-tuberculous mycobacteria*. Real-time PCR of exosomal apoptosis miR-20a-5p, miR-29a, miR-let7e showed significant statistics in *non-tuberculous mycobacteria* compared to *Mtb* (P≤0.0001). However, no significant analysis was determined for miR-155 in Ntb compared to *Mtb*. Data represent mean and SD from 25 Ntb and 30 *Mtb* patients.

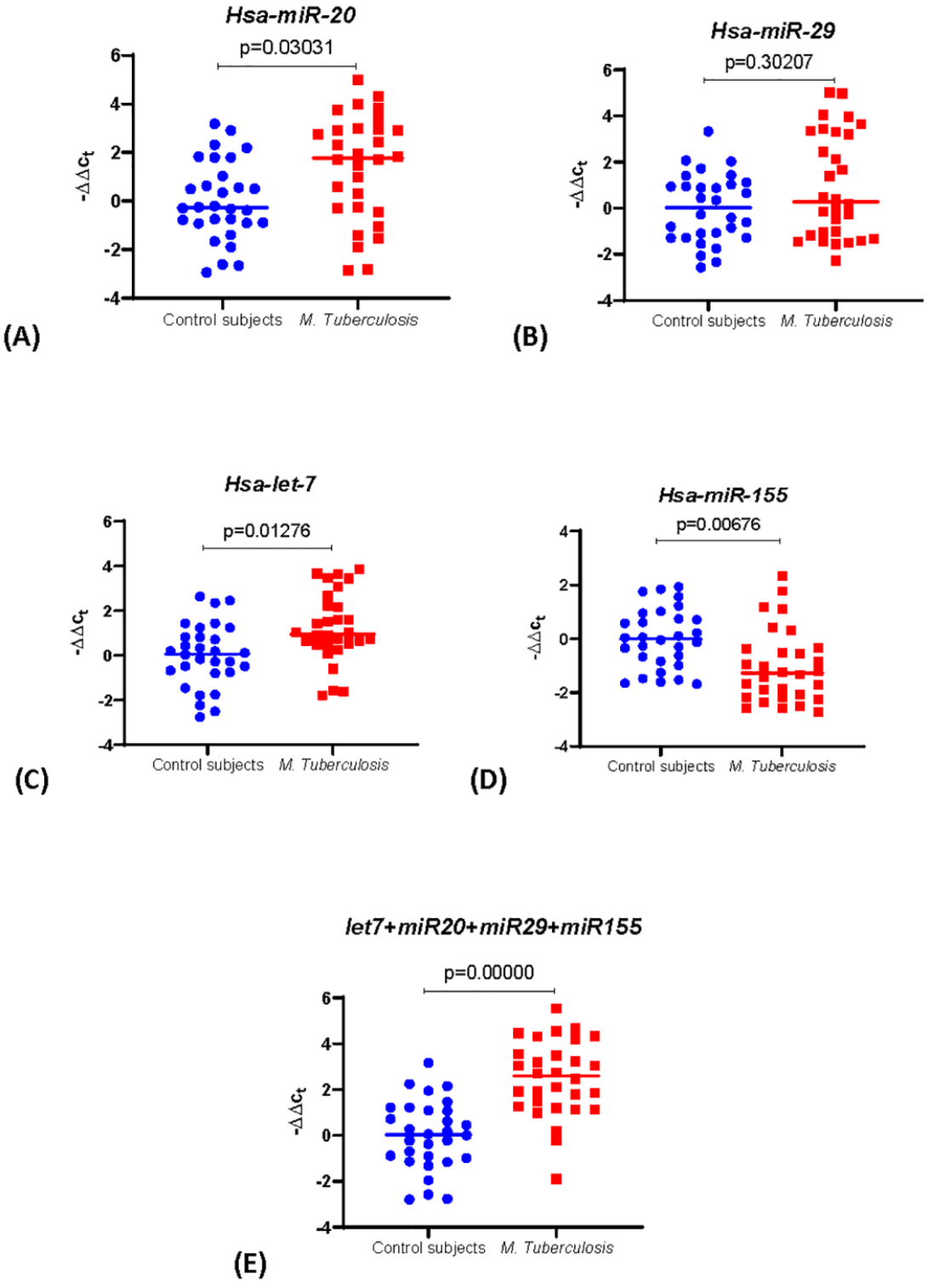

Figure 6. The expression level of serum exosomal miRNAs, (a) miR-20a-5p, (b) miR-29a, (c) miR-let7e, and (d) miR-155 of patients with *Mycobacterium tuberculosis* compared to healthy subjects reconstructed using mean –ΔΔCt. The expression of miR-20a-5p, miR-29a, miR-let7e overexpressed in the patients (–ΔΔCt: 1.38 $p \leq 0.03$, –ΔΔCt: 0.95, $p \leq 0.30$, –ΔΔCt: 1.23 $p \leq 0.01$, respectively). Downregulation was found in miR-155 (–ΔΔCt: -1.038 $p \leq 0.006$). (e) A combination of the miRNAs showed a –ΔΔCt 2.48 with $p \leq 0.0001$.

