Int J Clin Exp Pathol 2016;9(9):8784-8790
www.ijcep.com /ISSN:1936-2625/IJCEP0028272

Review Article

Downregulation of miR-26b impact on lens epithelial cells in cataract rat

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Received March 15, 2016; Accepted July 12, 2016; Epub September 1, 2016; Published September 15, 2016

Abstract: Lens epithelial cells (LECs) membrane damage is one of the main pathogeneses of cataract. MiRNA-26b is found to play an important role in LECs proliferation and growth. This study aimed to explore miR-26b upregulation impact on rat oxidative cataract LECs to provide basis for clinical cataract treatment and clarifying mechanism. SD rat transparent lens was cultured in vitro to separate LECs cells. LECs were randomly divided into three groups: normal control, H_2O_2 induced cataract model group, and miR-26b inhibition group. MiR-26b expression was detected by real time PCR. LECs proliferation was determined by MTT. LECs apoptosis was tested by Caspase-3. NF-κB mRNA and protein expression were detected by real time PCR and Western blot. Inflammatory factor TNF-α and IL-1β secretion was measured by ELISA. MiR-26b overexpressed, LECs proliferation was suppressed, and caspase-3 activity enhanced after H_2O_2 treatment. Furthermore, NF-κB mRNA and protein elevated, and TNF-α and IL-1β secretion increased in H_2O_2 group compared with normal control ($P < 0.05$). After miR-26b inhibitor transfection, miR-26b expression downregulated, LECs proliferation weakened, Caspase-3 activity reduced, NF-κB mRNA and protein declined, and TNF-α and IL-1β secretion decreased obviously compared with cataract model group ($P < 0.05$). Downregulation of miR-26b can delay oxidative cataract progression by inhibiting NF-κB expression, suppressing inflammatory factors, restraining LECs apoptosis, and regulating cell proliferation.

Keywords: miR-26b, cataract, lens epithelial cell, NF-κB

Introduction

Following aging of population and lifestyle changes, cataract incidence increases day by day [1, 2]. Cataract shows high incidence worldwide, and occupies the first cause of acquired blinding eye disease [3]. Cataract has numerous reasons, while age and ocular tissue degeneration are important inducement. In addition to age, physical and chemical injury, inflammation, surgery, and tumor can cause lens abnormity, thus affecting normal metabolism to form cataract [4, 5]. Cataract extracapsular extraction combined IOL implantation is the main treatment method. However, surgery may lead to iatrogenic astigmatism and posterior capsule opacity, seriously affecting postoperative quality of life and brings spirit and economic burden to the society and family [6]. Therefore, founding the determinant factor of cataract is the key to reduce cataract incidence and blinding rate. At present, lens epithelial cells (LECs) membrane oxidative damage by free radical is considered as the main pathogenesis [7, 8].

MicroRNA, also known as miRNA, is a kind of non-coding short chain RNA widely existed in animals and plants [9, 10]. MiRNA can play its regulation function by degrading RNA and inhibiting protein translation through complete or incomplete matching target genes [11, 12]. MiRNAs were mostly studied in tumor, whereas they were less investigated in other diseases. Recent study showed that except cervical cancer and lung cancer, miR-26b also involved in LECs growth and proliferation [13, 14]. MiR-26b impacts on LECs in cataract rat and related mechanism have not been reported. Therefore, this study intends to explore the role of miR-26b upregulation impact on LECs in oxidative cataract rat and related mechanism, to provide basis for clinical treatment and clarifying cataract pathogenesis.

Table 1. Primer sequences used in PCR

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTGCCAGCCTCGTCTCATAG	CGTTGAACTTGCCGTGGGTAG
Mir-26b	AGCAAGCAGTCATCTCAATGG	GGCGCTTCACATCTCCGTA
NF-κB	CTCATCTAAGCGGAACAATGG	GCACATTCTCTCCGTAGCG

Materials and methods

Experimental animals

A total of 30 healthy male SD rats (60 eyes) at 2 months old and weighted 250 ± 20 g were purchased from Zhejiang Chinese Medical University experimental animal center and fed in SPF laboratory.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of The First Clinical College of Zhejiang Chinese Medical University.

