MiR-34a Protects Mesenchymal Stem Cells From Hyperglycaemic Injury Through The Activation of The Sirt1/FoxO3a Autophagy Pathway

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Research

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Abstract

Background

Autologous mesenchymal stem cells (MSCs) are favourable treatments for ischaemic diseases; however, MSCs from diabetic patients are not useful for this purpose. Recent studies have shown that the expression of miR-34a is significantly increased in patients with hyperglycaemia, the precise role of miR-34a in MSCs in diabetes need to be clarified.

Objective

The aim of this study is to determine the precise role of miR-34a in MSCs exposed to hyperglycaemia and in healing the heart after myocardial infarction in diabetes mellitus (DM) rats.

Methods

DM rats were established by STZ injection. MSCs were isolated from the bone marrow of donor rats. Chronic culture of MSCs under high glucose was used to mimic the DM microenvironment. The role of miR-34a in regulating cell viability, senescence and paracrine effects were investigated using a cell counting kit-8 (CCK-8) assay, senescence-associated β-galactosidase (SA-β-gal) staining, and VEGF and bFGF ELISA, respectively. The expression of autophagy- and senescence-associated proteins in MSCs and silent information regulator 1 (SIRT1) and forkhead box class O 3a (FOXO3a) were analysed by western blotting. Autophagic bodies were analysed by transmission electron microscopy (TEM). The MI model was established by left anterior descending coronary artery (LAD) ligation, and then the mice were transplanted with differentially treated MSCs intramuscularly at sites around the border zone of the infarcted heart. Thereafter, cardiac function in mice in each group was detected via cardiac ultrasonography at 1 week and 3 weeks after surgery. The infarct size was determined through a 2,3,5-triphenyltetrazolium chloride (TTC) staining assay, while myocardial fibrosis was assessed by Masson staining.

Results

The results of the current study showed that miR-34a was significantly upregulated under chronic hyperglycaemia exposure. Overexpression of miR-34a was significantly associated with impaired cell viability, exacerbated senescence and disrupted cell paracrine capacity. Moreover, we found that the mechanism underlying miR-34a-mediated deterioration of MSCs exposed to high glucose involves the activation of the Sirt1/Foxo3a autophagy pathway. Further analysis showed that miR-34a inhibitor-treated MSC transplantation could improve cardiac function and decrease the scar area in DM rats.

Conclusions

Our study demonstrates for the first time that miR-34a mediates the deterioration of MSC functions under hyperglycaemia. The underlying mechanism may involve the Sirt1/Foxo3a autophagy signalling
pathway. Thus, inhibition of miR-34a might have important therapeutic implications in MSC-based therapies for myocardial infarction in DM patients.

**Background**

Type 2 diabetes mellitus is a whole-body disease, and cardiovascular diseases (CVDs), especially myocardial infarction (MI), are the major causes of mortality in patients with type 2 diabetes mellitus (T2DM). Although the treatment of patients with MI has improved, many myocardial infarction patients with diabetes die from acute MI or suffer from heart failure after MI, which suggests that some vital pathophysiological mechanisms have been neglected.

With the development of cell therapy research, stem cell-based cell therapy is an effective intervention for myocardial infarction. The transplantation of MSCs plays a vital role in paracrine functions and contributes to cardiac repair through mechanisms involving cytoprotection, neovascularization and inhibition of apoptosis, all of which minimize ischaemic reperfusion injury[1, 2]. However, the therapeutic potential of MSCs is impeded by their poor survival rate after transplantation in the harsh hyperglycaemic microenvironment of the infarcted myocardium in the context of diabetes. Autologous MSC therapies have superiority in appropriately matched donors with immunological complications [3]. Therefore, improving the function of MSCs in diabetic patients will become a hot topic.

