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MiR-20a ameliorates diabetic angiopathy in streptozotocin-induced diabetic rats by regulating intracellular antioxidant enzymes and VEGF

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Abstract. – OBJECTIVE: We evaluated the beneficial effect of miR-20a mimic against diabetic angiopathy (DA) in rats by regulating intracellular antioxidant enzymes and vascular endothelial growth factor (VEGF).

MATERIALS AND METHODS: Diabetes was induced by intraperitoneal administration of streptozotocin (STZ; 65 mg/kg). Rats were then treated with miR-20a mimic (250 nmol/kg orally) for 8 weeks after STZ administration. The effect of miR-20a mimic against DA in rats was evaluated by estimating serum glucose concentration, lipid profile, Lp-a, kidney function test, inflammatory mediators, and markers of endothelial cell function. Markers of oxidative stress in the aortic tissue were estimated in rats treated with miR-20a mimic. Western blot assay, RT-PCR, and histopathology of kidney and myocardial tissues were also performed.

RESULTS: Serum levels of blood glucose and markers of renal function were significantly lower, and the lipid profile improved in the miR-20a mimic group compared to the DA group. Treatment with miR-20a mimic ameliorated the altered markers of endothelial function and oxidative stress, as well as mediators of inflammation, in the DA rats. Protein expressions of ERK1/2, JNK, and p38 MAPK, as well as mRNA expressions of TLR-4 and NF-κB, in aortic tissues were lower in the miR-20a mimic group than in the DA group. The miR-20a mimic group had fewer histopathological changes in kidney and myocardial tissues than the DA group.

CONCLUSIONS: MiR-20a mimic can protect against DA in rats by regulating vascular endothelial function and oxidative stress.

Key Words:
Diabetes, MiR-20a mimic, Oxidative stress, Streptozotocin, Vascular endothelial growth factor.

Introduction

Diabetes mellitus is a chronic metabolic disorder commonly diagnosed with glucose intolerance and hyperglycaemia. More than 60% of those suffering from diabetes are from Asia[1]. Uncontrolled diabetes results in the development of micro- and macroangiopathy. Diabetic angiopathy (DA) causes neuropathy, retinopathy, renal failure, and atherosclerosis. There are several pathogeneses involved in angiopathy in diabetic patients, including oxidative stress and vascular endothelial growth factor (VEGF)[2]. Inflammation enhances neo-vascularisation induced by ischaemia[3]. Additionally, ischaemia activates macrophages and monocytes, which in turn activate further production of matrix metallopeptidases, VEGF, tumour necrosis factor alpha (TNF-α), and interleukin 18 (IL-18)[4]. These inflammatory cytokines enhance the proliferation, migration, and survival of cells by activating phosphatidylinositol-3-kinase/Akt[5]. It is important to identify clinical targets to manage DA effectively.

It was recently discovered that small non-coding RNAs, also called microRNAs (miRs), play a major role as regulators of cellular functions, including metabolism, migration, proliferation, differentiation, and apoptosis[6]. Several clinical and preclinical studies have suggested that miR expression alters the diabetic condition and complications associated with diabetes[7]. Thus, miRs could be a target for managing cardiovascular and renal disorders. MiR-20 is also reported to have beneficial effects on the management of several cancers[8]. Therefore, we evaluated the effects of miR-20a against DA in a streptozotocin (STZ)-induced DA rat model.

Materials and Methods

Animals

Male Sprague Dawley rats weighing 200-230 g were purchased from the Chinese Academy of

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Medical Sciences (Beijing, China). Animals were maintained under a 12-h light/dark cycle with 60 ± 5% humidity and a temperature of 24 ± 3°C. All study protocols were approved by the Institutional Animal Ethical Committee of Wuhan University of Science and Technology, Wuhan, China (IAEC/WUST/2017/07).

Experimental Design

DA develops during the early stages of diabetes; therefore, diabetes was induced by intraperitoneal administration of STZ (65 mg/kg). Induction of diabetes was confirmed by estimating the blood glucose level 72 h after STZ administration. Animals with a blood glucose level > 200 mg/dL were considered diabetic and used for further study. The miR-20a mimic used in this study (Jima Pharmaceutical Technology, Shanghai, China) was delivered with an RNA delivery system (MaxSuppressor™ *In Vivo* RNA-LANCEr II; Bioo Scientific, Austin, TX, USA) following the manufacturer's protocol. Animals were separated into three different groups: the control group; a group that received an STZ and saline solution orally for 8 weeks (DA group); and a group treated with miR-20a mimic (250 nmol/kg orally) for 8 weeks (miR-20a mimic group).