Main instruments and materials

DMEM medium, MEM medium, and penicillin-streptomycin were from Hyclone. MTT was from Gibco. Enzyme-EDTA and epidermal growth factor were from Sigma. PVDF membrane was from Pall Life Science. Western blot related reagents were from Beyotime. ECL reagents were from Amersham Biosciences. Rabbit anti-rat NF-κB and β-actin primary antibodies, and HRP tagged IgG secondary antibody were from Cell Signaling. RNA extraction kit, reverse transcription kit, TNF-α, and IL-1β ELISA kits were from RD. Caspase 3 activity kit was from R & D. Eye surgery microscopic instruments were from Suzhou medical apparatus factory. Microplate reader was from BD. DNA amplifier was from PE Gene Amp PCR System 2400. Other reagents were from Sangon.

LECs isolation and cultivation

SD rats were anesthetized by 10% chloral hydrate intraperitoneal injection. The eye was quickly extracted after euthanasia and washed by cold PBS containing penicillin-streptomycin for 10 min. Lens was extracted as follows: eyeball wall was isolated from cross intersections, and the connected suspensory ligament was clipped on the equator to take out the complete lens. The lens was put into the 24-well plate and incubated in MEM medium at 37°C and 5% CO_2. LECs were randomly divided into three groups: Normal control; Cataract model group (H_2O_2 group): 200 μM H_2O_2 was added to LECs; MiR-26b inhibition group: H_2O_2 were given to LECs after miR-26b inhibitor transfection.

MiR-26b inhibitor transfection

MiR-26b inhibitor was synthetized by Gene Pharma. MiR-26b inhibitor sequence: 5'-UAAUACGUGCCUGUAAUGAUGAC-3'; miR-26b negative control sequence: 5'-CCUGUUAACGUGAAUUGAAUGAC-3'. MiR-26b inhibitor was transfected by lipo2000 to LECs cultured in H_2O_2. LECs in logarithmic phase were collected and seeded in 6-well plate at 3×10^6/ml. 5 μl lipo 2000 was mixed with 200 μl serum free medium, while miR-26b was mixed with 200 μl serum free medium for 15 min under room temperature. Then they were mixed at room temperature for 30 min. After cultured at 5% CO_2 and 37°C for 12 h, the cells were washed by PBS and added with 1.6 ml serum free medium. Next, the mixture was added to each well and cultured for 6 h. After changing the medium, LECs were continued cultured in 200 μM H_2O_2.

Real-time PCR

Total RNA was extracted from LECs by Trizol and reverse transcripted to cDNA using the related primers (**Table 1**). Real-time PCR was applied to detect the target genes. The reaction consists one cycle of 52°C for 1 min, followed by 35 cycles of 90°C for 30 s, 58°C for 50 s, and 72°C for 35 s. GAPDH was used as reference, and the results were calculated $2^{-\Delta Ct}$ method.

MTT assay

LECs in logarithmic phase were seeded in 96-well plate at 3000/well with five repetition in each group. After the cell adhering to the wall, 20 μl MTT at 5 g/L was added to each well and incubated for 4 h. 150 μl DMSO was added after removing the supernatant and vibrated for 10 min. The plate was read at 570 nm on microplate reader for cell proliferation calculation. All experiments were repeated for three replicates.

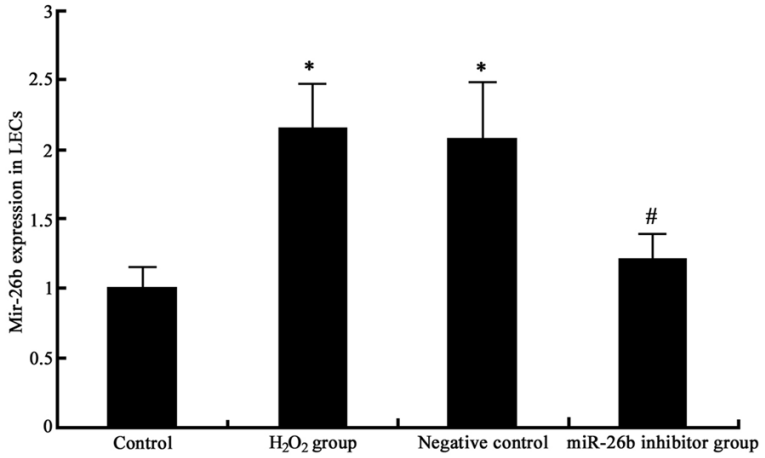

Figure 1. MiR-26b expression in LECs. *P < 0.05, compared with normal control; #P < 0.05, compared with H_2O_2 group.