MicroRNAs (miRNAs), a class of ~21-23 nucleotide-long noncoding RNAs, are critical repressors of gene expression through binding to the 3′-untranslated region (UTR) of target mRNAs[4]. miRNAs have been reported to be involved in mediating multiple biological processes in stem cells, including cell division, differentiation, and survival [5]. Recent studies have shown that the expression of miR-34a was significantly increased in patients with hyperglycaemia [6]. MiR-34a mediates the inhibitory effect of metformin on pancreatic tumours[7], and overexpression of miR-34a exacerbates endothelial dysfunction and decreases angiogenesis under high glucose conditions[8]. Clinical studies have shown that compared to that of control patients, miR-34a was significantly elevated in the serum of patients with myocardial infarction and diabetes mellitus, suggesting that miR-34a may be involved in the occurrence and progression of myocardial infarction and diabetes mellitus.

With the development of functional studies, the maladjustment of energy metabolism in stem cells under high glucose stress leads to impaired stem cell functions and weakened abilities to repair myocardial infarction. Related studies have shown that autophagy is an important mechanism for regulating homeostasis and energy metabolism in patients with hyperglycaemia. Recently, autophagy activation was observed in a fructose-induced mouse model of IR, which was associated with increased myocardial peroxidation products, fibrosis and cell death[9]. Increasing evidence has revealed that autophagy dysfunction is involved in cell death in diabetic mice. Some studies showed that autophagy could promote cell survival under hyperglycaemic conditions, while the opposite result was observed in the hearts of DM mice administered isometric corn oil by gavage[10]. These variable results prompted us to explore the precise role of autophagy in diabetic hearts.
The present study aimed to explore the dynamic relationship between functional changes of MSCs, autophagy and miR-34a under high glucose conditions. The initiation and stability of autophagy are very complex and can be controlled by many factors in the microenvironment. Foxo3a is an important autophagy factor. Foxo3a can be activated by AMPK, and activated Foxo3a can induce the expression of the autophagy-related proteins lc3b-ii, Gabarapl1 and Beclin1 and promote the expression of autophagic proteins at the site of autophagy initiation. Foxo3a can also be activated by Sirt1/Sirt3, thereby activating its downstream factor PINK1, which can strongly activate PARKIN, causing mitochondrial division and activating mitochondrial autophagy, thus playing a cardioprotective role[11]. Activation of autophagy by the FoxO transcription factor may be an important way to reduce the myocardial infarction area, slow the negative remodelling of the left ventricular myocardium, promote the survival of cardiomyocytes, and maintain left ventricular function[12].

Based on these background studies, we hypothesized that miR-34a influences the energy metabolism of stem cells by regulating autophagy, thereby affecting stem cell survival, secretion and senescence, which affect the efficacy of stem cell therapy in diabetic myocardial infarction.

Materials And Methods

Reagents

Penicillin, streptomycin and HRP-conjugated goat anti-mouse IgG (H+L) were obtained from Zhongshan Golden Bridge Biotechnology (Beijing, China), the senescent cell histochemical staining kit was obtained from Beyotime Institute of Biotechnology (Haimen, China), and the cell counting kit-8 (CCK-8) assay was obtained from Tongren Institute of Chemistry (Japan). VEGF and bFGF enzyme-linked immunosorbent assay (ELISA) kits were purchased from Wuhan Yunkelong Technology Co., Ltd. (Hubei, China). Sirt1 and Foxo3a were both from Cell Signaling Technology (Danvers, MA, USA), LC3 was from Abcam (Cambridge, MA, USA), Beclin1 was from Affinity Biosciences (OH, USA), p21 and p16 were obtained from Hua’an Biotechnology Co., Ltd., (Zhejiang, China).

Experimental diabetes model

All animals were housed in an accredited facility with appropriate temperature and humidity. The rats were fed standard chow and provided water ad libitum. The experimental protocols were approved by Xuzhou Medical University. To induce the DM animal model, the rats were fed a high-fat diet for 8 weeks, intraperitoneally injected with streptozotocin (STZ) (25 mg/kg/d in 0.1 mM sodium citrate buffer; Sigma, St. Louis, MO, USA) and then fed a high-fat diet for 4 weeks. Blood samples were then collected to determine the blood glucose (BG) levels using an automatic BG monitor (LifeScan Inc., Milpitas, CA, USA). Animals with fasting glycaemia levels higher than 300 mg/dl and presenting signs of polydipsia and polyuria were considered diabetic and were selected for subsequent study.