Determination of Biochemical Parameters

Blood serum glucose was determined using an autoanalyzer (UN6030; Wuhan Union Medical Technology Co., Ltd., Hubei, China) following the glucose oxidase-peroxidase method. Serum endothelin and VEGF levels using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc., Minneapolis, MN, USA) per the manufacturer's directions. The immunonephelometric method was used to determine the level of lipoprotein(a) (Lp-a) with N Latex Lp (a) reagent (R&D Systems, Inc., Minneapolis, MN, USA). Colorimetric analyses were conducted to estimate serum concentrations of triglycerides, high-density lipoprotein (HDL), creatinine, and blood urea nitrogen (BUN). A nitric oxide (NO) kit (R&D Systems, Inc., Minneapolis, MN, USA) was used to determine NO levels *via* the Griess reaction.

Determination of Inflammatory Cytokines

Concentrations of inflammatory cytokines, such as TNF-α, NF-κB, IL-6, and IL-18, in the serum of all animals were estimated using ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) per the manufacturer's directions.

Preparation of Tissues

All animals were sacrificed at the end of the treatment protocol, and kidney, cardiac, and aortic tissues were washed with saline solution immediately after isolation. Cardiac and kidney tissues were kept in 4% and 10% formaldehyde solutions, respectively. Aortic tissues were kept at −80°C for further investigation.

Determination of Oxidative Stress

The levels of malondialdehyde (MDA) and reduced glutathione (GSH), as well as activities of xanthine oxidase (XO), catalase (CAT), and superoxide dismutase (SOD), were estimated in aortic tissue following the manufacturers' protocols of the respective test kits (Siemens Healthcare GmbH, Erlangen, Germany). Nitric oxide synthase (iNOS) levels in aortic tissue were determined using an ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA).

Western Blot Assay

Extraction of total protein from isolated aortic tissue was performed after treatment with NP40 protein lysis buffer. Total protein concentration was estimated using a DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Isolated protein was separated using a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 10%) and filtered with a polyvinylidene difluoride (PVDF). membrane. The membrane was then treated with 5% fresh non-fat dry milk to block the reaction, incubated at 4°C overnight with primary antibodies, such as p-JNK, p-ERK1/2, p-p38 MAPK, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and thereafter incubated with secondary antibodies for 60 min at room temperature. Enhanced chemiluminescence (ECL) was used to enhance the blots; densitometric analysis of the blots was performed using Image Lab software (Bio-Rad, Hercules, CA, USA).

RT-PCR

RNA was isolated from separated aortic tissues using TRIzol reagent. A RevertAid First Strand cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to reversely transcribe the RNA. The primers listed in Table I were mixed with RT 2 SYBR Green Mastermix to determine gene expression using the Quantitative SYBR Green PCR assay (Qiagen, Villebon-sur Yvette, France). The procedure used for all samples was as follows: 98°C for 2 min and then 25-40 cycles of 98°C for 10 s, 55°C for 5 s,

Table I. Primer Sequences.

Genes	Sequence
mTLR-4 Forward:	T AG ACACTGTCGCCATGCCT
Reverse:	CGCTGGTGCCTTCGCTATGGT
NF-κB Forward:	5′-GCAGATGGCCCATACCTTCA-3′
Reverse:	5′-CACCATGTCCTTGGGTCCAG-3′
mGAPDH Forward:	GGCATATGTGAAGCAGACGC
Reverse:	GGAACACTGCTGGTAGGAGAG

and 72°C for 20 s. mRNA expression levels were calculated according to relative standard curves. The curves were generated by plotting the quantification cycle (Cq) against the logarithm of total cDNA added to the reaction. The relative target gene-expression levels were determined using the $2^{-\Delta\Delta Cq}$ equation.