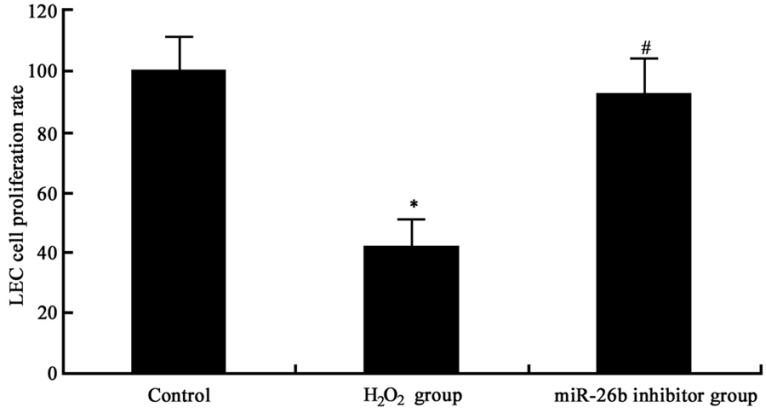

Figure 2. MiR-26b impact on LECs proliferation. *P < 0.05, compared with normal control; #P < 0.05, compared with H_2O_2 group.

Western blot

LECs in each group were cracked on ice for 15-30 min and ultrasonicated for 4×5 s to extract protein. After centrifuged at 10000 g and 4°C for 15 min, the protein was moved to a new Ep tube and store at -20°C. The protein was separated by 10% SDS-PAGE electrophoresis and transferred to PVDF membrane. After blocked by 5% skim milk for 2 h, the membrane was incubated in primary antibody at 1:1000 and 4°C over night. Then the membrane was incubated with secondary antibody at 1:2000 for 30 min and washed by PBST. At last, the membrane was treated with chemiluminescent agent for 1 min and imaged on X-ray. Protein image processing system and Quantity one software were used for data analysis. All experiments were repeated for four times.

Caspase 3 activity detection

Caspase-3 activity was detected according to the kit instruction. LECs were digested by enzyme and centrifuged at 600 g and 4°C for 5 min. After removing the supernatant, the sediment was treated with cell lysis buffer on ice for 15 min. After centrifuged at 20000 g and 4°C for 5 min, the solution was added with 2 mM Ac-DEVD-pNA and detected at 405 nm for Caspase-3 activity calculation.

ELISA

ELISA was applied to test inflammatory factors TNF-α and IL-1β level in cell supernatant according to the manual. 50 μl standard substances were diluted and added to five repeat holes at each concentration to draw the standard curve. 50 μl sample was added to each well with three replicates. The plate was vibrated at room temperature for 30 s and washed for 5 times. After incubated at 37°C for 30 min, the plate was washed for 5 times. 50 μl color developing agent A and 50 μl color developing agent B were added and incubated at 37°C for 10 min. After added by 50 μl stop buffer, the plate was read at 405 nm on microplate reader for OD value within 15 min. OD value and standard substance concentration were used to draw the standard curve for TNF-α and IL-1β content calculation.

Statistical analysis

SPSS16.0 was adopted for data analysis. All data were presented as mean ± standard deviation ($\bar{x} \pm S$). One-way ANOVA was applied for comparison. P < 0.05 was considered as statistical significance.