Cell culture and treatments
MSCs were isolated from the bone marrow of Sprague-Dawley (SD) rats (weighing 60–80 g), as previously described. Briefly, the femurs and tibias were removed from the SD rats, and the bone marrow was washed out using 10 ml of Dulbecco's modified Eagle's medium/low glucose (DMEM/L; HyClone, Waltham, MA, USA) supplemented with 1% penicillin/streptomycin (Beyotime Institute of Biotechnology, Haimen, China). The cells were centrifuged at 300 ×g for 5 min. The resulting cell pellets were resuspended in 6 ml of DMEM/L supplemented with 10% foetal bovine serum (HyClone) and 1% penicillin/streptomycin and plated in a 25 cm² plastic flask at 37°C in a humidified atmosphere containing 5% CO2 to allow the MSCs to adhere. After culturing the cells for 3 days, the medium was changed, and the nonadherent cells were removed. The medium was replaced every 2 days. Upon reaching 80–90% confluence, the adherent cells were detached from the dishes using 0.25% trypsin (Beyotime Institute of Biotechnology) and expanded at a dilution of 1:2 or 1:3. For the normal glucose group, the cells were cultured with 5.5 M glucose, while the high glucose group was cultured with 25 M glucose. Cells were cultured for as long as 28 days.

**Cell transfection**

Before transfection, MSCs were replated into six-well plates at a density of 2 × 10^5 cells/well and incubated overnight. For overexpression or inhibition of miR-34a, cells were transfected with 20 nM of miR-34a mimic or miR-34a inhibitor (both from GenePharma Co., Ltd, Shanghai, China), respectively. For Sirt1 inhibition, 100 nM Akt siRNA (GenePharma Co., Ltd, Shanghai, China) was transfected into cells. All miRNAs and siRNA were transfected into MSCs using a commercial transfection reagent (X-treme siRNA transfection reagent; Roche Applied Science, Penzberg, Germany) according to the manufacturer’s protocol. Forty-eight or seventy-two hours after transfection, the cells were harvested for further analysis.

**Senescence-associated β-galactosidase staining**

MSC senescence was determined by in situ staining for senescence-associated β-galactosidase (SA-β-gal) using a senescent cell histochemical staining kit (Beyotime Institute of Biotechnology, Haimen, China). Briefly, after treatment, MSCs were first fixed for 30 min at room temperature in fixation buffer. The cells were washed with PBS and incubated with β-galactosidase staining solution for 16 h at 37°C without CO2. The reaction was stopped by the addition of PBS. Statistical analysis was performed by counting 600 cells for each sample.

**Cell proliferation assay**

Cell proliferation was assessed with the cell counting kit-8 (CCK-8) assay (Tongren Institute of Chemistry, Japan). For the CCK-8 assay, cells were plated onto 96-well plates (3×10^3 cells/well). Assays were performed from 1 to 4 days after plating with the addition of 100 µl of fresh medium and 10 µl of CCK-8 solution for another 2 h at 37°C. The optical density (OD) at 450 nm was measured. The assay was repeated three times.

**Cytokine measurement via ELISA**
Using 500 μl of supernatant containing $5 \times 10^5$ cells, the VEGF and bFGF concentrations were assessed by a standard sandwich ELISA (Wuhan Yunkelong Technology Co., Ltd., Hubei, China) according to the manufacturer's instructions. The VEGF and bFGF concentrations are expressed in nanograms per millilitre and were calculated from calibration curves constructed from serial dilutions of human recombinant standards. The sensitivity of the VEGF and bFGF assays was 2 pg/ml.

