

## Original Article

# MicroRNA-155 mimics ameliorates nerve conduction velocities and suppresses hyperglycemia-induced pro-inflammatory genes in diabetic peripheral neuropathic mice

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**Abstract:** Background: MicroRNA-155 (miR-155) regulates inflammatory cytokines, however its role in Diabetic neuropathy (DN) remains unexplored. Methods: A strain of mice (db/db) having type II diabetes were studied for expression of miR-155 in plasma and in sciatic nerves. The miR-155 mimic treated mice were studied for effect on motor and sensory nerve conduction velocities along with blood perfusion in sciatic nerves and response to thermal stimuli test. The mice were evaluated for density of blood vessels, quantity of intra-epidermal nerve fibers (IENF), diameters of axons & thickness of myelin sheath of sciatic nerves. Bioinformatics analysis was done to confirm target genes of miR-155. Results: The db/db mice showed significant suppression of miR-155 in sciatic nerves. The treatment of miR-155 mimic elevated levels of miR-155 in both sciatic nerves and plasma; it also enhanced the blood flow in sciatic nerves and velocities of conduction for both sensory and motor nerves. The treatment showed significant decrease in the threshold to thermal stimuli in db/db mice. A significant improvement in density of perfused blood vessels was observed, along with elevation of IENF and thickness of myelin and axon diameters of sciatic nerves. The treatment attenuated levels of TNF- $\alpha$ , iNOS, IL1- $\beta$  and Ym1. Microarray analysis showed that the treatment decreased the expression of proinflammatory genes TRAF2 and Notch2, SORT1 and were identified as target by *in silico* studies. Conclusion: Treatment of miR-155 mimic in db/db mice attenuated DN, suppressed diabetic associated proinflammatory genes and confirmed miR-155 mimic as therapeutic strategy for treating DN.

**Keywords:** miR-155 mimic, diabetic neuropathy, inflammatory cytokines, db/db mice

## Introduction

Diabetes has been identified as a dangerous health issue encountered globally [1]. Diabetic neuropathy also called as diabetic peripheral neuropathy (DN) has been found to affect at least two third of total diabetes affected public and have also contributed majorly accounting for their disability. DN progresses with symptoms of pain, allodynia and hyperalgesia, which leads to severe abnormalities of neurovascular system [2]. Dysfunction of vascular system mediated by diabetes begins at initial stages and predates the deficits in conduction velocities of nerves [3, 4]. The nerve fibers get impaired due to the damage caused to the micro-vasculature which supplies blood flow to the peripheral nerves resulting in painful symptoms of DN which also include foot ulcer and

loss of sensitivity [3, 5]. Literatures report number of studies suggesting strategies which target the neurovascular function for improving the nerve function in experimental induced DN in animal models [6-8].

So far studies related to DN have suggested involvement of TNF- $\alpha$ , IL-6, IL-1 $\beta$  and IL-8 which are the pro-inflammatory cytokines and MCP-1 which is chemokine in the micro-vascular complications of DN, among them IL-6 and TNF- $\alpha$  have been reported to have major involvement in DN [10, 12]. In a recent report, sciatic nerves showed elevated levels of IL-6 and TNF- $\alpha$  in subject with DN and streptozotocin (STZ) induced DN mice [10]. It was also found that, the mice having deficit of TNF- $\alpha$  failed to acquire changes in nerve conduction velocities and nociceptive behavior [11]. Exposure of sciatic

nerves in DN mice to TNF- $\alpha$  caused decreased motor nerve conduction velocity (MNCV) [10, 13]. These studies suggest that, proinflammatory pathways as valuable targets in reversing the harmful consequences of pro-inflammatory mediators acting on functioning of neurovascular system and hence halting the development of DN.

The small non-coding RNAs also called as microRNAs (miRNAs) have recently discovered to play major role in regulating the development and progression of diabetes induced vascular damage [13]. miR-155 has been recently discovered to be linked with inflammatory response by their property to up-regulate multiple immune cells via inflammatory cytokines, toll-like ligands and some antigens [14-16]. We also found reports suggesting role of miR-155 in inflammation-mediated glomerular endothelial injury in diabetic nephropathy [17]. However, therapeutic involvement of miR-155 in DN remains unclear. In the current work we developed an animal model of type II diabetes which was clinically relevant in features of peripheral neuropathy [18]. The study suggested that upon exposing the DN mice with miR-155 mimic resulted in elevation of blood flow locally in sciatic nerves and caused suppression of DN.

## Material and methods

### *Experimental animals*

For the study, Male BKS.Cg-m $^{+}/^{+}$ Leprdb/J (db/db) mice aging 20 weeks (Jackson Laboratories, Bar Harbor, Maine) (The strain is reported to have the spontaneous diabetes mutation (DB) in the leptin receptor (LEPR) gene) were used. All the animal protocols were in accordance to the draft of Animal protection law of the People's Republic of China-2009 for experimental animals, the study received prior approval from institutional ethical committee of The First Affiliated Hospital of University of South China, Hunan province, China with approval number 6781157A. The control mice comprised of non-penetrant genotype age-matched heterozygotes mice (db/m).

### *Treatment of mice with MiR-155 mimics*

The miR-155 mimic used in the study was brought from Sigma Aldrich USA. The obtained miR-155 mimic was delivered by incorporating it in a RNA delivery system MAXSUPPRESS-OR™ In Vivo RNA-LANCER II. (Bioo Scientific,

Austin, USA) was used according to supplier's protocol. The miR-155 mimic oligos were delivered at two concentration 500 nmol/kg and 1000 nmol/kg of body weight (Once in a week for next four weeks) into the diabetic mice (db/db) and non-diabetic mice (control mice) through tail vein route under isoflurane anesthesia. The Cel-miR-67 mimic, confirmed to poses the least sequence identity with miRNAs in mice and humans and has no any effect on tested miRNA function and was hence selected as negative control (NC). Each treatment group comprised of 10 mice. All the mice after 4 weeks of treatment were sacrificed by ketamine and xylazine anesthesia.

### *Thermal stimulation-production test*

After 4 weeks of treatment the thermal sensitivity test was done in mice. For the same, tail flick and plantar test was done with the help of thermal stimulation meter. The plantar surface was kept directly on the activated stimulator for performing the plantar test. The response time for paw withdrawal against the latent heat was measured. For the tail flick test, the cut off time was adjusted for 10 sec with 40% heating intensity. Six individual test were carried for each experiment/animal at a interval of 20 min and mean readings were reported [19].

### *Test for tactile sensitivity*

To evaluate tactile sensitivity test, we used Von Frey filaments for stimulating the paw withdrawal as discussed earlier [19]. The experimental setup consisted of number of filaments in series having the force ranging from 0.02 to 1.4 g the left hindpaw plantar surface was applied against the filament causing it to buckle. After each stimulus, the paw withdrawal response was measured followed by calculation of 50% paw withdrawal threshold using the formulas as mentioned in published reports [19, 20].

### *Neurophysiology for nerve conduction velocity*

The velocity of sciatic nerve conduction was measured by following the orthodromic procedure, as published earlier [21]. Briefly, model 2100 isolated pulse stimulator (Micro control Instruments Systems, UK) was opted to deliver trigger single square wave current pulses. Two electrodes previously sterilized were planted in the intrinsic foot muscles for recording the electromyography's. The animal rectal temper-

ature was recorded during the experiment and was kept constant at  $37 \pm 1.0^\circ\text{C}$  with the help of a precision water bath. The sensory nerve conduction velocity (SCV) and motor nerve conduction velocity (MCV) and were computed as reported earlier [21].

