

## Research Paper

## The Effect of Sialic Acid on MiR-320a and Let-7e Expression in Human Glial Cell Line

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**Citation** Noorbakhsh, N., Galehdari, H., & Shafiei, M. (2022). The Effect of Sialic Acid on MiR-320a and Let-7e Expression in Human Glial Cell Line. *Basic and Clinical Neuroscience*, 13(3), 315-324. <http://dx.doi.org/10.32598/bcn.2022.2090.1>

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**Article info:****Received:** 30 Sep 2019**First Revision:** 05 Jan 2020**Accepted:** 10 Oct 2020**Available Online:** 01 May 2022**Keywords:**Inflammation,  
Neuroinflammatory Diseases,  
Sialic Acids, Neuroglia,  
MicroRNAs**ABSTRACT**

**Introduction:** Sialic acid is pivotal in various critical physiological events at molecular and cellular levels and pathological processes. Changes in sialic acid concentration are observed in many pathological processes; for example, some available data exist on the evaluated level of sialic acid and neurodegenerative prevalence. Presumably, sialic acid can play a significant role in regulating a diverse range of uncovered neurodegeneration factors and downstream targets. matrix metalloproteinases 9 (MMP9) is one factor that changes the exposure of different concentrations of sialic acid solution. Hence, we aimed to examine the possible effect of sialic acid solution exposure on the glial cell line in the expression patterns of miR-320a and let-7e as two upstream factors.

**Methods:** Human glial cell line was prepared from the Pasteur Institute of Iran and cultured in a dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS). The IC<sub>50</sub> value of sialic acid was obtained by colorimetric assay for assessing cell metabolic activity 3-(4,5-Dimethylthiazol-2-yl) (MTT), and the glial cell line was treated with sialic acid in 300, 500, 1000 µg/mL for 24 h to investigate the effect of the sialic acid ligand on the expression pattern of the miR-320a and let-7e. Total RNA was isolated from approximately 10×10<sup>6</sup> glial cells and was used from each sample for complementary dna (cDNA) synthesis. For quantitative analysis of miR-320a and let-7e, we used real-time polymerase chain reaction (PCR), and for statistical analysis, the SPSS v. 21 software was applied.

**Results:** Analyzing the real-time data revealed that the expression of miR-320a and let-7e was significantly increased (P<0.0001) in 300, 500, and 1000 µg/mL treated glial cells by sialic acid compared to the control group.

**Conclusion:** A possible linkage of sialic acid on miR-320a and let-7e regulation was observed in the glial cell line as proinflammatory factors in the inflammation pathway.

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## Highlights

- Differing in sialic acid concentration is seen in various pathological states.
- MicroRNAs play a role in numerous biological processes and human disorders.
- miR-320a and let-7e expression levels displayed a significant increase in different sialic acid concentrations.

## Plain Language Summary

Inflammation in the nervous system occurs because of numerous factors. Sialic acid is an inflammatory factor that promotes cellular inflammation, particularly in the glial cells. That is why it could serve as a useful model for simulating several neurodegenerative diseases, including Parkinson's and Multiple sclerosis. Changes in sialic acid concentration are observed in many pathological states, which could be a useful marker for identifying the inflammatory process. The present study was carried out to examine the impact of different concentrations of sialic acid on two non-coding RNAs in glial cells. Our research shows that these two microRNAs greatly increased when responding to sialic acid. We suggest that these two microRNAs are contributed to the neuroinflammatory pathways related to sialic acid.

### 1. Introduction

Inflammation is the biological reaction of body tissues and a complex protective response mechanism (Ferrero-Miliani, Nielsen, Andersen, & Girardin, 2007). Inflammation in the nervous system occurs because of various reasons and is the component of the pathogenicity of neurodegenerative diseases, particularly Parkinson disease, multiple sclerosis, and Alzheimer disease (Fung, Vizcaychipi, M., Lloyd, Wan, & Ma, 2012; Giovannoni and Quintana 2020). It is necessary to know more about inducing inflammatory responses within the Central Nervous System (CNS) and the mechanisms that eventually lead to pathogenicity (Zipp and Aktas 2006; Glass, Saijo, Winner, Marchetto, & Gage 2010).