Histopathology

Isolated myocardial and kidney tissues were rehydrated with ethanol and seeded into liquid paraffin wax. The wax cubes with embedded tissues (kidney and myocardial) were cut into 4-µm-thick sections a microtome. Tissues were stained with haematoxylin–eosin (H&E) stain. A trinocular microscope was used to observe pathological changes in the tissue sections.

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD; n = 10), and statistical analyses were performed using Graph Pad Prism 5.0 software (San Diego, CA, USA). The groups were compared using Dunnett's post-hoc test, and *p*-values < 0.05 were considered to indicate statistical significance.

Results

MiR-20a Mimic Ameliorates Blood Glucose Level

Figure 1 shows the estimated blood glucose levels of rats treated with STZ and miR-20a mimic. Serum concentrations increased to 294 and 310 mg/dL 72 h after administration of STZ in the DA and miR-20a mimic groups, respectively. After 8 weeks, blood glucose level was greater in the DA group than in the control group (328 *vs.* 101 mg/dL), whereas blood glucose level in the miR-20a mimic group was significantly lower than that of the DA group (117 mg/dL; $p < 0.01$).

MiR-20a Mimic Ameliorates Markers of Endothelial Cell Function

Serum concentrations of markers of endothelial cell function were determined in DA rats treated with STZ and miR-20a mimic (Figure 2). Serum endothelin and VEGF levels in the DA group (197 and 206 pg/mL, respectively) were significantly higher than those of the control group. Furthermore, the serum NO level in the DA group (81.7 pg/mL) was significantly lower than that in the control group. Treatment with miR-20a mimic for 8 weeks ameliorated the serum levels of endothelin, NO, and VEGF in rats with DA.

MiR-20a Mimic Ameliorates Biochemical Parameters

Figure 3 shows the effect of miR-20a mimic on the serum concentrations of biochemical markers, including the lipid profile, Lp-a, and markers of renal function, in rats with DA. The estimated HDL and triglyceride levels were higher in the DA group than in the control or miR-20a mimic groups (Figure 3A). Serum levels of Lp-a, creatinine, and BUN were also significantly greater in the DA group than in the control group and miR-20a mimic group (Figures 3B and C; $p < 0.01$).

MiR-20a Mimic Ameliorates Mediators of Inflammation

Figure 4 shows the effect of miR-20a mimic on the serum levels of inflammation mediators in rats with DA. Serum concentrations of TNF-α, IL-6, IL-18, and NF-κB were greater in the DA group

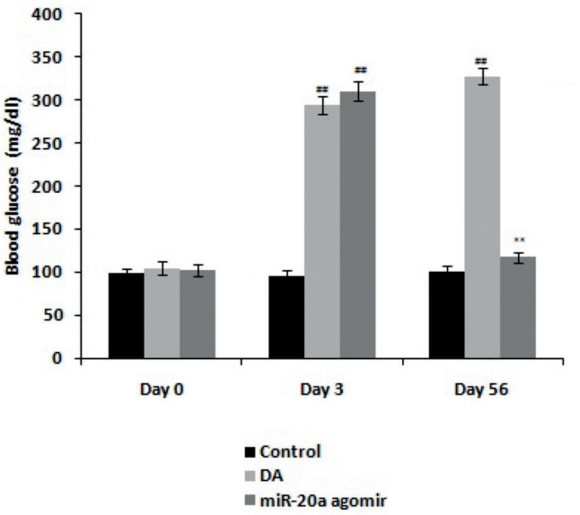

Figure 1. MiR-20a mimic ameliorates the serum glucose level of a diabetic angiopathy (DA) rat model. Mean ± SD (n = 10), $^{\#\#}p < 0.01$ *vs.* control group; $^{**}p < 0.01$ *vs.* DA group.