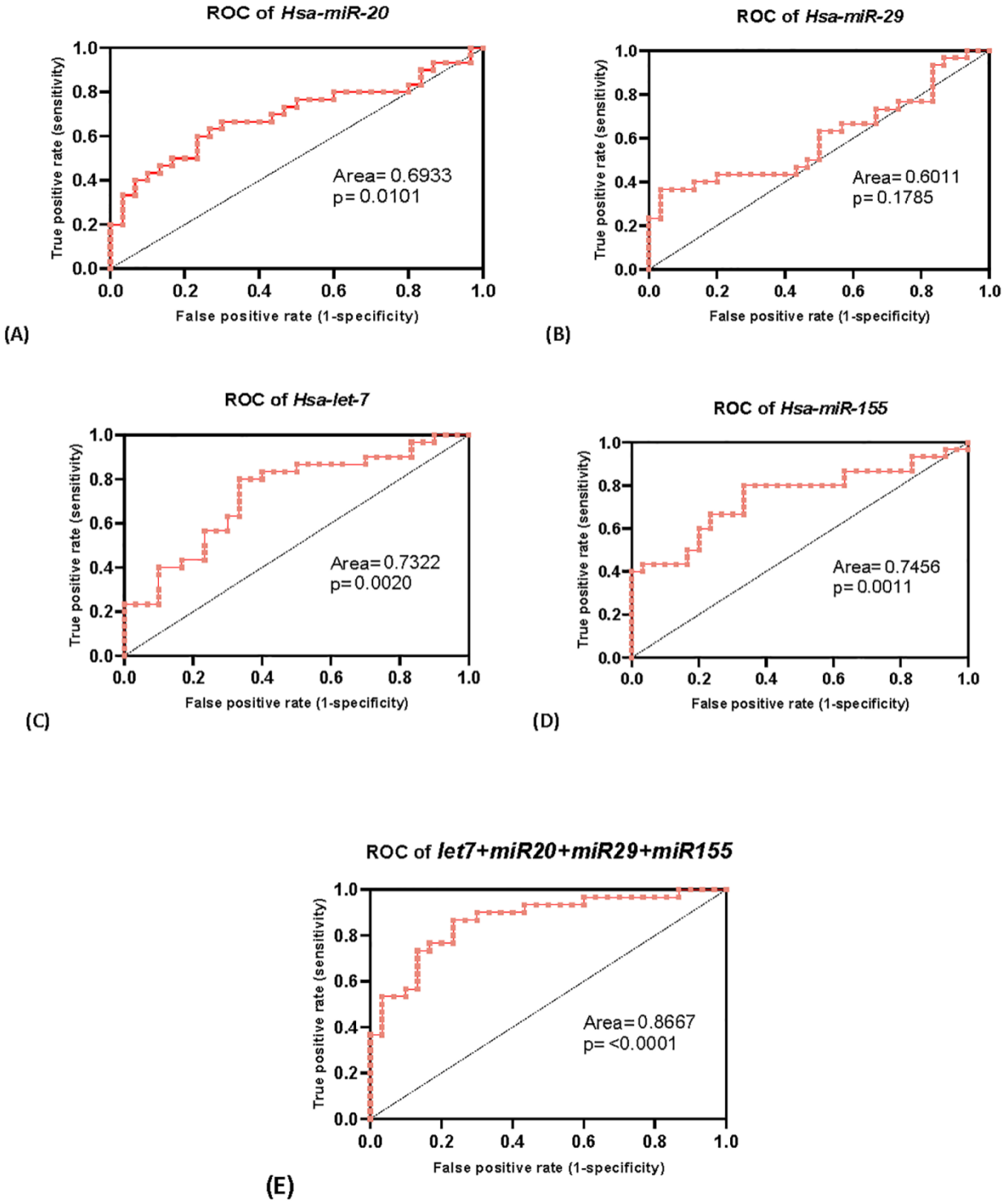

Figure 7. ROC curve analysis of serum exosomal miRNA(a) miR-20a-5p, (b) miR-29a, (c) miR-let7e and (d) miR-155 level in patients with *Mycobacterium Tuberculosis*. Receiver operator characteristic (ROC) curves were established using each microRNA expression value to determine the diagnostic power of the exosomal apoptosis miRNAs in *MTB* patients compared to healthy subjects. The area under the curve (AUC) with 95% CI and the P values were computed and presented for each ROC curve (miR-20a-5p: AUC=0.6933, p ≤ 0.01, miR-29a: AUC=0.6011, p ≤ 0.17, miR-let7e: AUC=0.7322, p ≤ 0.002, miR-155: AUC=0.7456, p ≤ 0.001). The optimal diagnostic point was determined at cutoff values with significant Youden's index (sensitivity and specificity -1). (e) Combination of miR-20a-5p, miR-29a, miR-let7e and miR-155 with an AUC 0.86 and p ≤ 0.0001.

DISCUSSION

Exosomes play as a pathogenic origin that induce host defense and immunity, or regulators mediators of immune evasion such as programmed cell death (17). The current study shows the role of serum-derived exosome from patients with *Mycobacerium tuberculosis (Mtb)* and diverse *non-tuberculous mycobacteria species (NTM)* on THP-1 monocyte cell death and attempts to determine any association between apoptosis miRNAs expression level including miR-20a, miR-29a, miR-let7e, and miR-155 with monocyte cell death. The main finding of this study was that *Mtb* and *NTM* exosomes may associate with accelerating THP-1 monocyte cell death. The main finding of this study was that Mtb and NTM exosomes may associate with accelerating THP-1 monocyte cell death and could be due to the overexpression level of apoptosis miRNAs. Thus, exosomes might not be considered as a valuable candidate in vaccine development and its other appliances. In 2015, Wang et al evaluated the association between exosomes from *M. avium*-infected macrophages and cell death. Exosomes from *M. avium*-infected macrophages induce immune responses in response to *M. avium* infection, but cell death was not accelerated (18). Cytokine assay was also assessed and only *M. avium* infection causes overexpression of TGF-β1 that resulted in cell death dysfunction. Exosomes increase the inflammatory response within macrophages infected with bacterial antigens but could not reduce macrophage viability suggesting that exosomes would make a good vehicle for vaccine delivery (18). But, a limitation of their study could be the cells have alternated in different pathways and protein dysfunctions might increase or decrease apoptosis in vivo, compared to in vitro condition.