## *Evaluation of regional sciatic blood perfusion and number of plasma-perfused blood vessels*

The blood perfusion of regional sciatic nerve region was recorded by a laser Doppler flowmeter (Perimed AB, Sweden), as per described process [19] perfusion units (PU), which are values of relative blood flow measured after subjecting the animals to anesthesia. The values of blood flow in sciatic nerve of control mice i.e db/m were considered as control values for comparing results calculated as perfusion ratios. Laser Doppler perfusion method was employed to study blood perfusion in the foot pads, the images were recorded using PIMSoft software. The sensor was located 10 cm above the target foot. The sensor showed signals of blood perfusion in the form of a colored image, the dark red indicated high degree of perfusion whereas the dark blue color showed lower degree of perfusion. For evaluating the blood flow, we recorded the images at particular time periods defining them as regions of interest (ROI) for each mouse. To evaluate perfusion in the blood vessels, 0.2 ml of FITC-dextran (50 mg/ml) was injected intravenously [19]. To determine the vascular density, the FITC-dextran perfused vessels density was divided by total tissue area.

## *The blood glucose test, glycosylated hemoglobin and insulin tolerance test*

The levels of blood glucose were recorded one time in a week with a digital gluco-meter (Bayer, Switzerland), the glycosylated hemoglobin (HbA1C) was done after 2 weeks. For insulin tolerance test, the mice were fasted for 6 h and were intraperitoneally injected with glucose (2 g/kg body weight) or insulin (0.75 U/kg body weight), the blood was withdrawn at different time points by tail vein method.

## *Separation of peripheral blood mononuclear cells and of splenocytes*

The peripheral blood mono-nucleocytes were collected using a sterile density gradient medium (Ficoll-Paque PLUS density gradient media, GE healthcare). For isolation of splenocytes,

the spleens tissues were dissected and were passed by a nylon cell strainer (Fischer scientific USA). The obtained tissues samples after filtering were suspended in lysis buffer and centrifuged. The spleen cells were evaluated for viability and were studied for expression of proteins by Immunoblotting study or RT-PCR.

## *Fluorescence-activated cell sorting (FACS) of live cells*

To isolate cells and to obtain suspension of single cells from spleen tissue, a tissue dislocation enzyme was added (MACS, CA). The tissue were passed through a cell strainer (70  $\mu\text{m}$ ) and added to lysis buffer for obtaining the suspension. Single spleen cells were subjected to staining using anti-mouse anti F4/80 [IQF4/80 Polyclonal] Fluorescein (Immuquest, USA) and anti-mouse CD11b-PE mAb (Invitrogen, USA). The cells labeled with antibodies were subjected to flow cytometry analysis for sorting.

## *Real-time quantitative (RT-PCR) analysis*

RNA isolation kit (ThermoFisher USA) was used for isolating RNAs from the obtained cells, followed by RT-PCR. The  $2^{-\Delta\Delta\text{Ct}}$  method as discussed earlier [22] was used for calculating relative levels of miRNAs compared to U6 snRNA as control. The RNA isolation kit (ThermoFisher USA) was employed to isolate RNAs from sera and control i.e cel-miR-39 mimic was used for normalization of proteins. Quantitative detection of mRNA transcripts was done by RT-PCR, Universal SYBR green quantitative PCR (Sigma Aldrich, USA). The expression of proteins was normalized using  $\beta$ -actin as loading control. The primers used for the study are depicted in **Table 1**.

## *miR-155 target prediction algorithm and PCR array analysis for target genes*

The mouse miR-155 PCR array (Qiagen) was used for profiling the expression of 63 mmu-miR-155-5p target genes. The procedure described in suppliers manual was followed. The expression of mRNAs was studied against the levels of housekeeping genes from the microarray selected as control. The fold changes for every mRNA were evaluated by using the Ct values obtained from PCR array data in the software. The fold regulation was calculated as the negative inverse of the obtained values for fold change  $[-1/2^{-\text{RRT}}]$  which suggested the changes of miR-155-5p target genes compared to the

**Table 1.** Sequence of oligonucleotides primers for real-time PCR

Primer	Sequence (5' to 3')
Notch2 forward primer	ATGACTGCCCTAACCACAGG
Notch2 reverse primer	TGCAGTCATCTCCACTCCAG
TRAF2 forward primer	CACCGGTACTGCTCCTTCTG
TRAF2 reverse primer	TGAACACAGGCAGCACAGTT
SORT1 forward primer	CTACTCCATCCTGGCAGCCAAT
SORT1 reverse primer	GACTTGGAGTAGACAATGCCTCG
iNOS forward primer	TGGTGGTGACAAGCACATT
iNOS reverse primer	AAGGCCAAACACAGCATACC
TNF- $\alpha$ forward primer	ACGGCATGGATCTCAAAGAC
TNF- $\alpha$ reverse primer	GTGGGTGAGGAGCACGTAGT
IL-1 $\beta$ forward primer	GGGCCTCAAAGGAAAGAATC
IL-1 $\beta$ reverse primer	TACCAGTTGGGGAAGTCTGC
YM1 forward primer	GAAGGAGCCACTGAGGTCTG
YM1 reverse primer	CACGGCACCTCCTAAATTGT

samples obtained from control (db/db+miR control). The fold regulation values >1 indicated down-regulation.

#### Immunohistochemical analysis

The immuno-histochemical analysis was done for analyzing sciatic nerves and epidermal foot pads of db/db mice. Sections of 2  $\mu$ m were prepared using Thermo Scientific™ HM 325 Rotary Microtome (ThermoFisher, USA), the sections were studied for myelin thickness after staining with toluidine blue [23]. The morphology of obtained sections was studied using Halo™ image analysis solutions (PerkinElmer, USA). The tissue sections of foot pad were exposed to antibodies against protein gene product 9.5 (1:1000) (Merck Millipore USA) to measure IENFs. All the images were captured at resolution of 40  $\times$  using Carl Zeiss Primo Star Microscope (Carl Zeiss, Germany). The density of nerve fibers at the junction of dermal and epidermal layer were counted and represented as number of fibers/mm of section [19]. The images of the intra-epidermal nerve fibers were captured with the help of LSCM, Olympus FV2000 microscope (Olympus Corporation, Japan).

#### Expression of proteins by Immunoblotting studies and enzyme-linked immunosorbent assay (ELISA)

Immunoblotting studies were done for expression of proteins. Antibodies were selected as described earlier [24]. The antibodies used for

the study were anti-YM1 (Abcam, USA), anti-TNF- $\alpha$  (ThermoFischer, USA), anti-iNOS, anti-PGP9.5 (Abcam, USA), anti-IL-1 $\beta$ , anti-NF- $\kappa$ B p65 and anti- $\beta$ -actin (Abcam, USA), anti-TRAF2, anti-Notch2, anti-Arginase-1 and anti-SORT1 (Santa Cruz Biotech, USA). ELISA was done using ELISA reader (ThermoFisher, USA) for studying expression of IL-1 $\beta$  and TNF- $\alpha$  in samples of sera.