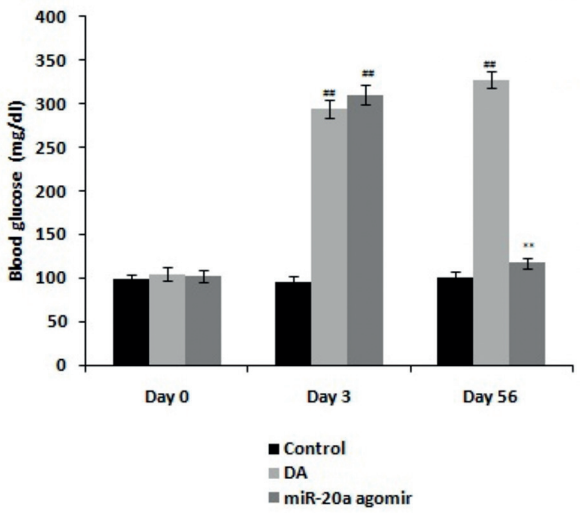

Figure 2. MiR-20a mimic ameliorates the concentration of markers of endothelial cell function in the serum of a diabetic angiopathy (DA) rat model. NO, nitric oxide; VEGF, vascular endothelial growth factor. Mean ± SD (n = 10), $^{\#\#}p < 0.01$ *vs.* control group; $^{**}p < 0.01$ *vs.* DA group.

than in the control group. Treatment with miR-20a mimic reduced the serum concentrations of TNF-α, IL-6, IL-18, and NF-κB compared to levels in the DA group.

MiR-20a Mimic Ameliorates Oxidative Stress

Parameters of oxidative stress, such as levels of MDA and GSH and activities of SOD, CAT, XO, and iNOS, from the aortic tissue homogenate of rats with DA are shown in Table II. MDA and GSH levels were significantly higher and lower, respectively, in the DA group than in the control group. SOD and CAT activities were lower, and XO and iNOS activities higher, in the tissue homogenate of the DA group compared with those of the control. The levels of MDA and GSH and activities of SOD, CAT, XO, and iNOS in the miR-20a mimic group fell in between those of the control and DA groups.

MiR-20a Mimic Ameliorates Protein Expression

Expressions of several proteins, including JNK, ERK, and NF-κBp38, were measured in the aortic tissue homogenate of rats treated with STZ and miR-20a mimic (Figure 5). Relative expressions of p-JNK, p-ERK1/2, and NF-κB-p38 in the aortic tissue homogenate were greater in the DA group than the control and miR-20a mimic groups.

MiR-20a Mimic Ameliorates the mRNA Expression of TLR-4 and NF-κB

Figure 6 shows the effect of miR-20a mimic on the mRNA expression of NF-κB and TLR-4 in the aortic tissues of the DA rat model. The relative mRNA expression of genes for TLR-4 and NF-κB in the aortic tissue homogenate was greater in the DA group than in the control and miR-20a mimic groups.

Effect of MiR-20a Mimic on Kidney and Myocardial Tissues

The histopathology of renal and myocardial tissues was observed in a DA rat model using H&E staining (Figures 7A and B). The transverse section of kidney tissue showed normal morphology in the control and miR-20a groups, whereas that of the DA group showed leukocyte infiltration and swelling, congestion, haemorrhage, and tubules in the glomeruli (Figure 7A).

Figure 7B shows the histopathological changes determined by H&E staining. The transverse sec-

Table II. MiR-20a mimic ameliorates the markers of oxidative stress in the aortic tissue of a diabetic angiopathy rat model.

Sr. No.	Group	MDA (nmol/mg protein)	GSH (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	XO (mU/mg protein)	iNOS (U/mg protein)
1	**Control**	1.16 ± 0.02	9.62 ± 0.26	2.36 ± 0.04	0.72 ± 0.03	1.26 ± 0.05	1.28 ± 0.07
2	**DA**	7.41 ± 0.06$^{\#\#}$	3.74 ± 0.09$^{\#\#}$	1.14 ± 0.01$^{\#\#}$	0.21 ± 0.01$^{\#\#}$	2.13 ± 0.07$^{\#\#}$	2.14 ± 0.16$^{\#\#}$
3	**miR-20a agomir**	2.82 ± 0.03**	4.49 ± 0.14**	1.39 ± 0.03**	0.57 ± 0.02**	1.59 ± 0.09**	1.52 ± 0.09**

Abbreviations: DA, diabetic angiopathy; MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; XO, xanthine oxidase; iNOS, nitric oxide synthase. Mean ± SD (n = 10), $^{\#\#}p < 0.01$ *vs.* control group; $^{**}p < 0.01$ *vs.* DA group.