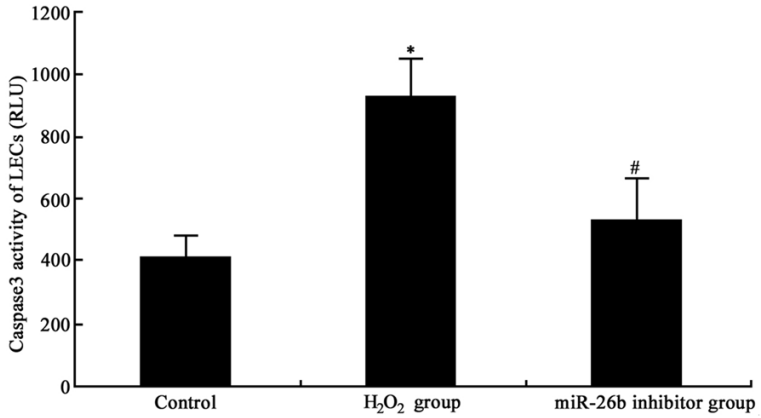

Figure 3. MiR-26b impact on Caspase 3 activity in LECs. *P < 0.05, compared with normal control; #P < 0.05, compared with H_2O_2 group.

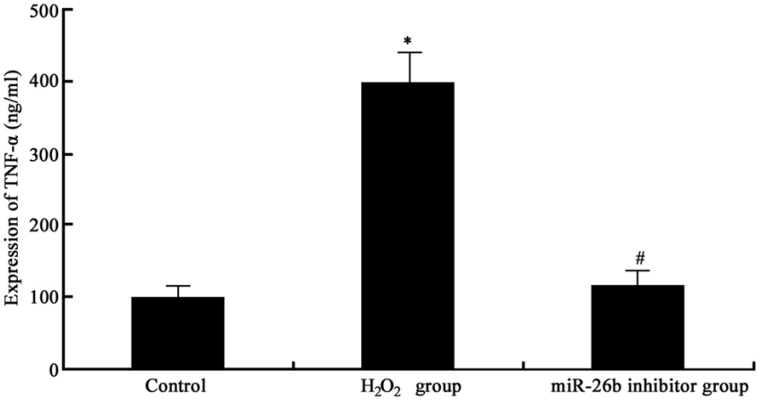

Figure 4. MiR-26b impact on TNF-α secretion in LECs. *P < 0.05, compared with normal control; #P < 0.05, compared with H_2O_2 group.

Results

MiR-26b expression in LECs

Real time PCR was applied to detect miR-26b expression in LECs in cataract rat. The results showed that miR-26b significantly overexpressed in LECs after H_2O_2 treatment (2.16 ± 0.35) compared to control (1.03±0.15) (P < 0.05). MiR-26b negative control transfection failed to change miR-26b elevation induced by H_2O_2 (2.08 ± 0.43). However, miR-26b inhibitor transfection obviously suppressed H_2O_2 induced miR-26b overexpression (1.25 ± 0.19) (P < 0.05) (**Figure 1**).

MiR-26b impact on LECs proliferation

MTT assay was used to determine miR-26b impact on LECs proliferation under H_2O_2 environment. It was revealed that H_2O_2 markedly inhibited LECs cell viability (42.7 ± 13.6) compared to control (100.2 ± 14.5) (P < 0.05). MiR-26b inhibitor transfection obviously promoted LECs proliferation cultured in H_2O_2 (92.3 ± 12.3) (P < 0.05) (**Figure 2**). These results indicated that H_2O_2 can upregulate miR-26b expression in LECs and suppress LECs proliferation, while downregulation of miR-26b can enhance LECs cell viability.

MiR-26b impact on caspase 3 activity in LECs

Caspase 3 activity detection showed that H_2O_2 significantly enhanced Caspase 3 activity in LECs (926.3 ± 186.1) compared to control (400.1 ± 87.6) (P < 0.05). MiR-26b inhibitor transfection markedly decreased Caspase 3 activity in LECs induced by H_2O_2 (543.3 ± 159.8) (P < 0.05) (**Figure 3**). It suggested that miR-26b can promote LECs apoptosis induced by H_2O_2 through regulating apoptotic protein.