**Western blotting**

After the designated treatments, the cells were lysed in RIPA buffer. The protein concentration was determined with a bicinechonic acid (BCA) kit (Beyotime, Haiman, China) according to the manufacturer's instructions. For western blot analysis, 50-80 μg of denatured protein was separated on SDS-PAGE gels and transferred onto PVDF membranes. The membranes were blocked with 5% skim milk in TBS containing 0.5% Tween 20 (TBST) for 1 h at room temperature (RT) on a shaking table. The membranes were then incubated overnight at 4°C with primary antibodies diluted in TBST. The membranes were subjected to three 10-min washes with TBST (TBS containing 0.5% Tween 20) and then reprobed with HRP-conjugated secondary antibodies at RT for 1 h. The membranes were then washed three times with TBST as described previously and visualized with an ECL detection system and Beyo ECL Plus reagent (Beyotime, Haimen, China), and the images were analysed using Image Lab software (version 4.1, Bio-Rad). The following antibody dilutions were used: antibodies against p21 (1:1000), p16 (308) (1:1000), Sirt1 (473) (1:1000), FoxO3a (1:1000), LC3 (1:750) and Beclin1 (1:1000), and HRP-conjugated secondary mouse, rabbit and goat antibodies (1:5000).

**Transmission Electron Microscopy (TEM)**

MSCs were fixed, soaked, dehydrated stepwise in an alcohol series, displaced, embedded, polymerized, sectioned, stained, and observed using an electron microscope (JEM-2000EX TEM, JEOL, Tokyo, Japan).

**MI model and MSC transplantation**

To induce an acute MI model, the left anterior descending coronary artery (LAD) was ligated as previously described (Liang et al., 2019). After LAD ligation, the rats randomly received one of the following treatments: (a) sham; (b) MI; (c) $3 \times 10^5$ LG MSCs; (d) $3 \times 10^5$ HG MSCs; (e) $3 \times 10^5$ HG + anti-miR-34a-HG MSCs. All MSCs were suspended in 100 μl of PBS and injected intramuscularly at four sites around the border zone of the infarcted heart. Another group of mice that underwent thoracotomy without LAD ligation served as the control group. Cardiac function in each mouse was assessed by transthoracic echocardiography (Ultramark 9; Soma Technology) at baseline (before MI), and at 1 week or 3 weeks following MI. LVEF and LVFS were calculated as previously described (Liao et al., 2019).

For the MSC-treated group, the MSCs were transfected with different treatments, suspended in 100 μl of PBS and injected intramuscularly at four sites around the border zone of the infarcted heart.
**Masson's staining**

After echocardiography assessment at 3 weeks post-MI, all mice were sacrificed, and the heart tissues were harvested, embedded, and sectioned. The infarction size of the mouse heart, as evidenced by fibrosis, was examined by a Masson's staining kit according to the manufacturer's protocol (HT15, Sigma). The percent infarct size was calculated as the ratio of the fibrosis area to the total LV area ×100%.

**TTC staining**

2,3,5-Triphenyltetrazolium chloride (TTC) staining was performed after the heart was removed, rinsed and weighed. Next, the heart was frozen at -20°C for 20 min and cut into 5 sections (approximately 1-2 mm in thickness). Thereafter, the sections were subjected to a water bath containing 1% TTC phosphate buffer (pH 7.4) at 37°C for 15 min (protected from the light and shaken) and fixed with 10% formalin. Finally, a fluorescence microscope was utilized for to capture photographs.

**Data analysis and statistics**

The data are expressed as the means ± SD of at least three independent experiments. When only two value sets were compared, the statistical analysis was performed with Student's t-tests. One-way ANOVA followed by Dunnett's test was used when the comparison involved three or more groups. Values of P < 0.05, P < 0.01, and P < 0.001 were considered statistically significant, and these values are indicated by *, ** or ***, respectively.

**Results**

**Hyperglycaemia damages the proliferation and paracrine abilities of MSCs**

We first evaluated the effects of hyperglycaemia on MSCs. To mimic the DM microenvironment, we isolated and cultured MSCs from normal rats with high glucose for 28 days. We compared the self-renewal potential and senescence of MSCs cultured with normal and high glucose using CCK-8 assays and SA β-gal staining respectively, as well as the effects on MSCs from DM rats.

As shown in Fig 1a and b, MSCs obtained from DM rats and high glucose-cultured MSCs exhibited decreased viability and increased senescence. The secretion of cytokines and growth factors to promote neovascularization is one of the major mechanisms underlying MSC-based therapy for cardiac repair after myocardial infarction[13]. In our study, we measured the level of cytokine release and found that high glucose reduced the production of VEGF and bFGF (Fig. 1c).