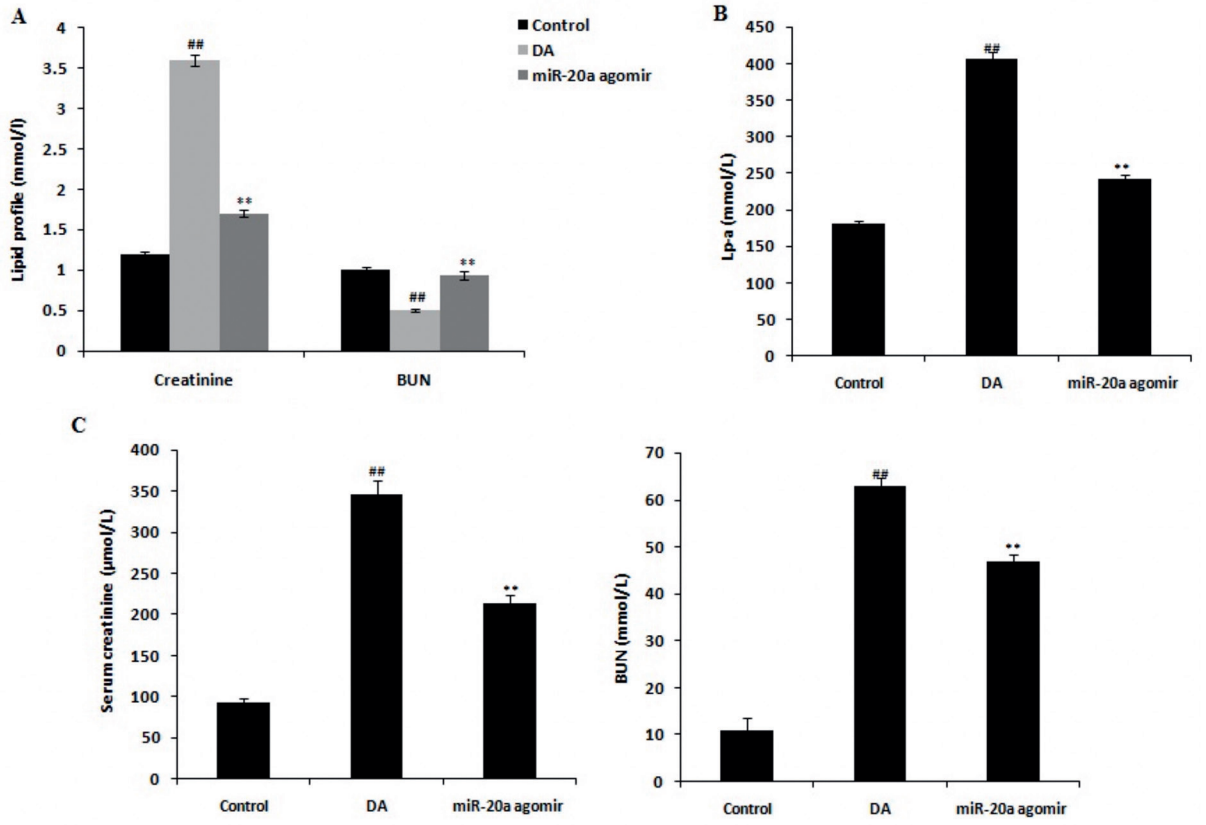

Figure 3. MiR-20a mimic ameliorates the concentration of biochemical markers in the serum of a diabetic angiopathy (DA) rat model. **A**, Lipid profile; **B**, Level of lipoprotein(a) (Lp-a); **C,** Markers of renal function. BUN, blood urea nitrogen. Mean ± SD (n = 10), $^{\#\#}p < 0.01$ *vs.* control group; $^{**}p < 0.01$ *vs.* DA group.

tion of myocardial tissue had normal morphology and structure in control group; however, tissue from the DA group showed erythrocytes and neutrophil-infiltration damage to the myocardial fibres, as well as myocyte necrosis, oedema, and an enlarged intracellular space. Treatment with miR-20a mimic attenuated the damage to myocardial tissue in rats with DA.

Discussion

Diabetic complications commonly associated with micro- and macroangiopathy and various pathogenic pathways are responsible for the development of DA. Research suggests that miR-20a plays a role in the management of ischaemia-induced cardiac injury[9]. The present study found a beneficial effect from using a miR-20a mimic in rats with STZ-induced DA. This effect was evaluated by estimating serum concentrations of glucose, the lipid profile, Lp-a, markers of renal function, inflammatory mediators, and markers of endothelial cell function. Additionally, markers of oxidative stress in aortic tissue were estimated in rats treated with miR-20a mimic, and Western blot assay, RT-PCR, and histopathology of kidney and myocardial tissues were also performed.