#### Statistical analysis

All the results are represented as mean  $\pm$  %RSD (Relative standard deviation). The comparisons between the groups were done by t-test. Further, one way analysis of variance was done followed by Student-Newman-Keuls test using multiple sample analysis, the value of  $P < 0.05$  was considered as significant.

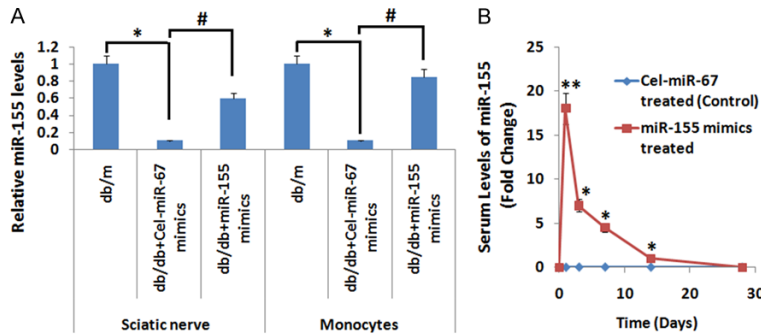
## Results

#### MiR-155 mimics attenuated neurological function in diabetic mice

The outcomes of RT-PCR of sciatic nerve tissues and monocytes suggested major reduction (88%) in levels miR-155 in diabetic mice (db/db) against control mice (db/m) (**Figure 1A**). We further studied the consequence of exogenous miR-155 on DN by studying the serum levels of miR-155, for the same chemically modified miR-155 mimics (1000 nmol/kg body weight) [25] in a single dose were administered to control group mice (db/m) via tail vein, a group of mice was also injected with cel-miR-67 mimics. After injecting miR-155 mimics, the blood was collected on 1<sup>st</sup>, 3<sup>rd</sup>, 7<sup>th</sup>, 14<sup>th</sup> and on 28<sup>th</sup> day, the experiment was repeated thrice for each point. The levels of miR-155 increased in mice receiving treatment of miR-155 mimics compared to those receiving cel-miR-67 over the period of 14 days, maximum increase was observed on the 1<sup>st</sup> day after receiving the treatment (**Figure 1B**).

The study was done to evaluate effects of miR-155 mimics on levels of miR-155 in monocytes and sciatic nerves of db/db mice. The diabetic mice were administered miR-155 mimics (1000 nmol/kg body weight) for one in week for four consecutive weeks. The monocytes from blood and sciatic nerve tissues were recovered 24 h after the last treatment of miR-155 mimic in db/db mice. The results of qRT-PCR showed





**Figure 1.** Expression of miR-155 in sciatic nerves and monocytes of experimental mice. A: qRT-PCR analysis demonstrated that levels of miR-155 decreased in both monocytes as well as sciatic nerves of (diabetic) db/db mice versus the tissues of (non diabetic) db/m mice. B: The results of time serum miR-155 levels with time in db/m mice given single dose of miR-155 mimics versus the levels in db/m mice (control) treated with miR-67 mimics. \*P<0.05, \*\*P<0.01 compared to control group of mice (db/m).

that, the exposure of miR-155 mimics resulted in elevation of miR-155 levels significantly in monocytes as well as in sciatic nerve tissues compared to animals injected with cel-miR-67 mimics (**Figure 1A**). All together, outcomes of our experiment demonstrated that treatment of miR-155 mimics caused elevation of miR-155 expression in blood and sciatic nerve tissues of both diabetic and non-diabetic experimental mice.

We then evaluated the effect of exogenous miR-155 on diabetic neuropathy. The results suggested that, the diabetic mice of 20 weeks old showed a significant decrease (P<0.05) in SCV and MCV (**Figure 2A** and **2B**) and had increased latency to thermal response when evaluated by tail flick method and hot plate test (**Figure 2C** and **2D**) suggesting presence of DN in diabetic mice at the age of 20 weeks. Hence, the diabetic mice (db/db) aged 20 weeks were given treatment of miR-155 mimic at two different doses i.e 500 nmol/kg and 1000 nmol/kg of body weight one time in a week for next four weeks, the cel-miR-67 mimic injected in diabetic mice were the control group as the mice do not express cel-miR-67 [26], we also added similar aged non-diabetic (db/m) mice as an another control group in the study.

The total duration of experiment was eight weeks. We found that the treatment of miR-155 mimic at the dose of 1000 nmol/kg showed a significant improvement in SCV and MCV after the fourth and the eighth week

against the mice injected with cel-miR-67 mimic control. With the 500 nmol/kg dose of miR-155 mimic, we observed significant improvement in both SCV and MCV only after the eighth week of treatment (**Figure 2A** and **2B**). With the thermal response test, the treatment of miR-155 at the dose of 1000 nmol/kg caused a significant decrease in latency of thermal response when recorded by hot plate method and tail flick test, on the other hand the dose of 500 nmol/kg of miR-155 failed to produce significant thermal response

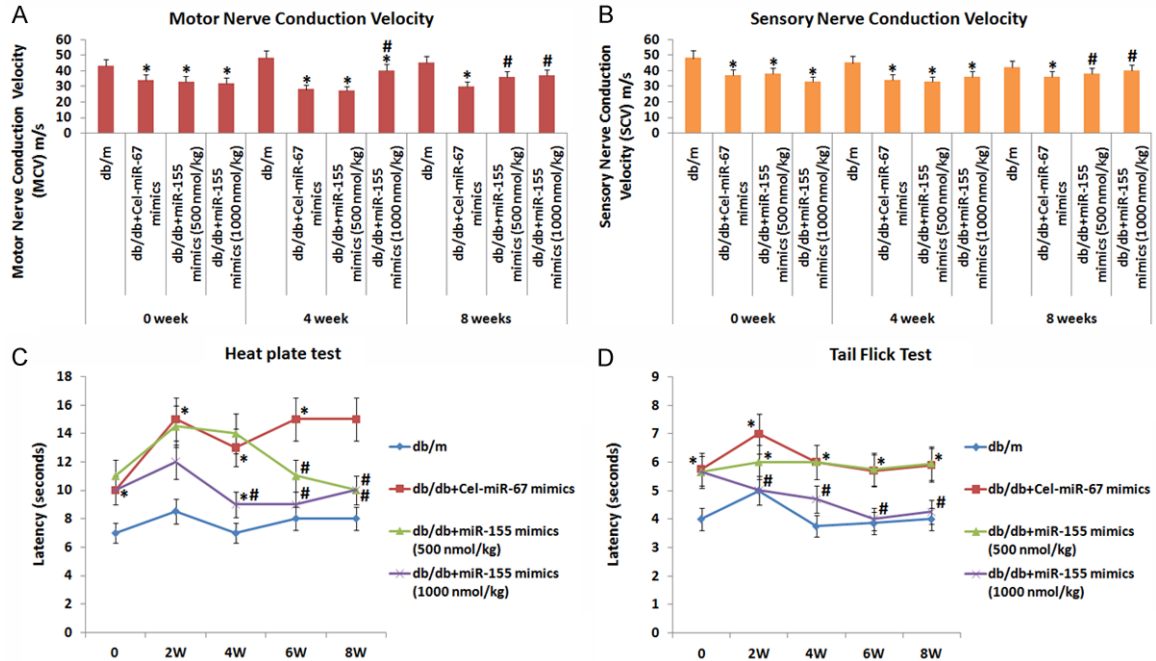
(**Figure 2C** and **2D**). The mice at the end of experiment i.e after the eighth weeks were sacrificed by Ketamine anesthesia intraperitoneally (100 mg/kg body weight). The outcomes of the experiment suggest that, miR-155 mimic at a dose of 1000 nmol/kg of body weight attenuated the neuronal response of diabetic neuropathy mice, hence we selected dose of 1000 nmol/kg for miR-155 mimic further in our study.