MiR-26b impact on inflammatory factors secretion in LECs

ELISA was adopted to measure inflammatory factors secretion by LECs. It was found that TNF-α (405.3 ± 65.3) and IL-1β (245.3 ± 60.5) levels obviously increased after H_2O_2 treatment compared to control (100.5 ± 25.6) (78.2 ± 28.1) (P < 0.05). MiR-26b transfection markedly inhibited their secretion compared with H_2O_2 group (119.6 ± 43.3) (85.6 ± 40.3) (P < 0.05) (**Figures 4** and **5**). It indicated that miR-26b can affect H_2O_2 induced LECs cell viability by regulating inflammatory factors secretion.

MiR-26b impact on NF-κB mRNA expression in LECs

Real time PCR was performed to test NF-κB mRNA expression in LECs. The results demon-

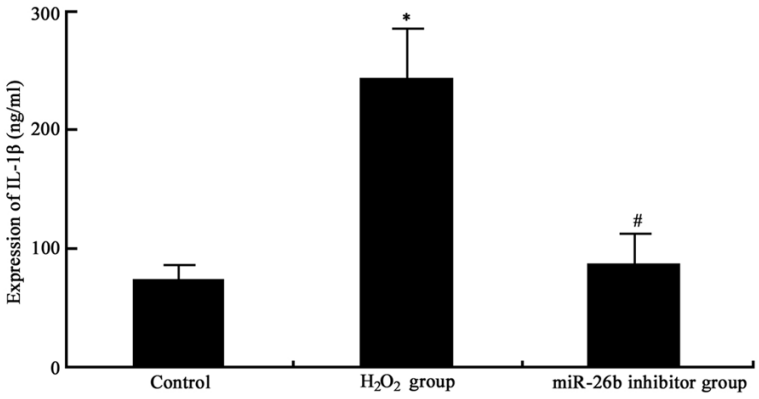

Figure 5. MiR-26b impact on IL-1β secretion in LECs. *P < 0.05, compared with normal control; #P < 0.05, compared with H_2O_2 group.

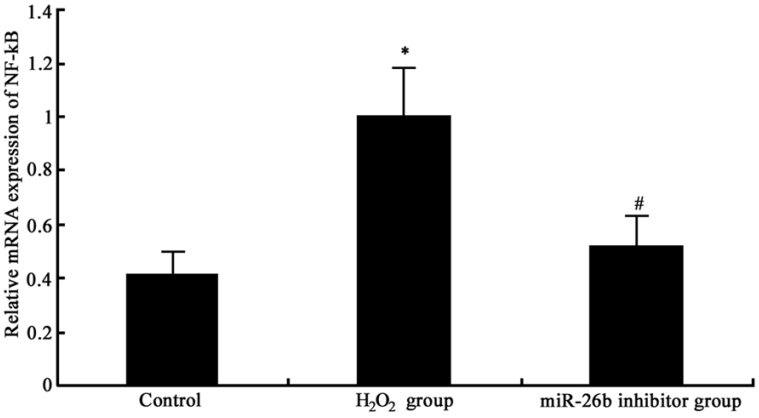

Figure 6. MiR-26b impact on NF-κB mRNA expression in LECs. *P < 0.05, compared with normal control; #P < 0.05, compared with H_2O_2 group.

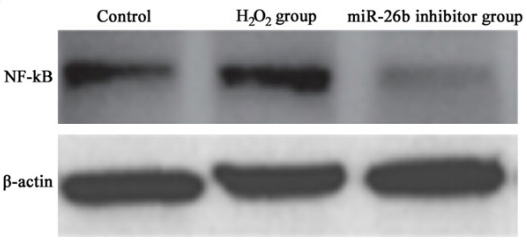

Figure 7. MiR-26b impact on NF-κB protein expression in LECs.

strated that NF-κB mRNA level elevated in LECs under H_2O_2 effect (1.02 ± 0.26) compared with normal control (0.41 ± 0.11) (P < 0.05). MiR-26b inhibitor transfection obviously downregulated NF-κB mRNA expression (0.52 ± 0.21) compared with H_2O_2 group (P < 0.05) (**Figure 6**).

MiR-26b impact on NF-κB protein expression in LECs

Western blot experiment revealed that similar with NF-κB mRNA results (1.32 ± 0.15 (H_2O_2) vs 0.78±0.13 (control) vs 0.61 ± 0.26 (inhibitor)), NF-κB protein enhanced in H_2O_2 group compared with control (P < 0.05). MiR-26b inhibitor transfection significantly inhibited NF-κB protein compared with H_2O_2 group (P < 0.05) (**Figures 7** and **8**). It suggested that miR-26 can affect LECs proliferation and cell viability by regulating NF-κB mRNA and protein expression.