Several factors contribute to the development of DA, including uncontrolled glucose levels and hyperlipidaemia[10]. Hyperglycaemia leads to changes in lipid metabolism to compensate for the energy needs of the body[11]. Hyperlipidaemia contributes to endothelial cell dysfunction, which further plays a role in developing vascular complications[12]. In diabetes, controlling blood glucose levels and the lipid profile are reported to protect against diabetic complications associated with microangiopathy[13]. Our results showed that miR-20a mimic reduced blood serum glucose levels and improved the lipid profile compared to those in the DA group. Apoptosis of endothelial cells has been reported to be inhibited by VEGF, whereas endothelin, a strong vasoconstrictor with proinflammatory effects, impacts vascular function by acting on proliferative endothelial cell-derived peptides[14]. Moreover, endothelin and

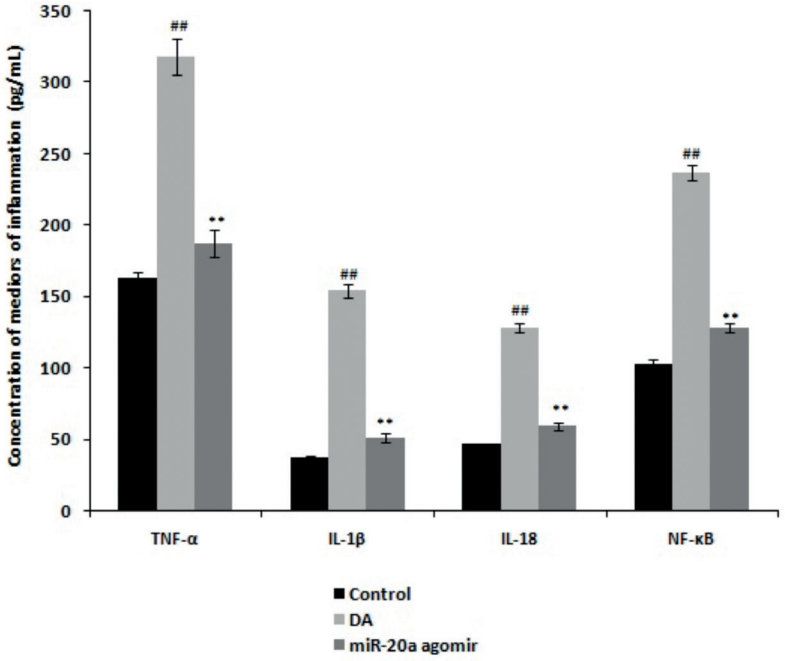

Figure 4. MiR-20a mimic ameliorates the level of mediators of inflammation in the serum of a diabetic angiopathy (DA) rat model. Mean ± SD (n = 10), $^{\#\#}p < 0.01$ *vs.* control group; $^{**}p < 0.01$ *vs.* DA group.

NO are responsible for the progression of endothelial dysfunction. DA and atherosclerosis occur due to overexpression of endothelin receptor[15]. The results of this study suggest that treatment with miR-20a mimic can attenuate the altered serum levels of VEGF, NO, and endothelin in rats with DA.

Inflammatory mediators are a major risk factor contributing to the development of DA[16], and therapeutic intervention can reduce the occurrence of diabetic complications. In this study, treatment with miR-20a mimic reduced the levels of inflammatory mediators in the serum of rats with DA. Inflammatory mediators and the lipid profile are also reported to be elevated under diabetic conditions, which further increases oxidative stress[17]. Our results showed that the miR-20a mimic treat-