#### *Treatment of miR-155 attenuates peripheral tissue perfusion and vascular function in diabetic mice*

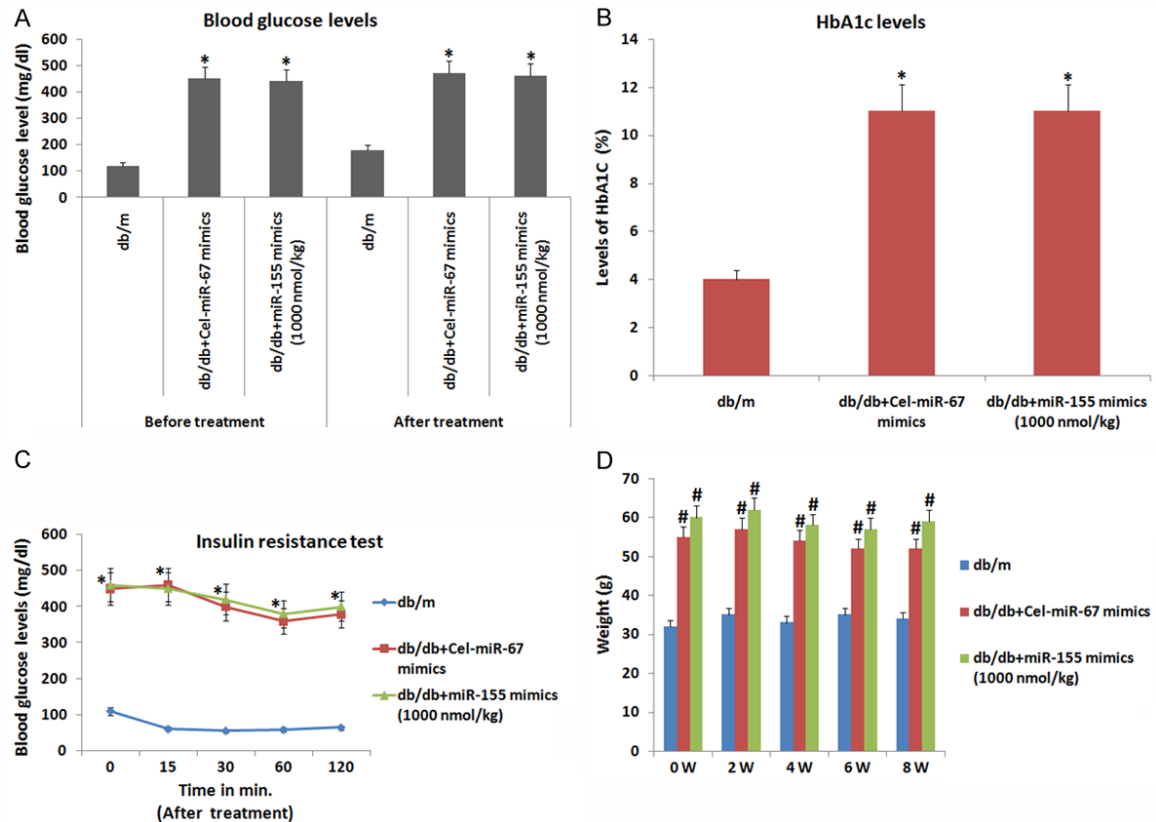
The analysis of blood glucose, HbA1C and insulin resistance suggested no significant alteration produced by treatment of miR-155 mimics in diabetic mice compared to animals receiving treatment of cel-miR-67 mimic after eight weeks (**Figure 3A-C**). The results clearly indicated therapeutic potential of exogenous miR-155 on DN is not mediated by regulating levels of glucose. We also evidenced that, treatment of miR-155 mimics did not produced any potential change in the body weight of diabetic mice compared to the those receiving treatment of miR-67 mimics, on the other hand we found a significant gain in body weight in db/db mice versus the db/m control mice (**Figure 3D**).

Local blood perfusion in sciatic nerves and tissues of foot pad is not well explored in animal model of diabetes. In the present study we discovered that the db/db mice aged 20 weeks showed a significant decrease in blood flow of

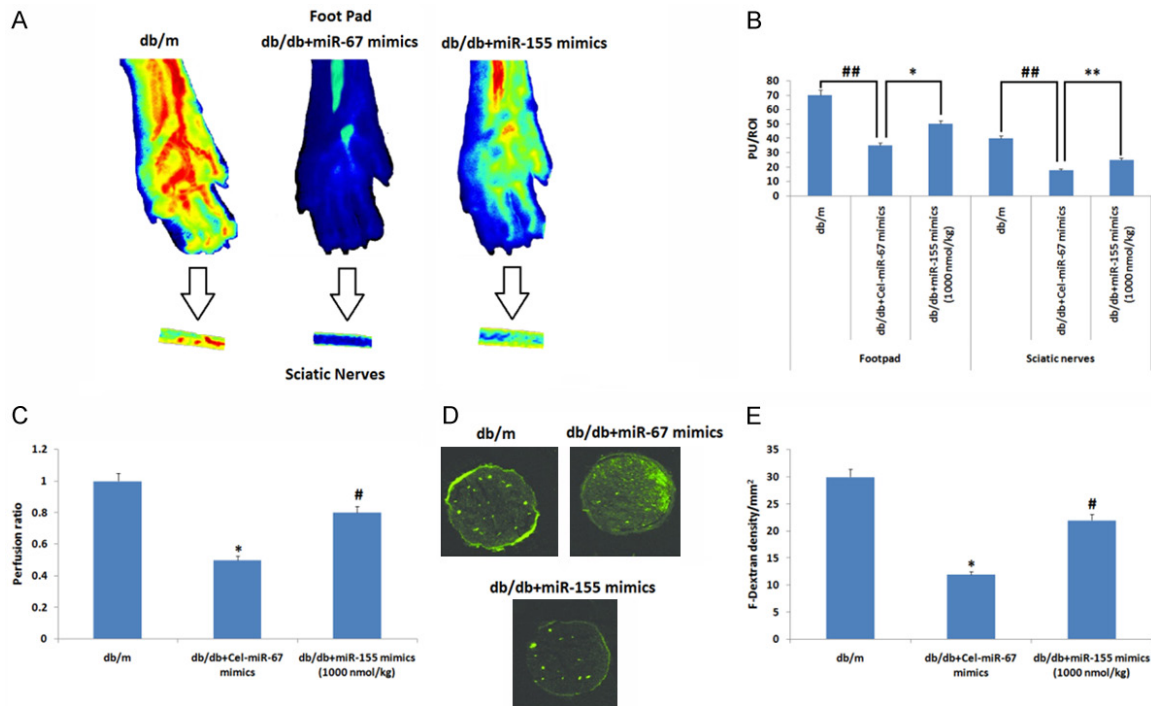
# MicroRNA-155 ameliorates diabetic peripheral neuropathy



**Figure 2.** Treatment of miR-155 mimics attenuates nerve functions. A and B: The test for motor nerve conduction velocity and sciatic nerve conduction velocity shows improvement in neurological functioning. C and D: Significant improvement in latency was observed for heat plate and tail flick test in db/db mice injected with miR-155 mimics at both doses (500 nmol/kg and 1000 nmol/kg) \*P<0.05 compared to db/m mice (non diabetic), #P<0.05 compared to diabetic mice injected with miR-67 mimics.