These results showed that during high glucose culture, MSCs become senescent, have poor proliferation and produce fewer proangiogenic factors, which is similar to the characteristics of MSCs obtained from DM rats. In addition, to determine whether miR-34a mediates hyperglycaemic injury in MSCs, we performed qRT-PCR to measure miR-34a expression in serum from normal rats and DM rats and in LG- and HG-cultured MSCs. As shown in Fig.1d, there was a dramatic increase in the expression of miR-34a in
the DM and high glucose groups, suggesting a potential relationship between miR-34a and high glucose-induced damage.

**miR-34a mediates MSC dysfunction under hyperglycaemic conditions**

To further analyse the relationship between miR-34a and hyperglycaemia, MSCs were transplanted a miR-34a mimic and inhibitor into MSCs and examined the function of MSCs exposed to hyperglycaemia. We first performed qRT-PCR to measure miR-34a transfection efficiency. As shown in Fig.2a, when the miR-34a mimic was transfected into MSCs, the expression of miR-34a was 8 times higher than that in the control group, and when the miR-34a inhibitor was transfected into MSCs, a significant reduction in the miR-34a level was observed. Then, we performed a CCK-8 assay to examine the connection between miR-34a and cell viability under hyperglycaemia. As shown in Fig.2b, the miR-34a mimic exacerbated the high glucose-induced damage to cell viability, while the miR-34a inhibitor alleviated high glucose-induced damage. In addition, ELISA was performed to determine whether the paracrine ability of MSCs exposed to hyperglycaemia can be regulated by miR-34a. The results showed that high glucose affected the paracrine abilities and that the miR-34a mimic exacerbated the damage, while the miR-34a inhibitor reversed this injury (Fig.2c). To investigate the role of miR-34a in MSC senescence, SA-β-gal staining and WB were performed. Compared with miR-control treatment, miR-34a mimic treatment greatly enhanced the level of SA-β-gal activity (Fig. 2d) and the protein expression levels of p21 and p16 (Fig. 2e). On the other hand, miR-34a inhibitor treatment led to an obvious increase in cell viability and decreases in the level of SA-β-gal activity (Figure 2d) and the protein expression levels of p21 and p16 (Figure 2e) in MSCs. Collectively, these findings suggest that the miR-34a mimic exacerbates high glucose-induced damage, while the miR-34a inhibitor protects against hyperglycaemic injury.

**miR-34a influences the expression of autophagy factors under hyperglycaemic conditions**

Increasing evidence has revealed that autophagy dysfunction is involved in cell death in diabetic mice. As shown in Fig. 3a, autophagy was induced in high glucose-cultured MSCs, as evidenced by increased expression levels of Beclin-1 and LC3II/LC3I. The miR-34a mimic promoted the expression of autophagy-related proteins, as shown by western blotting (Fig.3a), and autophagic corpuscle expression, as shown by transmission electron microscopy (TEM) (Fig.3c), while the miR-34a inhibitor ameliorated Beclin-1 and LC3II/LC3I expression compared with that of the control group (Fig. 3a). Collectively, these observations suggest that hyperglycaemia induces autophagy and that the miR-34a mimic exacerbates this effect, while the miR-34a inhibitor decreases autophagic protein expression, which suggests that miR-34a promotes the negative effect of hyperglycaemia by regulating autophagy.

Foxo3a was reported to regulate autophagy. Our previous studies showed that the Sirt1-Foxo3a signalling pathway plays a critical role in regulating apoptosis and senescence in MSCs, and we proved that Sirt1 could be negatively regulated by miR-34a expression[14]. In our study, we further examined the role of miR-34a and Sirt1/Foxo3a under hyperglycaemic conditions. The results showed that the miR-34a mimic could inhibit the expression of Sirt1 and Foxo3a. However, whether Sirt1/Foxo3a mediates the effect of miR-34a on autophagy requires further study.
miR-34a induces MSC dysfunction under hyperglycaemic conditions by regulating Sirt1-Foxo3a autophagy dynamics