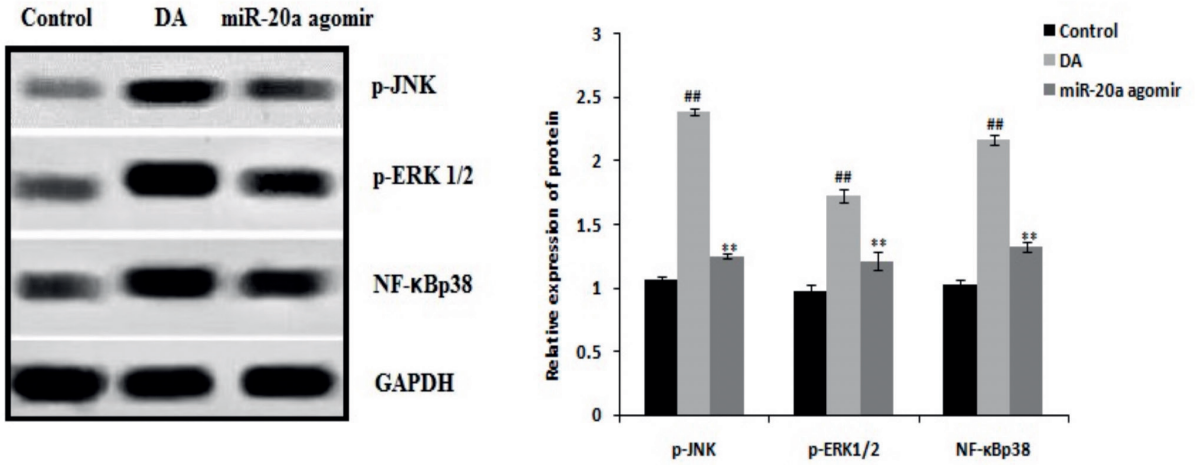

Figure 5. MiR-20a mimic ameliorates the expression of proteins such as JNK, ERK, and NF-κBp38 in the aortic tissue of a diabetic angiopathy (DA) rat model. Mean ± SD (n = 10), $^{\#\#}p < 0.01$ *vs.* control group; $^{**}p < 0.01$ *vs.* DA group.

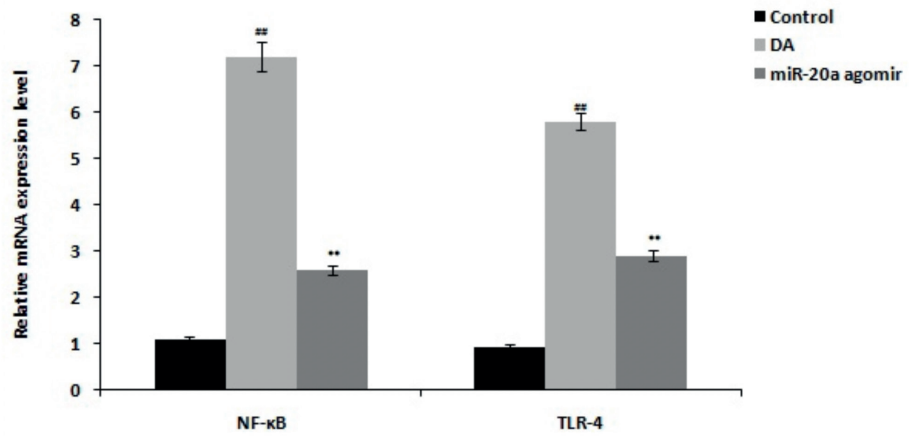

Figure 6. MiR-20a mimic ameliorates the mRNA expression of genes such as NF-κB and TLR-4 in the aortic tissues of a diabetic angiopathy (DA) rat model. Mean ± SD (n = 10), $^{\#\#}p < 0.01$ *vs.* control group; $^{**}p < 0.01$ *vs.* DA group.