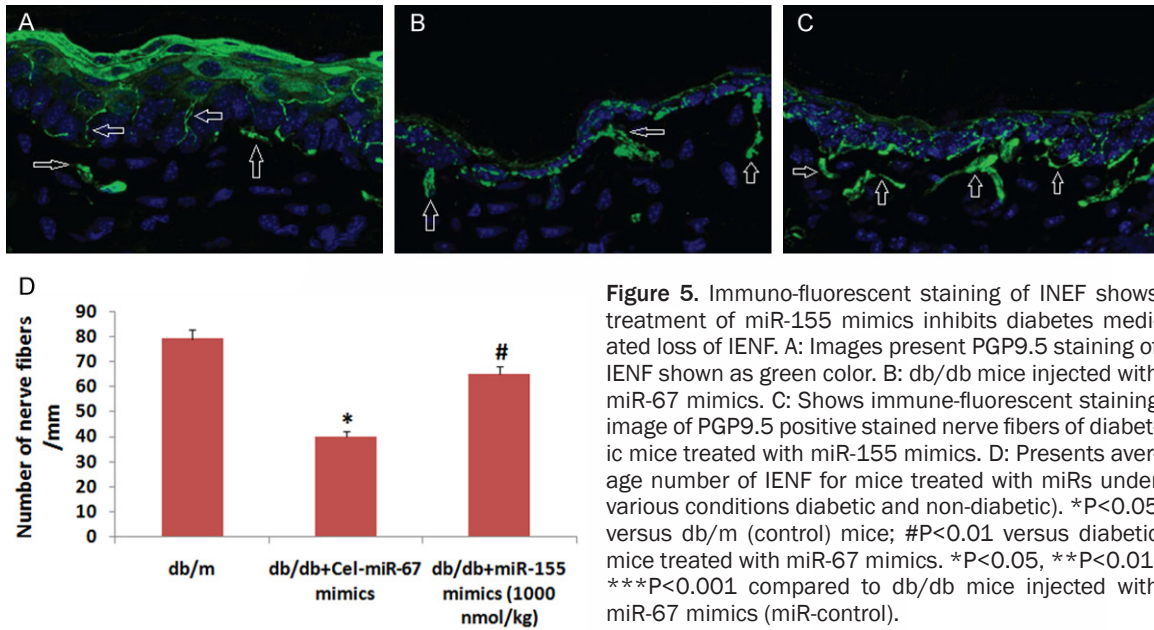
**Figure 3.** The effect of miR-155 mimics blood glucose levels, HbA1C, body weight and insulin-sensitivity. A-C: The results suggested that no significant effect was observed on levels of glucose, HbA1C and insulin resistance test in control (db/m) mice and (diabetic) db/db after eight weeks of the initial dose of miR-155 mimics (1000 nmol/kg) or miR-67 mimics. C: The results of insulin resistance suggested that the tolerance of insulin (1 U/kg) lasted for about 120 min. D: The treatment of miR-155 mimics (1000 nmol/kg) and miR-67 mimics caused significant increase in body weight in diabetic mice compared to non diabetic mice (db/m). \* $P < 0.05$  and # $P < 0.01$  compared to non diabetic mice (db/m).



**Figure 4.** Treatment of miR-155 mimics attenuates neurovascular function. A: The images show blood flow in the region of foot pad along with sciatic nerve tissues eight weeks after the mice received treatment of miR-155 mimics. The blue region marked the low blood flow pattern, the green and yellow indicated intermediate perfusion whereas maximum was observed with portion in red. B: Quantitative evaluation of blood perfusion using Doppler imaging technique used for measuring perfusion. The db/db mice injected with miR-155 mimics showed significant enhancement of blood flow of both foot pad and sciatic nerves. C: Perfusion ratio indicated by blood flow compared to db/m (non diabetic) in sciatic nerve fibers. D: Images of vessels perfused with FITC-dextran. E: Quantitative data of perfused vascular area of non-diabetic (db/m), diabetic mice (db/db) treated with miR-155 mimics or treated with miR-67 mimics called to as control group. # $P < 0.05$  compared to db/db control mice i.e mice treated with cel-miR-67 mimics. \* $P < 0.05$  compared to non-diabetic (db/m) mice.

foot pad tissues compared to same aged db/m mice when analyzed by LDF. We also found that the blood perfusion in db/db mice was further reduced significantly at the age of 28 weeks compared to mice aged 20 weeks. The results hence suggested that diabetes leads to progressive decrease in peripheral tissue blood flow. However, when db/db mice were treated with miR-155 mimic at the age of 20 weeks resulted in a significant rise in regional blood perfusion in the foot pads as well as the sciatic nerve tissues at the age of 28 weeks against the mice treated with miR-67 mimic (**Figure 4A-C**).

Further, to find whether, increase in blood flow mediated by miR-155 mimics was involved in correction of perfusion in blood vessels, we injected the mice with FITC-dextran for circulation in the blood for 5 minutes before sacrificing. The FITC-dextran is reported to circulate in all functional blood vessels [27]. The results of study suggested that miR-155 mimics caused a significant increase in perfusion in sciatic nerves when compared to those treated with miR-67 mimics (**Figure 4D and 4E**). The results suggest that miR-155 mimics resulted in improvement of blood perfusion in peripheral tissue in diabetic mice (db/db) mice.



**Figure 5.** Immuno-fluorescent staining of IENF shows treatment of miR-155 mimics inhibits diabetes mediated loss of IENF. A: Images present PGP9.5 staining of IENF shown as green color. B: db/db mice injected with miR-67 mimics. C: Shows immune-fluorescent staining image of PGP9.5 positive stained nerve fibers of diabetic mice treated with miR-155 mimics. D: Presents average number of IENF for mice treated with miRs under various conditions diabetic and non-diabetic). \* $P < 0.05$  versus db/m (control) mice; # $P < 0.01$  versus diabetic mice treated with miR-67 mimics. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to db/db mice injected with miR-67 mimics (miR-control).

#### *Treatment of miR-155 mimics improves nerve function*

Density of Intra-epidermal nerve fibers (IENF) is reported to be an important diagnostic tool for peripheral neuropathy, it is regarded as a criteria for measuring the effectiveness of any treatment for correcting DN [28]. Here in this study we evaluated the density of IENF in foot pads and observed that the protein gene product 9.5 positive IENFs in diabetic mice (db/db) aged twenty and twenty eight weeks decreased significantly compared to the control mice of similar age (db/m). We also found that, diabetic mice (db/db) showed a significant reduction in IENF densities after eight weeks of treatment compared to their initial state i.e eight weeks before (at the start of miR-155 mimics treatment). On the contrary, the treatment of miR-155 mimics resulted in a complete suppression of diabetes mediated decrease of IENFs when compared to mice treated with miR-67 mimics in diabetic mice at the end of eight weeks (**Figure 5A-D**).