To further determine the relationship between autophagy and Sirt-Foxo3a and the effect on cell function after exposure to hyperglycaemia, a Sirt1 inhibitor was used. As shown in Fig.4a, treatment with siRNA-Sirt1 partially reversed the downregulation of Foxo3a by the miR-34a inhibitor. The Sirt1-inhibitor alleviated hyperglycaemia-induced senescence, as evidenced by the protein expression of P21 and P16 and SA-β-gal activity. In addition, the LC3II/LC3I ratio was further decreased in the Sirt1-inhibitor group (Fig.4a), and Beclin 1 expression showed an increasing trend. Moreover, paracrine functions were also reversed by the Sirt1 inhibitor.

Overall, miR-34a regulates MSC functions under hyperglycaemic conditions in MSCs by targeting the Sirt1/Foxo3a signalling pathway.

Transplantation of anti-miR-34a-MSCs improves cardiac function following infarction in diabetic mice

To examine whether the inhibition of miR-34a in MSCs can improve the therapeutic effects of MSCs, we transplanted anti-miR-34a MSCs into infarcted rat hearts. The heart function of rats in the different groups was measured by echocardiography at baseline (before MI) and 1 week and 3 weeks post-MI. Representative echocardiography images were taken at 1 week and 3 weeks after MI in mice. For DM rat model, high blood glucose were determined after the STZ was injected and maintained high level compared with the normal group. ECG showed elevated ST segment and echocardiography revealed that compared with those of the control group, left ventricle ejection fraction (LVEF) and LVFS were robustly reduced at 1 week and 3 weeks post-MI, indicating that the rat model of MI was successfully established (Fig.5b and c). At 1 week and 3 weeks post-MI, the LVEF was enhanced in MSC-transplanted groups (besides HG MSCs treated group) compared with the MI group (Fig.5b). However, the LVEF was significantly reduced in the HG MSC group compared with the LG MSC group, but this effect was partially restored in the anti-miR-34a-MSC HG group, indicating that HG+anti-miR-34a-MSCs were superior to HG MSCs group in improving heart function following MI (Fig.5b). Similarly, the infarct size, as determined by Masson's trichrome staining, was much higher in the HG MSC group than in the LG MSC group, while anti-miR-34a-MSCs showed improved recovery after myocardial infarction compared with the HG MSC group. The same results were determined by TTC staining. Collectively, these findings suggest that anti-miR-34a-MSC transplantation inhibits cardiomyocyte apoptosis and enhances heart recovery in infarcted DM rat hearts, indicating miR-34a inhibitor treatment is a favourable way for autologous MSCs treatments for ischaemic diseases of DM rat.

Discussion

The current study presented several major findings. First, hyperglycemic culture damaged the self-renewing and paracrine functions of MSCs and exacerbated MSC senescence. Second, miR-34a mediated MSC senescence and paracrine functions by regulating autophagy. Third, miR-34a regulated autophagic dynamics by targeting the Sirt1/Foxo3a signalling pathway under hyperglycemic conditions.
Finally, the inhibition of miR-34a restored MSCs and increased cell survival in infarcted DM rats, thereby promoting the cardioprotective effects of MSCs.

Over the past few decades, transplantation of MSCs has shown promising results in MI recovery in animal studies and early clinical trials due to the availability of cells from numerous sources and the multilineage potential and immune privileged status of these cells[15].

Autologous MSCs are assumed to be favourable because patient-derived cells are readily available and do not entail sustained immunosuppressive therapy, and it has been proven that compared with those of allogeneic MSC transplantation, the long-term effects of autologous MSCs are better[16]. However, MSCs isolated from DM patients become dysfunctional, as shown by changes in angiogenesis/vasculogenesis, altered pro-inflammatory cytokine secretion, increased oxidative stress markers, impaired cellular differentiation and decreased proliferation[17]. Furthermore, compared with transplanted healthy MSCs, transplanted DM MSCs exhibit impaired abilities to improve cardiac function after myocardial infarction[18].