Sialic acid belongs to the family of 50 naturally monosaccharides that differ from other glycan molecules. It is a sugar with a nine-carbon backbone that can be enzymatically modified inside the cell (Schauer 2009). This sugar plays an important role in many physiological processes such as protein folding, neural development, cell-cell interactions, and pathological processes like autoimmunity and cancer. Changes in sialic acid concentration are shown in many pathological states that might be a valuable marker for determining the inflammatory process (Noorbakhsh, Zamani, & Galehdari, (2020); Citil, Gunes, Karapehliyan, Atalan, & Marasli 2004; Varki 2008; Büll, Heise, Adema, & Boltje 2016). The biological function of sialic acids exerts through multiple mechanisms that participate directly and indirectly in multiple

events at molecular and cellular levels (Schauer 2009; Büll, et al. 2016). Sialic acid is a sugar that is frequently located on the cell surface. For unknown reasons, the brain has the highest sialic acid concentration in the body (Schauer, Srinivasan, Wipfler, Kniep, & Schwartz-Albiez 2011; Sbardella, Fasciglione, Gioia, Ciaccio, C., Tundo, & Marini 2012; Wielgat and Braszko 2012; Linnartz-Gerlach, Mathews, & Neumann 2014). These sialic acid features make it a suitable candidate for investigating neuroinflammatory pathways.

MicroRNAs (miRNAs) are a class of single-stranded non-coding RNA molecules with a length of 20-24 nucleotides. Individual miRNA can control the expression of more than one target mRNA and is estimated to regulate up to one-third of all human genes, such as genes involved in immunity, immune cell differentiation, and the correct functioning of CNS (Fenoglio, Ridolfi, E., Galimberti, & Scarpini 2012; Bhalala, Srikanth, & Kessler 2013).

MiRNAs are contributing to several biological processes and human diseases. Their involvement in a broad range of cellular processes makes them a powerful cellular tool in numerous biological processes such as cell development, proliferation, differentiation, inflammation, immunity, metabolism, apoptosis, and angiogenesis (Coolen and Bally-Cuif 2009; Eacker, Dawson, & Dawson, 2009).

Dysregulating the miRNAs' expression and function is associated with several human diseases such as neurodegeneration, autoimmunity, and cancer. Also, miRNAs' involvement in the pathogenesis of some neurodegen-

erative diseases such as Alzheimer and Parkinson diseases have been demonstrated. After the CNS damage, the expression pattern of miRNAs is changed and affects the downstream factors of these miRNAs (Bhalala, et al., 2013). Moreover, new studies emerged for making new tools for diagnosing and treating several human diseases (Mattes, Collison, et al. 2008; Hébert and De Strooper 2009; Guan, et al. 2013; Liu, et al. 2014).

miRNAs can downregulate gene expression at the post-transcriptional level, leading to degrading or translational inhibition of the target by directing the three prime untranslated region (3'-UTR) of mRNAs through sequence-specific binding. It is essential to know the miRNAs' targets to understand their functions. Moreover, miRNAs regulate gene expression through post-transcriptional repression by binding to the 3'-UTR of their target mRNA (Akhtar, Micolucci, et al., 2016).

We previously demonstrated in our laboratory that sialic acid could alter the expression of inflammatory genes like *MMP-9* and *TIMP-1* in the presence of sialic acid in the human glial cell line. We revealed that the expression of *MMP-9* and *TIMP-1* genes in all three concentrations (300, 500, and 1000 µg/mL) increased compared to the control group (Shabani Sadr, Shafiei, Galehdari, & Khirolah, 2020). In this study, we examine two miRNAs (miR-320a and *let-7e*) that contribute to some neurodegenerative diseases directly and can affect *MMP-9* (Guan, et al., 2013; Aung, Mouradian, Dhib-Jalbut, Balashov, 2015).

Signaling pathways influencing the expression of miR-320a and *let-7e* are still incompletely understood, so we aimed to explore the potential roles and molecular mechanisms of miR-320a and *let-7e* in the glial cells in response to sialic acid because both components are embedded in inflammation regulation.

## 2. Materials and Methods

### Culture of 1321N1 cells for sialic acid treatment

The human glial cell line (1321N1) was obtained from the Pasture Institute of Iran. The glial cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Bio Idea Company, Tehran, Iran) supplemented with 10% FBS (Gibco), streptomycin (100% µg/mL), and penicillin (100 U/mL). All cells culture were maintained at 37°C in an incubator containing 95% air and 5% CO<sub>2</sub> atmosphere. The glial cell line was treated with sialic acid (300, 500, 1000 µg/mL) for 24h to evaluate the effects of this ligand on miR-320a and *let-7e* transcript expression.