#### *Treatment of miR-155 mimics enhances the myelin thickness*

Disfunctioning of microvasculature is associated with damage of myelin sheath of neurons and axons in peripheral nerves which is contributing factor for progression of diabetic neuropathy [3]. To evaluate the effect of miR-155 mimics on myelin thickness of neurons and axons,

the transverse sections of nerves were stained with toluidene blue and were studied for morphomeric changes using microscope [23]. The thickness of myelin sheath was recorded using Halo™ image analysis solutions (PerkinElmer, USA), the degree of myelination was calculated by g-ratio analysis [19]. The diabetic mice aged 20 weeks showed a substantial decrease in myelin thickness along with neuronal diameter, which worsened more after 8 weeks of study compared to similarly aged control mice (**Table 2**). The db/db mice injected with miR-155 mimics showed increase in thickness of myelin sheath of neurons and also had lower g-ratio compared to miR-67 mimics injected mice after the 8<sup>th</sup> week of study (**Figure 6; Table 3**).

#### *miR-155 mimics regulates Notch2/TRAF2 and their target genes*

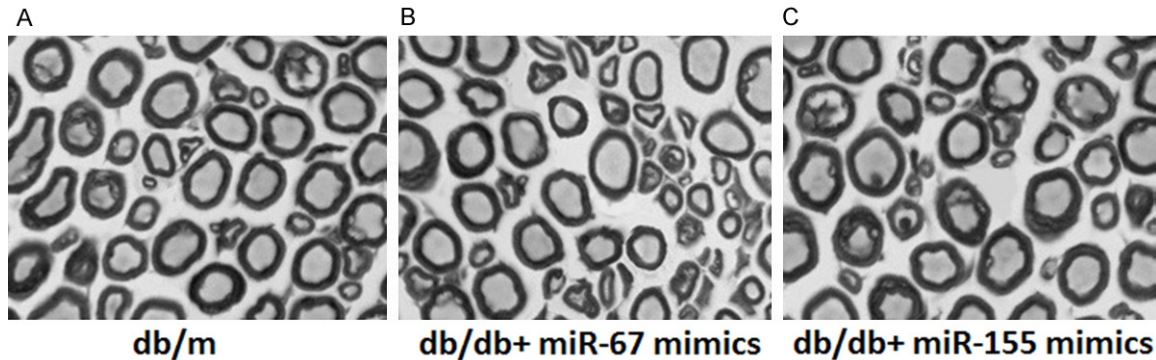
MiRNAs regulate the translation of target gene and its stability. Here in this study using a new miR-155 target PCR array, consisting about 89 experimentally and in silico discovered genes regulated by miR-155, we studied the target genes in sciatic nerves and the monocytes. We discovered that, treatment of miR-155 mimics to db/db mice caused suppression of number of target genes; finally, genes having roles reported for DN, inflammation and immunity were identified through literature survey. Using these criteria, we focused on 13 candidate targets which included Sema3G, IRAK1, BRCA2, DOCK1, ADAMTS3, IRAK2, FADD, Notch2, TRAF2, BCORL1, SORT1, TRAF6 and FAS.



**Table 2.** Morphomeric alterations of myelinated sciatic nerves

Property	db/m (aged 20 Weeks)	db/m (aged 28 weeks)	db/db (aged 20 weeks)	db/db+miR control	db/db+miR-155 mimics
Diameter of Axon ( $\mu\text{m}$ )	4.70 $\pm$ 0.21	4.65 $\pm$ 0.22	4.71 $\pm$ 0.18	4.18 $\pm$ 0.06##	4.64 $\pm$ 0.11**
Diameter of fiber ( $\mu\text{m}$ )	8.12 $\pm$ 0.29	8.20 $\pm$ 0.22	7.4 $\pm$ 0.15@	7.01 $\pm$ 0.11###	8.25 $\pm$ 0.21***
Myelin thickness ( $\mu\text{m}$ )	1.45 $\pm$ 0.05	1.42 $\pm$ 0.07	1.38 $\pm$ 0.05@	1.16 $\pm$ 0.04###	1.44 $\pm$ 0.02***
g ratio	0.57 $\pm$ 0.01	0.56 $\pm$ 0.01	0.64 $\pm$ 0.01	0.59 $\pm$ 0.01#	0.56 $\pm$ 0.005**

The presented values are  $\pm$  SE, @P<0.05 compared to db/m (20 weeks); #P<0.05, ##P<0.01, ###P<0.001 compared to db/m (28 weeks); \*\*P<0.01, \*\*\*P<0.001 compared to db/db+miR-control.



**Figure 6.** Effect of miR-155 mimics on histomorphometric parameters sciatic nerves. A: The images are transverse sections of sciatic nerves stained with toluidine blue of non-diabetic mice. B: db/db mice treated with miR-67 mimics. C: db/db mice treated with miR-155 mimics (1000 nmol/kg).

**Table 3.** Effect of miR-155 mimics on alterations in histomorphometric parameters of sciatic nerves of mice

Property	db/m	db/db+ miR-control	db/db+miR-155 mimics
Diameter of Axon ( $\mu\text{m}$ )	4.65 $\pm$ 0.22**	4.18 $\pm$ 0.06	4.64 $\pm$ 0.11**
Diameter of fiber ( $\mu\text{m}$ )	8.20 $\pm$ 0.22***	7.01 $\pm$ 0.11	8.25 $\pm$ 0.21***
Myelin thickness ( $\mu\text{m}$ )	1.42 $\pm$ 0.07***	1.16 $\pm$ 0.04	1.44 $\pm$ 0.02***
g ratio	0.56 $\pm$ 0.01*	0.59 $\pm$ 0.01	0.56 $\pm$ 0.005**

The presented values are  $\pm$  SE, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 compared to db/db+miR control.