Consistent with these observations, in the current study, MSCs isolated from DM rats displayed increased levels of SA-β-gal activity and decreased proliferative and paracrine capacities. We also observed that high glucose-cultured MSCs exhibited almost the same behaviour as DM MSCs, and so in the following studies, cells were treated with chronic exposure to high glucose for 28 d to mimic the behavior of DM MSCs. However, the potential mechanisms underlying DM MSC dysfunction remain unclear.

Recently, a variety of miRNAs have been reported to be involved in regulating MSC senescence via multiple pathways[18]. Among the known miRNAs, miR-34a has been implicated in cardiovascular diseases such as atherosclerosis, ischemic cardiomyopathy, and myocardial infarction. Recent studies have shown that the expression of miR-34a was significantly increased in patients with hyperglycaemia [6]. MiR-34a mediates the inhibitory effect of metformin on pancreatic tumours [7], and overexpression of miR-34a exacerbates endothelial dysfunction and decreases vasculogenesis under high glucose exposure [8]. Our previous results showed that the expression of miR-34a was significantly increased in the ischemic/hypoxic environment (used to simulate the hypoxic and ischemic microenvironment in myocardial infarction), the activity of MSCs with miR-34a overexpression was decreased, the apoptosis rate was significantly increased, and cell senescence was also increased in the ischemic and hypoxic environment. However, it has not been reported whether miR-34a affects the functional changes in MSCs exposed to high glucose.

In the present study, miR-34a was markedly increased under hyperglycaemic conditions. We further found that overexpressing miR-34a in MSCs exposed to hyperglycaemia enhanced the senescent phenotype, including increased SA-β-gal activity and expression of p21 and p16. Besides, miR-34a mimic treated MSCs under hyperglycaemia also shown decreased proliferative and paracrine capacities. In contrast, inhibition of miR-34a in MSCs exposed to hyperglycaemia reduced SA-β-gal activity, increased cell proliferation and promoted the secretion of pro-survival growth factors, including VEGF and bFGF.
Transplantation of anti-miR-34a-MSCs had better effects in attenuating cardiac remodelling and restoring heart function in DM rats following infarction than transplantation of MSCs cultured in high glucose.

These findings confirmed that miR-34a accelerated MSC dysfunction in hyperglycaemia and that inhibition of miR-34a restored MSC abilities. The exact mechanism underlying miR-34a-mediated regulation of MSC function, however, is still largely unknown.

Autophagy is an intracellular metabolic pathway. The role of autophagy in the development of DCM is controversial, especially in type 2 diabetic hearts. Suppression of autophagy, in combination with cell death, fibrosis and dysfunction, is well characterized in type 1 diabetic hearts [19]. Other study showed that promoting autophagy expression can express more VEGF for MSCs treatment to better ameliorate erectile dysfunction[20]. Cardiomyocytes isolated from db/db mice[21] and HFD-induced obese mice exhibit blunted autophagic responses[22].

The initiation and stabilization of autophagy is a complex process regulated by many factors in the microenvironment, of which Foxo3a is an important factor. Foxo3a is a member of the FoxO transcriptional family. With the development of studies on Foxo3a, the relationship between Foxo3a and autophagy has attracted increasing attention. When the expression of Foxo3a is increased and nuclear translocation is enhanced, the expression of the autophagy-related genes ATG5, ATG12, BECN1 and LC3 is increased, which further activates autophagy and increases the anti-apoptotic ability of cells[23]. Foxo3a can be activated by Sirt1/Sirt3, thereby activating downstream PINK1, which can strongly activate PARKIN, cause mitochondrial division and activate mitochondrial autophagy, thus playing a cardioprotective role [11]. Our previous study showed that miR-34a could regulate the expression of Sirt1 and Foxo3a. Whether miR-34a mediates autophagic fusion by regulating Sirt1 signalling remains unclear.

In the current study, we found that the expression of Sirt1 was greatly increased in high glucose-induced MSCs compared with the normal MSCs. Upregulated Beclin 1 and LC3II/LC3I levels were observed in high glucose-cultured MSCs, suggesting that excessive autophagy contributes to hyperglycaemic damage. Furthermore, miR-34a mimic treatment greatly downregulated the expression of Sirt1 and upregulated Foxo3a, which hinted miR-34a-induced excessive autophagy may be regulated by Sirt1/Foxo3a signalling. Moreover, we found that siRNA-SIRT1 partially rescued miR-34a-induced excessive autophagy. These results further confirmed that miR-34a induces autophagy by partially targeting Sirt1/Foxo3a signalling.