### Cell viability assay

Cell viability in response to different treatments was evaluated using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl (MTT) tetrazolium bromide assay. Cells were seeded in a 96-well plate (7000 cells/well) and 200 µL of culture medium treated with different concentrations (between 20-1500 µL) of sialic acid. Cell viability was evaluated with the control group after 24h using the MTT assay (Autocell) at 570 nm with a wavelength of 630 nm. ELISA reader calculated the optical absorption values for each well plate, and the results were analyzed by Excel software.

### Ribonucleic acid extraction, reverse transcriptase, and real-time quantitative reverse transcription

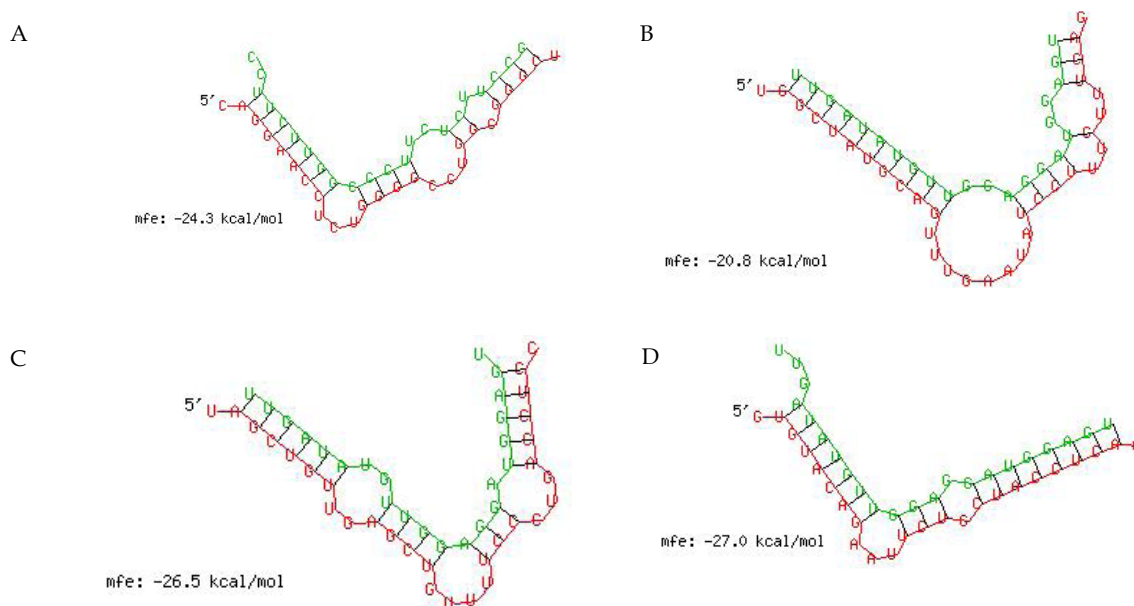
Total RNA of the cocultured cell was extracted by miRNA isolation kit from approximately 10×10<sup>6</sup> glial cells using TRIzol-Sigma-Aldrich (TRI Reagent®, USA) following the manufacturer's protocol.

The yield and purity of the extracted RNAs were evaluated by 2% agarose gel electrophoresis and NanoDrop N 1000 spectrophotometer (Thermo Scientific).

Gene expression changes of microRNA were assessed using a BON-miR kit (Bon Yakhteh, Tehran, Iran) according to the manufacturer's instructions; U6 small nuclear RNA was used to normalize the data. U6 has been commonly used for miRNA normalization due to its RNA stability and abundant expression (Peltier and Latham 2008).

Five thousand nanograms of total RNA from each sample were used in cDNA according to the 'manufacturer's protocol. Real-time detection was performed with the SYBER Green master mix using primers for miR-320a and *hsa-let-7e*. The primers were purchased from BON-miR Research Center. The sequences of the used forward primers are as follows: miR-320a Forward primer: AAGGCTGGGTTGAGAGG, and *let-7e* Forward primer: GATGGGCTGTGAGGTAGG. Universal reverse primers were obtained from Bon Yakhteh Company, Tehran, Iran.

Polymerase Chain Reaction (PCR) amplification was performed on the Applied Biosystems 7500 stepOnePlus instrument (Applied Biosystems). Real-time Quantitative PCR (qRT-PCR) conditions were as follows: 95°C for 2 min (1 cycle), 95°C for 5s, and 62°C for 30s (40 cycles). All samples were duplicated and repeated at least in two independent biological repeats.