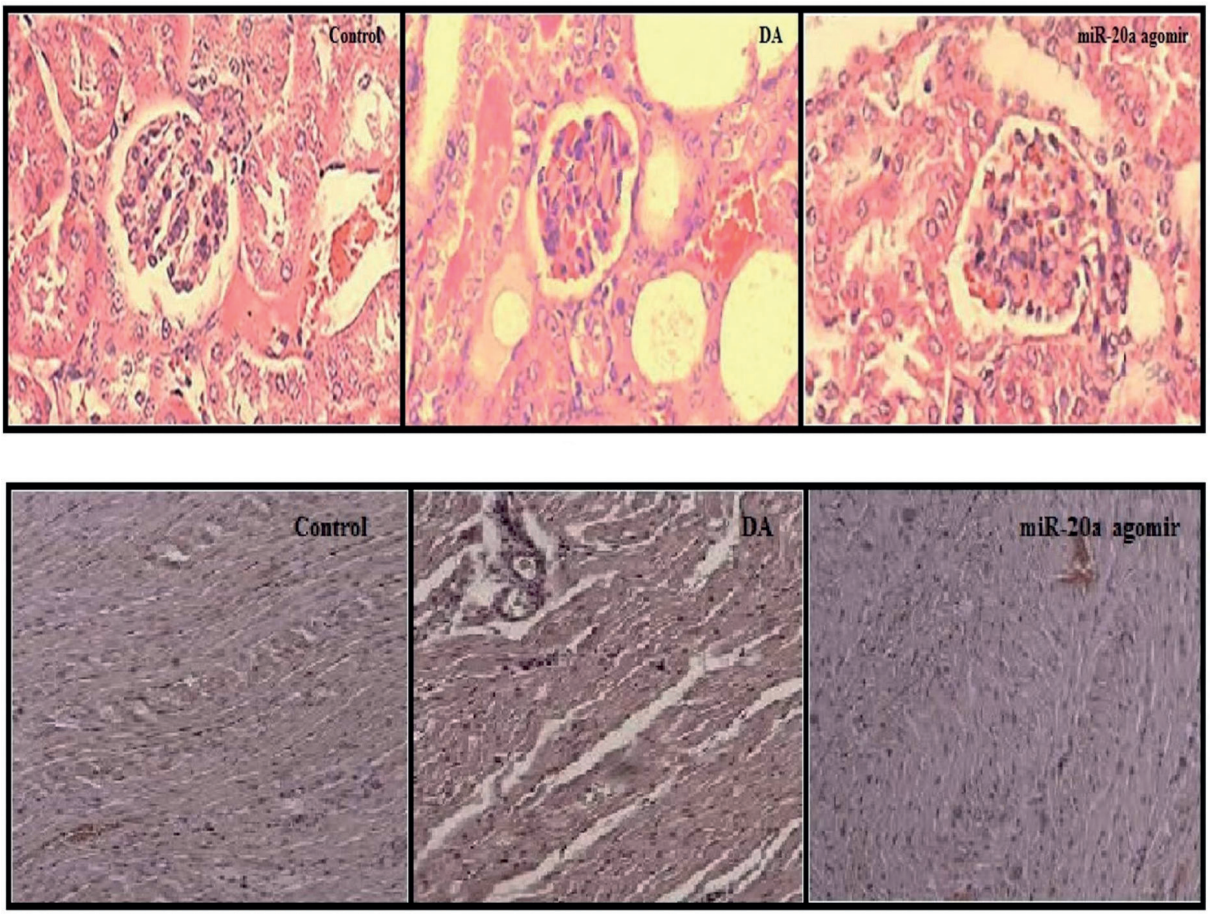

Figure 7. MiR-20a mimic ameliorates the altered pathological physiology of kidney and myocardial tissues of a diabetic angiopathy (DA) rat model (200_X). **A**, Transverse section of kidney tissue; **B**, Transverse section of myocardial tissue.

ment helped to reduce the markers of oxidative stress in rat aortic tissue.

JNK, nuclear factor-κB, TLR-4, ERK, and MAPK proteins play a role in the regulation of endothelial function and oxidative stress[18]. Our results showed that, in rats with DA, expressions of JNK, nuclear factor-κB, TLR-4, ERK, and MAPK proteins were altered but could be ameliorated by treatment with miR-20a mimic, which also helped to improve the histopathology of kidney and myocardial tissues. DA is related to histopathological alteration of blood vessels, which contributes to the development of diabetic complications, such as diabetic nephropathy, neuropathy, retinopathy, cardiomyopathy, and gastroparesis. Therefore, although we focused on the effect of miR-20a mimic on DA-induced pathological complications in kidney and myocardial tissues, the results may also be applicable to DA-induced retinal and gastroparesis complications.

Conclusions

The present study showed that miR-20a mimic helped to protect against DA in rats by regulating vascular endothelial function and oxidative stress. The miR-20a mimic also reduced inflammatory mediators and improved the lipid profile and markers of renal function in the serum of rats with DA.

Conflict of Interests

The Authors declare that they have no conflict of interests.

Acknowledgements

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