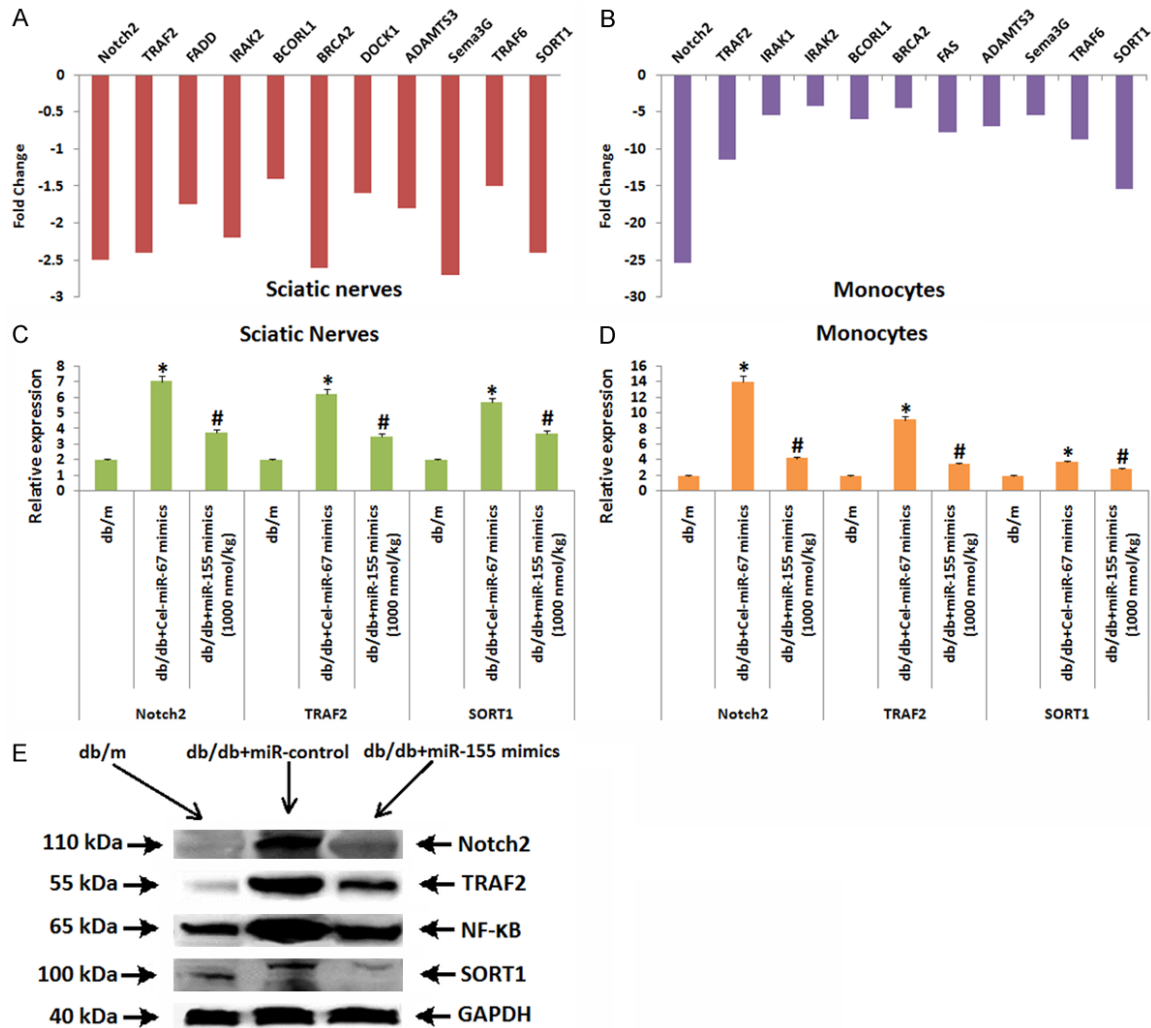
To further affirm the results of microarray analysis, qRT-PCR was performed making use of gene-specific primers. The results showed that miR-155 mimic down-regulated other genes including TRAF2 and Notch2 in both monocytes and sciatic nerve tissues (**Figure 7A, 7B**). Further using qRT-PCR and Immunoblotting technique we validated the mRNA expression along with expression of proteins (**Figure 7E**) of genes which included TRAF2 and Notch2, SORT1 was identified from in silico data (Targets can 4 data base) in monocytes and sciatic nerve tissues of diabetic mice. In a study earlier TRAF2 [29] and Notch2 [30] have been confirmed earlier as favorable targets of miR-

155 and as in silico predicted target of miR-155 and is also reported to play a potential role in inflammation [31]. The diabetic mice receiving treatment of cel-miR-67 mimics showed elevation in TRAF2, Notch2 and SORT1 mRNAs in monocytes (**Figure 7D**) and sciatic nerves (**Figure 7C**). The expression of TRAF2, Notch2 and SORT1 proteins was up-regulated in sciatic nerves (**Figure 7E**)

against control mice, however, the treatment of miR-155 mimics suppressed the expression of TRAF2, Notch2 and SORT1 against the mice treated with miR-67 mimics (**Figure 7C-E**). We also found that, miR-155 mimics suppressed the expression of p65 and NF- $\kappa$ B in sciatic nerves of db/db mice (**Figure 7E**).

#### *Treatment of miR-155 mimics suppresses inflammatory reaction and alters macrophage inflammatory polarization in diabetic mice*

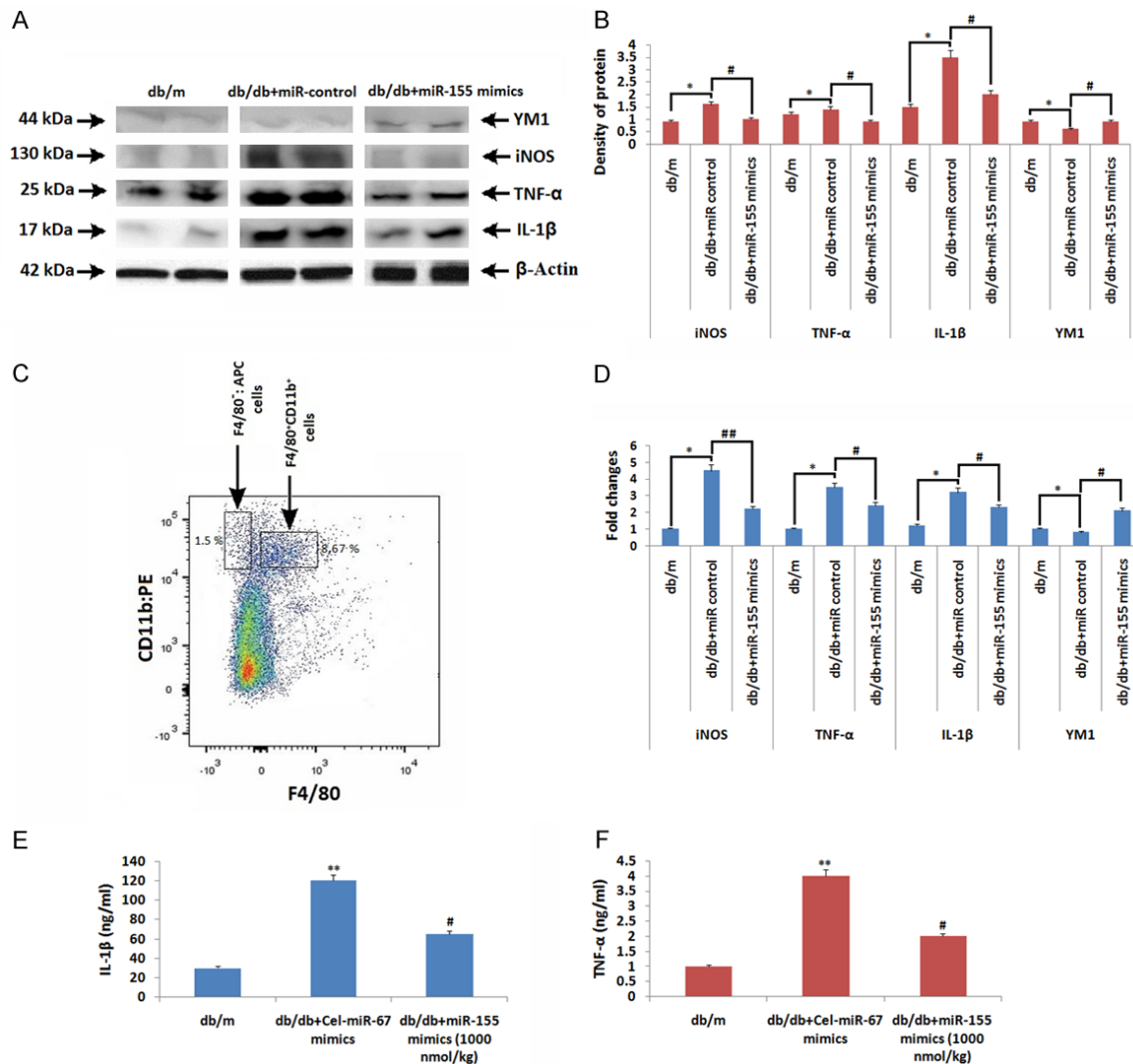
A report recently has suggested that, diabetes is associated with elevated M1 macrophages [32]. To establish correlation between miR-155