In 2013, one study showed that exosomes that shuttle mycobacterial antigens are capable to protect mice from *Mtb* infection. Exosomes from macrophages treated with *Mtb* culture filtrate protein–treated (CFP-treated) were determined as an induction for antigen-specific IFN-γ and IL-2-expressing CD4+ and CD8+ T cells. The immunity was better than BCG. So, exosomes might be used as a novel cell-free vaccine against *Mtb* (14). But, further studies on the role of cell death in *Mtb* and *NTM* patients should be evaluated for vaccine preparation. Recently, reports have suggested that mycobacterial species inhibit cell death as a virulence mechanism that is directly related to multiplicity of infection (19). Hence, Keane et al. indicated that minimum multiplicities of *Mtb* infection results less macrophage cell death than avirulent *Mtb* or other mycobacteria (20). A few studies have suggested that a variety of mycobacterial species increase macrophages cell death in vitro. Most reports discuss the role of macrophage cell death in host defense against mycobacterial infection (21). Both monocytes and alveolar macrophages infected with *Mtb* increase cell death in vitro, and accelerating cell death has been found in alveolar macrophages of active tuberculosis patients (22). Also, low numbers of *Mtb* bacilli inhibits cell death in monocytes (20, 23). Hence, an increase in monocyte cell death by exosome released from serum samples of mycobacteria patients could be due to direct infection of THP-1 monocyte cell that may result to TGF-β1 dysfunction and changes in level of TGF-β1 expression. Besides, multiplicity of infection could be also crucial as lower multiplicities of mycobacterial infection cause minimum cell death than maximum multiplicities that result in necrosis-like cell death by a caspase-independent mechanism.

The current study also shows that both 10 and 5 µg/ml, which are maximum multiplicities of infectious exosomes from mycobacterial species, cause an increase in THP-1 cell death comparable with lower multiplicity including 2.5 and 1.2 µg/ml. The most cells death were determined in 10 µg/ml of exosome released from *M. chelonae*; however, lowest cells death were found in 2.5 µg/ml of the exosome released from *M. chelonae*.