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**Figure 1.** The predicted structures

A) miRNA320a-mRNA IL4 Hybrids; B) *Let-7e*-mRNA IL-6; C) *Let-7e*-mRNA IL10; D) *Let-7e*-mRNA IL-13

The specificity of all the primers used in these experiments was confirmed with a single product peak in the melt curve analysis. Data analysis was performed using the  $2^{-\Delta\Delta CT}$  method between the treatment and control groups to compare the expressions of miR-320a and *let-7e*.

### Statistical analysis

The real-time Quantitative Reverse Transcription (qRT-PCR) data were analyzed by SPSS 21.0, and the normalization was conducted with U6 small nuclear RNA. Each reaction was performed in triplicate, then miRNA contents were calculated by the Equation 1: . Statistical analyses were done by SPSS v. 21 software, and data were expressed as Means $\pm$ SEM.

$$1. \Delta Ct_{miRNA} = Ct_{miRNA} - Ct_{U6}$$

The significance level of the observed difference between the groups was measured by Analysis of Variance (ANOVA), and then Tukey's HSD test was used to compare the groups. The graphs were generated by GraphPad Prism v. 7 software (GraphPad Software Inc. La Jolla, CA, USA). The t-test was performed to compare groups, and  $P < 0.05$  was considered the statistically significant difference.

### Target gene prediction

To investigate the putative targets involved in the miR-320a and *Let-7e* mediated regulation of inflammation of

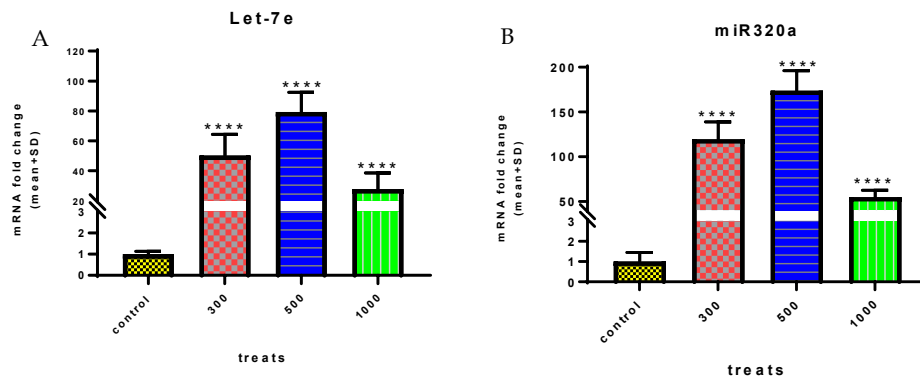
glial cells, four computational algorithms, including TargetScan (Agarwal, Bell, Nam, & Bartel 2015), miRDB (Liu and Wang, 2019), miRWalk database (Sticht, De La Torre, Parveen, & Gretz 2018), and miRmap (Vejnar, & Zdobnov, 2012) were used to analyze the possible anti-inflammatory cytokine binding sites of miR-320a and *let-7e*. The structures of the miRNA-320a-mRNA and *let-7e*-mRNA are displayed in Figure 1.

### 3. Results

The MTT assay results were analyzed by Excel software. Three concentrations of sialic acid (300, 500, and 1000  $\mu\text{g/mL}$ ) were utilized for the treatment of cells and the analysis of changes in gene expressions.

By analyzing qRT-PCR data, it was revealed that miR-320a and *let-7e* expressions were greatly influenced ( $P < 0.0001$ ) in response to different treatments (Figure 2). Three different concentrations of sialic acid (300, 500, and 1000  $\mu\text{g/mL}$ ) displayed a considerable increase in the expression of miR-320a and *let-7e* compared to the control group.

Therefore, we reject the hypothesis that miR-320a and *let-7e* directly affect *MMP-9* because by targeting 3'-UTR of mRNAs through sequence-specific binding, miRNAs can downregulate gene expression at the post-transcriptional level and lead to degradation or translational inhibition of the target. We have suggested that



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**Figure 2.** Change of expression in the presence of different (300, 500, 1000 µg/ml) concentrations of sialic acid

Relative expression of miR-320a and let-7e in the presence of different concentrations of sialic acid compared to endogenous control miRNA, validated by qRT-PCR. ANOVA test was implemented to determine the significance between groups.

(A) *Let-7e* was overexpressed in different concentrations (\*\*\*\* $P < 0.0001$ : one asterisk was used for moderate significance, and four asterisks indicate the maximum significance). (B) Change of expression of miR-320a in the presence of different concentrations of sialic acid compared to endogenous control miRNA. ANOVA test was implemented to determine the significance between groups. miR-320a was significantly overexpressed in different concentrations (\*\*\*\* $P < 0.0001$ ).

miR-320a and *let-7e* might affect anti-inflammatory cytokine to active cellular inflammation in the glial cells treated by sialic acid.