Furthermore, the stem cell-mediated repair of myocardial infarction under different treatment conditions was observed in rats. The effects of miR-34a on the function of MSCs cultured in high glucose, including proliferation, senescence and paracrine signalling, and the relationship between miR-34a and the Sirt1-Foxo3a-autophagy signalling pathway were also investigated. This project examined that miR-34a expression increased in stem cells under long-term high glucose exposure and affected the energy metabolism pathway of autophagy. Transplantation of miR-34a inhibitor treated MSCs will provide new ideas and has wide clinical applications for clinical stem cell treatment of patients with myocardial infarction and diabetes.
**Conclusion**

This study demonstrates that inhibition of miR-34a, which occurs partially via the Sirt1/Foxo3a autophagy-signalling pathway, restores damaged MSCs exposed to hyperglycaemia and provides a possible target to enhance the cardioprotection of MSCs in the DM heart following infarction.

**Abbreviations**

MSCs: mesenchymal stem cells

CCK8: cell counting kit-8

DM: diabetes mellitus

SA-β-gal: senescence-associated β-galactosidase

SIRT1: silent information regulator 1

FOXO3a: forkhead box class O 3a

TEM: transmission electron microscopy

LAD: left anterior descending coronary artery

TTC: 2,3,5-triphenyltetrazolium chloride

CVDs: cardiovascular diseases

MI: myocardial infarction

BG: blood glucose

SD: Sprague-Dawley

BCA: bicinechonic acid

TTC: 2,3,5-Triphenyltetrazolium chloride

TEM: transmission electron microscopy

LVEF: left ventricle ejection fraction

**Declarations**

**Ethics approval and consent to participate**
The animal were obtained from the Laboratory Animal Science Department of Xuzhou Medical University, Jiangsu, P.R. China. All of the study procedures were approved by the Institutional Animal Care and Use Committee of Xuzhou Medical University.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

FYZ designed the study and wrote the manuscript. FG, KW and XHL performed the experiments and data analysis. ZQZ managed, coordinated and was responsible for the research activity, planning and execution. All authors contributed to the revision of the manuscript and approved the final version.

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Tables

Table 1. Primers for qRT-PCR and oligonucleotide.
| Name       | Sequence                                                                 |
|------------|--------------------------------------------------------------------------|
| **qPCR**   |                                                                          |
| miR-34a    | 5'-UGGCAGUGUCUUAGCUGGUUGUU-3'                                           |
| SIRT1      | F 5'-AAGGCCACGGATAGGTCCATA-3'                                           |
|            | R 5'-CGCTTTGTTGTTCTGAAAGG-3'                                            |
| FOXO3a     | F 5'-TGCCGATGGGTTGGATT-3'                                               |
|            | R 5'-CCAGTGAAGTTCCACGT-3'                                               |
| U6         | F 5'-CCTGCTTCGGCAGCACA-3'                                               |
|            | R 5'-AACGCTTCACGAATTTGC-3'                                              |
| β-actin    | F 5'-CCAGCACAATGAAGATCAAGATCAT-3'                                       |
|            | R 5'-ATCTGCTGGAAGGTGTACAGC-3'                                           |
| **Oligonucleotide** |                                                  |
| miR-34a mimic | UGGCAGUGUCUUAGCUGGUUGUU CAACCAGCUAAGACACUGCCAUU                        |
| Negative Control (NC) mimic | UUCUCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT              |
| miR-34a inhibitor | UGGCAGUGUCUUAGCUGGUUGUU                       |
| NC inhibitor | CAGUACUUUUGGUAGUACA           |
| siRNA-SIRT1   | GCACCGAUCCUCGACAAUUTT AUUGUUCGAGGAUCGGUGCTT         |
| siRNA-NT     | UUCUCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT   |