One limitation of our study is that accelerating cell death is not only due to mycobacterium infection, since most of chronic disorders also enhance cell death. Based on above report findings, another cause of cell death could be either virulent mycobacteria that decrease cell death, or avirulent mycobacteria that increase cell death. So,

exosomes released from *M. chelonae* are able to cause minimum cell death, whereas different concentrations of exosome from *M. tuberculosis* have been shown maximum cell death among other mycobacteria.

The recent findings have shown the association of miRNAs regulation with bacterial infection (24). Studies have concluded that infected-macrophage cell by mycobacteria regulates the miRNAs of JNK-2 signaling in human macrophage that causes a negative regulation of cell death pathway (15). The upregulation of miR-20a-5p, as an apoptosis miRNA candidate, survives *M. tuberculosis* in the macrophages (15). But, other studies show that overexpressed MiR-20a-5p decreases *Mtb* survival within macrophages (15). Interestingly, a contrasting study reports that *Mycobacterium bovis Bacillus Calmette-Guerin (BCG)* downregulated miR-29 expression and induced IFN-γ expression in NK cells and T cells (25). This result indicates miR-29 inhibition may have facilitated IFN-γ production by T cells and expression of miR-29 is influenced by *Mycobacterium* species-specific virulence (26). Another study also reported serum miR-155 expression level was downregulated in relation to FOXO3 and TB-suppressing activity of cells (27-29). Thus, miR-155 inhibits cell death of monocytes in patients with active TB through negative regulation expression of FOXO3 (29). This study also presents an overexpression of miRs 20a, miR-29a, and miR-let7e of mycobacteria patients, while a downregulation was found in miR-155 level. The expression level of cell death miRs from each mycobacterial species in combination with other species was analysed. No significant differences were determined in the serum apoptosis miRs of each combination. For *Mycobacterium tuberculosi*s, the diagnostic potential of the 4 miRs was improved using combined miRNAs (AUC=0.86, P≤0.0001) compared to tested miRs individually (AUC=0.6-0.74, P≤0.01-0.001).

Another limitation of current study is poor sample size of *non-tuberculous mycobacterium (NTM)* species, therefore diagnostic potency of those apoptosis miRs was statistically uncomputed. This pilot study shows that there is a upregulation of serum exosomes-monocyte apoptotic miRs 20a, miR-29a, and miR-let7e in patients with mycobacterial diseases. Only miR-155 shows a decrease expression level in apoptotic exosomes of the patients. These may confirm that the level of exosomal miRNAs isolated from serum of mycobacteria patients was associated with increased cell death in THP-1 monocyte cell by the exosome released from serum of mycobacteria patients. Also, apoptosis miRNAs, miR-20a, miR-29a, and miR-let7e, indicate an increase in fold change expression level of both slow and rapid growth mycobacteria. ROC curve analysis shows that miR-20a, miR-let7e, and miR-155 have a predictive value for those of mycobacteria species. But, the fold change was reduced in miR-155 and no significant value was determined for miR-29a.

The cell death may be initiated and or increased by different mechanisms including miRNAs profiling functions, directly infecting the cell with exosome released from mycobacterial strains, multiplicity of infection with exosome directly released from *NTM* species and virulent or avirulent of the mycobacteria. Considering as a first trial, exosomes from serum samples of mycobacterium patients may be associated with increased cell death and this may result in making obstacles in TB and *NTM* vaccine preparation or even other exosomal benefits. We also believe that upregulation of apoptosis miRNAs may increase cell death of THP-1 monocyte. These putative miRNAs are not specific for only mycobacterial infection and the expression level might be up-regulated or down-regulated in different disease types including other infectious diseases. miRNAs expression profiling may provide beneficial clues for pathophysiological studies and improve diagnosis, prognosis, and therapeutic measures. Among other reasons, this be may considered as the most significant cause of increasing cell death of THP-1 monocyte cell.

Ethics approval and consent to participate

All participants provided written informed consent to participate in the study. Informed consent to publish data

of the current study was obtained from all participants. The protocol of the current study was approved by the institutional ethical board of Masih Daneshvari Hospital, Tehran, Iran (SBMU 1.REC1394.137).

Conflict of interests

The authors declare that they have no competing interests.

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Availability of data and materials

All data generated or analyzed during this study are available for further investigation.

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