To test our hypothesis, we utilized TargetScan, miRDB, miRWalk, and miRmap to predict the anti-inflammatory cytokines of miR-320a and *let-7e*, defined as potential target genes for future studies.

The anti-inflammatory cytokines are immune regulatory molecules, and their physiological functions in pathology in inflammation and inflammatory states are highly recognized. Various anti-inflammatory cytokines have been recognized and these include Interleukin (IL)-1ra, *IL-4*, *IL-6*, *IL-10*, *IL-11*, *IL-13*, and Transforming Growth Factor-Beta (TGF-β) (Opal, & DePalo 2000; Sultani, Stringer, Bowen, & Gibson 2012).

**Table 1.** Bioinformatics analysis of potential candidate targets of miR-320a and let-7e among anti-inflammatory cytokines by TargetScan, miRDB, miRWalk, miRmap, and RNA hybrid tools

MiR Transcript	Target	Position of Predicted Target Site	Predicted Consequential Pairing of Target Region (Top) and miRNA (Bottom)	Folding Energy (in kcal/mol)
hsa-miR-320a-5p	<i>IL-4</i>	23 of IL4 3' UTR	target 5' CUCUCCUC U 3' AGGAACC GGGG GG GGGC UUCUJGG CCCU UC UCCG miRNA 3' CC UC U 5'	-24.3 kcal/mol
has-let-7e	<i>IL-6</i>	254 of IL6 3' UTR	target 5' U UUUGAAUA UU UU G 3' GGCUAUGCAG UCCU G UCA UUGAUAUGUU AGGA U AGU miRNA 3' GG GG 5'	-20.8 kcal/mol
has-let-7e	<i>IL-10</i>	380 of IL10 3' UTR	target 5' U UG GUUU CUG C 3' AGCUGU AGCU UCC ACCUC UUGAUA UUGG AGG UGGAG miRNA 3' UG A U 5'	-26.5 kcal/mol
has-let-7e	<i>IL-13</i>	552 of IL13 3' UTR	target 5' G AAU G C 3' UGUACAG UCU CUACCUCA AUAUGUU GGA GAUGGAGU miRNA 3' UUG G 5'	-27.0 kcal/mol

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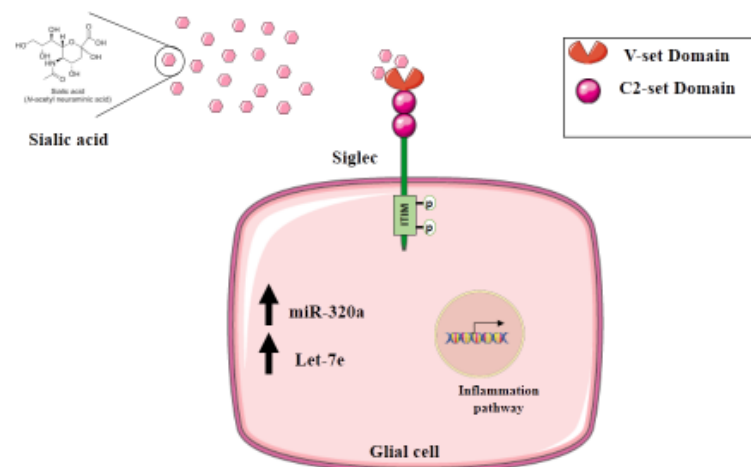


Figure 3. Schematic overview of the process

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We analyzed two microRNAs in bioinformatics tools to find potential anti-inflammatory cytokines. The results identified putative binding sites for miR-320a in the *IL-4* gene. We furthermore observed that *let-7e* contains binding for *IL-10* and *IL-13* (Table 1).

#### 4. Discussion

Numerous reports suggest that miRNAs are key therapeutic targets because they are involved in multiple essential pathways related to neurological networks and biological processes (Papagiannakopoulos, Shapiro, & Kosik 2008; Hébert, & De Strooper 2009). Therefore, any disruption would lead to complex neurological ailments. Hence, it is prudent to speculate on the role of these important molecules as a potential target for therapy and or early diagnosis of neurodegenerative diseases and their associated outcomes.

Here, we investigated the putative effect of sialic acid, as a versatile physiologically active molecule in various body processes, on the expression of two miRNAs, the miR-320a and the *let-7e*, to identify the network under these miRNAs control (Figure 3).