**Figure 7.** Treatment of miR-155 mimics decreases inflammatory response by altering expression of target genes. (A) The miR-155 PCR array analysis for target genes shows various target genes regulated by miR-155 in sciatic nerves, (B) monocytes in diabetic mice treated with miR-155 mimics and miR-67 mimics. (C) The results of RT-PCR suggested down regulation of target genes of miR-155 found by miR-155 target PCR array in sciatic nerves and (D) monocytes. Isolated from diabetic mice injected with miR-155 mimics or miR-67 mimics. (E) Expression of target genes by western blot analysis of Notch2, TRAF2 and in silico predicted target gene SORT1 of miR-155 in isolated sciatic nerves from diabetic mice injected with miR-155 mimics or miR-67 mimics and from db/m mice. \* $P < 0.05$ , \*\* $P < 0.01$  versus db/m mice. # $P < 0.05$  versus db/db mice injected with miR-67 mimics.

and macrophage inflammation in mice, we evaluated the levels of genes which served as important indicators of macrophages, classically activated (M1) and alternatively activated (M2) called as pro-inflammatory and anti-inflammatory respectively in the spleen tissues (Figure 8A and 8B) and spleen macrophages (Figure 8C and 8D). The lineage macrophages having F4/80 and CD11b markers were isolated from spleen cell suspension, the cells followed by labeling with antibodies against F4/80 and CD11b (Figure 8C). The results of Immunoblotting and qRT-PCR suggested that, as compared

to db/m mice the db/db showed a substantial elevation in levels of mRNA of M1 genes, iNOS, TNF- $\alpha$  and IL1- $\beta$ , on the other hand a significant decrease in M2 gene, and Ym1 gene in spleen macrophages. Interestingly we observed that, miR-155 mimics reversed the diabetes mediated increased levels of M1 and suppressed the M2 compared to mice treated with miR-67 mimics (Figure 8A, 8B and 8D), indicating that miR-155 is involved in alteration of macrophage phenotype and hence affects inflammation. The outcomes of ELISA suggested that, miR-155 mimics resulted in remarkable reduction



**Figure 8.** miR-155 regulates the polarization of macrophages. (A) Western blot images of expression of pro-inflammatory M1 markers, TNF- $\alpha$ , iNOS and IL1- $\beta$  and (B) their quantitative expression. The results show that levels of pro-inflammatory markers TNF- $\alpha$ , iNOS and IL1- $\beta$  were increased substantially, whereas the levels of Ym1 were suppressed in spleen tissue of db/db mice. The treatment of miR-155 mimics in diabetic mice inverted the expression of TNF- $\alpha$ , iNOS and IL1- $\beta$  along with Ym1 compared to mice receiving treatment of miR-67 mimics. (C) The spleen cells were labeled with antibodies against macrophages markers such as CD11b and F4/80, the FACS was used to sort the cells. (D) The results of RT-PCR for RNAs isolated from spleen cells for expression of TNF- $\alpha$ , iNOS, IL1- $\beta$  and YM1, all the values were normalized against  $\beta$ -actin. (E) The treatment of miR-155 mimics suppressed levels of IL-1 $\beta$  and (F) TNF- $\alpha$  evidenced by results of ELISA in diabetic mice versus mice treated with miR-67 mimics. \* $P < 0.05$  versus db/m mice (non diabetic). # $P < 0.05$  versus db/db mice (diabetic) injected with control miR-67.

of diabetes mediated levels of IL-1 and TNF- $\alpha$  (Figure 8E and 8F).

## Discussion

In the present study, we demonstrate that treatment of miR-155 mimics significantly improves functioning of peripheral nerves; vascular function of sciatic nerves along with myelination of axons in db/db mice, proposing that

exogenous miR-155 may poses therapeutic effect in treating diabetic neuropathy.

In the present research, we demonstrate that diabetes leads to progressive decrease in peripheral blood flow in tissues we also found that DN (db/db) mice when injected with miR-155 mimic showed a significant improvement in perfusion of peripheral nerve tissues myelin thickness of axons and IENF density. The results

suggest that attenuation of neurovascular functioning by miR-155 mimics may be prime cause in ameliorating DN. The findings of our study suggested that, diabetic mice (db/db) aged 20 weeks showed neurovascular dysfunction along with DN followed by impairment of peripheral nerve system as the time progressed during the study (next 8 weeks). The results of the study were parallel to findings reported earlier suggesting that dysfunctioning of microvasculature leads to thinning of myelin sheath and impairs the peripheral nerve system with the progression of DN [3, 4, 33, 34].

The treatment of miR-155 mimic in the db/db mice aged 20 weeks resulted in improvement of neurovascular function, we also evidenced that the treatment failed to reverse the neurovascular damage before treating the mice. Though the present study do not produced the etiology behind the dysfunctioning of vasculature system, damage of nerve tissues along with axonal damage, our findings suggest that improving vascular blood perfusion leads to improvement in functioning of nerve tissues in DN mice, this indicate that all the three events are interconnected in developing DN. The results suggested that dose of miR-155 in db/db mice at beginning of protocol (i.e on 20<sup>th</sup> week) with miR-155 mimics maximized the outcome of the treatment.

We evidenced that, treatment of miR-155 mimics do not decreased the blood glucose levels, HbA1C and the body weight, clearly suggesting that the DN corrective activity of miR-155 mimic do not involve decrease in levels of glucose in diabetic mice. The results of PCR array suggested that miR-155 mimics caused suppression of pro-inflammatory genes such as Notch2, TRAF2 and SORT1 which were target genes of miR-155. Notch2 [35], TRAF2 [36] and SORT1 [37] are reported to be downstream adaptors of a specific family receptors responsible for initiating immune and inflammatory response called to be as Toll like receptors (TLRs). The TLRs named TLR2 and TLR4 are reported to be associated in prolonging of inflammation specifically in DN [38, 39]. Notch2 and TRAF2 are associated with NF- $\kappa$ B, deregulation of TLRs in DN can lead to activation of Notch2, TRAF2 and their associated NF- $\kappa$ B pathway, which leads to synthesis of important chemokines and cytokines [38, 39]. High glucose levels in diabetic

patients leads to formation of thrombosis, also, up-regulation of NF- $\kappa$ B has a major function in diabetic mediated neurovascular dysfunction [40, 41]. The findings indicate that, miR-155 mimics leads to suppression of NF- $\kappa$ B mediated inflammation by targeting Notch2 and TRAF2, causing improvement in peripheral tissue perfusion. We also encountered a report suggesting miR-155 promotes angiogenesis [42]. Hence we suggest that role of miR-155 associated with vascularization specifically in sciatic nerve tissues in diabetic models requires more study.

In conclusion, we confirm that up regulation of levels of miR-155 in DN mice improve the neurovascular dysfunction and ameliorate the condition of peripheral neuropathy in diabetic mice.

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### Disclosure of conflict of interest

None.

### Abbreviations

DN, Diabetic neuropathy; miR-155, Micro RNA; IENF, Intra-epidermal nerve fibers; NC, Negative control.

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