Discussion

Oxidative stress is one of the important pathogenic factors of cataract. The lens itself does not have vascular distribution. Therefore, it is supplied by anaerobic metabolism, and the surrounding oxygen tension is low [15]. H_2O_2, hydroxyl radicals, and superoxide anion can cause lens oxidative damage, leading to lens transparency change. H_2O_2 has the most certainly damage effect to form cataract [16, 17]. The monolayer cells wrapping the anterior and equatorial lens capsule are called LECs. They can differentiate to lens capsule membrane and lens fibers. It shows the strongest metabolic ability in the lens and can maintain the lens function, metabolism, and transparency status [18]. Oxidative damage causes too much free radicals production, while weakens scavenging ability, resulting in LECs crosslinking, gathering, and protein and lipid peroxidation. LECs damage and apoptosis lead to lens structure and function changes to form acquired cataract [19, 20].

MiRNAs have multiple functions, such as regulating cell proliferation, apoptosis, signal transduction, differentiation, hormone secretion, fat

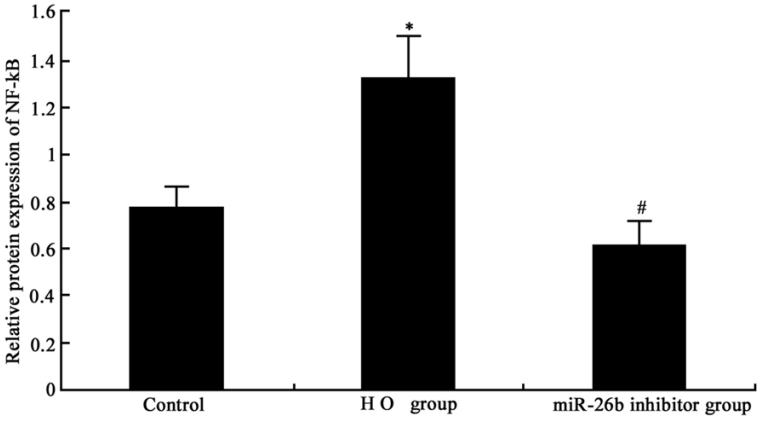

Figure 8. MiR-26b impact on NF-κB protein expression in LECs analysis. *P < 0.05, compared with normal control; #P < 0.05, compared with H_2O_2 group.

metabolism and maintaining embryonic stem cells potential. It enhances body adapt ability to the environment [21]. However, the role and related mechanism of miRNA in cataract are rare reported. In addition to tumor, miR-26b was confirmed to have negative regulatory function in LECs proliferation and metastasis [14, 22], suggesting that miR-26b may play a role in LECs lesions. Therefore, this study analyzed miR-26b impact in regulating LECs and related mechanism through culturing LECs cell and constructing H_2O_2 oxidative damage cataract model. The results showed that miR-26b overexpressed, LECs proliferation suppressed, and caspase-3 activity enhanced after H_2O_2 treatment. Furthermore, NF-κB mRNA and protein elevated, and TNF-α and IL-1β secretion increased in H_2O_2 group compared with normal control. After miR-26b inhibitor transfection, miR-26b expression downregulated, LECs proliferation weakened, Caspase-3 activity reduced, NF-κB mRNA and protein declined, and TNF-α and IL-1β secretion decreased obviously compared with cataract model group. It suggested that miR-26b downregulation had protective role on oxidative damaged LECs to delay cataract progression.

To sum up, downregulation of miR-26b can delay oxidative cataract progression by inhibiting NF-κB expression, suppressing inflammatory factors, restraining LECs apoptosis, and regulating cell proliferation. It provides theoretical basis and reference for selecting miR-26b as target for cataract prevention and treatment.

Acknowledgements

This project supported by the Projects of medical and health technology development program in Zhejiang province (NO. 2014KYA158).

Disclosure of conflict of interest

None.

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