#### 5. Conclusion

Sialic acid is an inflammatory factor that facilitates cellular inflammation, particularly in the glial cells. It would be an applicable model for mimicking some neurodegenerative diseases such as Parkinson disease and multiple sclerosis.

This research indicates that selected sialic acid concentrations induced significant changes in the expression of miR-320a and *let-7e* in treated glial cells compared

to nontreated groups. It is observed that miR-320a and *let-7e* were upregulated in the glial cells treated with sialic acid compared to the control group. This finding approved the potential role of miR-320a and *let-7e* on molecular pathways related to sialic acid.

Also, previous studies have shown that *let-7e* has been upregulated in some inflammation processes; for example, Coskun, Bjerrum, Seidelin, Troelsen, Olsen, & Nielsen (2013) demonstrated that *Let-7e* acts as a potential biomarker in Ulcerative Colitis (UC) (an inflammatory Bowel Disease (IBD)). They have identified that the expression level of *let-7e* was significantly upregulated in UC (Coskun, et al., 2013). Polikepahad et al. (2010), in their in vivo analysis, suggested the proinflammatory properties of let-7 miRNAs. Their studies indicate a powerful proinflammatory role for the let-7 family of miRNAs in allergic lung disease (Polikepahad, Knight, et al. 2010). *Let-7e* is significantly upregulated in multiple sclerosis patients, suggesting an active role for *let-7e* in MS pathogenesis (Guan, et al., 2013, Guan, et al., 2016).

Chen et al. (2015), in their in vivo studies, showed that the overexpression of miR-320a led to an increase in the serum inflammatory cytokines such as *IL-6*, MCP-1, TNF- $\alpha$ , and fibrinogen. (Chen, et al. 2015). Some studies have suggested that the miR-320 family is altered in patients with inflammatory bowel disease (Fasseu, et al., 2010). This evidence suggests the potential role of *let-7e* and miR-320A as proinflammatory factors in the inflammatory process.

Exploring the target genes and pathways of miR-320a, *let-7e*, and other differentially expressed miRNAs may contribute to understanding the potential role of miR-

NAs in this pathway. MicroRNAs perform functions through mediating translational repression or directing cleavage of target genes. Identifying the target genes of miR-320a and *let-7e* will be the next step toward uncovering their role in this pathway.

The present study has suggested that miR-320a over-expression was involved in the cellular inflammation of the glial cells. Because miR-320a regulates multiple genes, it could play an important role in the regulatory network underlying the inflammation of the glial cells (Noorbakhsh, et al., 2020).

Bioinformatics tools show that *IL-4* is the potential anti-inflammatory cytokine of miR-320a, and *IL-4* was selected for further experiments as it ranked the highest amongst the potential target genes. In addition, the association between *IL-4* and miR-320a was previously investigated in HTR-8/SVneo cells. The results suggested that *IL-4* was a direct target gene of miR-320a and could repress *IL-4* expression by binding to its 3'-UTR (Xie, Jia, & Li, 2019).

Bioinformatics tools show that *IL-10* and *IL-13* are the potential anti-inflammatory cytokines of *let-7e* and were selected for further experiments as they ranked the highest amongst the potential target genes. Furthermore, the links between *IL-10*, *IL-13*, and *let-7e* have previously been analyzed in patients with MS. The results identified that *let-7e* is directly targeted in *IL-10* and *IL-13*. (Guan, et al. 2013; Guan, Singh et al. 2016).

Based on in silico studies, it was determined that *let-7e* could inhibit *IL-10* and *IL-13*, and miR-320a can inhibit *IL-4*. Therefore, *IL-4*, *IL-10*, and *IL-13* were selected for further experiments as they ranked the highest amongst the potential target genes.

## Ethical Considerations

### Compliance with ethical guidelines

There was no requirement for ethical approval because our work has been limited to cell lines.

### Funding

The article was extracted from the MSc thesis of the second author in the Department of Genetics, Faculty of the Science at the Shahid Chamran University of Ahvaz.

## Authors' contributions

Conceptualization and supervision: Hamid Galehdari; Methodology, Investigation, Data analysis, writing-original draft, and writing-review & editing: Negar Noorbakhsh; Conceptualization and Writing-Review & Editing: Mohammad Shafiei

## Conflict of interest

The authors declared no conflict of interest.

## Acknowledgments

The authors would like to acknowledge all the contributors to this study for supporting this analysis, particularly Tahereh Seifi. We acknowledge the Support of Shahid Chamran University of Ahvaz for supporting this work

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