MiR-181a inhibits human trabecular meshwork cell apoptosis induced by H₂O₂ through the suppression of NF-κB and JNK pathways

Yuwen Wang^{A,D,F}, Heding Zhou^{B,D,F}, Xiaotian Liu^{C,D,F}, Yin Han^{B,F}, Suqi Pan^{B,F}, Yanyan Wang^{C,F}

Ningbo Eye Hospital, Ningbo, China

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D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence

Yuwen Wang
E-mail: wangyuwen12321@126.com

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Abstract

Background. The trabecular meshwork (TM) plays a critical role in the outflow of aqueous humor.

Objectives. In this study, we aimed to investigate the effect of miR-181a on H₂O₂-induced apoptosis in TM cells.

Material and methods. Human primary explant-derived TM cells were cultured in fibroblast medium and then treated with different concentrations of H₂O₂ for 2 h. We used a series of methods to carry out the research, such as MTT assay, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), apoptosis assay, and western blot methodology.

Results. The apoptosis assay and qRT-PCR showed that H₂O₂-induced apoptosis and cell viability were suppressed in a dose-dependent manner in TM cells. After the TM cells were treated with H₂O₂, miR-181a expression was significantly lower. The overexpression of miR-181a enhanced TM cells' viability, while the knockdown of miR-181a inhibited viability of cells. The overexpression of miR-181a suppressed TM cell apoptosis, while the knockdown of miR-181a induced apoptosis. H₂O₂ activated the nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK) pathways and induced cell apoptosis, while the overexpression of miR-181a suppressed both pathways and decreased the rate of apoptosis.

Conclusions. In conclusion, this study indicated that miR-181a could improve the survival rate of TM cells after H₂O₂ treatment by blocking the NF-κB and JNK signaling pathways. These findings might provide novel therapeutic opportunities in the treatment of glaucoma.

Key words: cell apoptosis, H₂O₂, miR-181a, trabecular meshwork, nuclear factor-κB, c-Jun N-terminal kinase pathway

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Introduction

Worldwide, glaucoma is the one of the leading causes of irreversible blindness.^{1,2} There are 60 million people diagnosed with glaucoma, of whom 8.4 million have been blinded due to glaucoma.² One of the major risk factors associated with glaucoma includes elevated hydrostatic pressure or intraocular pressure (IOP) in the eyes.³ Intraocular pressure depends on the rate of formation and drainage of the aqueous humor via the trabecular meshwork.³ The trabecular meshwork (TM), consisting of TM cells and located at the intraocular anterior chamber angle, plays a critical role in the outflow of aqueous humor.⁴ Decreased outflow of aqueous humor induces an elevation in IOP and ultimately leads to glaucoma.^{3,4} This in turn leads to increased oxidative DNA damage in the TM of glaucoma patients. The oxidative stress induces the activation of the nuclear factor- κ B (NF- κ B) and c-Jun N-terminal kinase (JNK) signaling pathways.⁵ Trabecular meshwork cells play an important role in maintaining the extracellular matrix synthesis (ECM) and phagocytosis of the debris in outflow tissues which control the aqueous-outflow facility. Thus, a shortage of TM cells might result in ocular hypertension. Therefore, the elucidation of the mechanism underlying the miR-181a and primary oxidative stress response signaling pathways might reveal promising opportunities for preventing TM cell apoptosis, which can prevent glaucoma.⁵ An understanding the biological functions of TM cells is then beneficial for understanding the pathogenesis of glaucoma and for developing novel drugs.

MicroRNAs (miRNAs) are a group of non-coding RNAs with 14–20 nucleotides, which are involved in the inhibition of endogenous gene expression through translational cleavage.⁶ MiRNAs are implicated in many biological progresses, such as cell proliferation, aging, apoptosis, and tumorigenesis.^{7–10} These miRNAs, either directly or indirectly, have been found to play an important role in the pathogenesis of glaucoma in both animal and human visual systems.^{11,12} The role of miRNAs in the molecular mechanisms of ECM provides potential for the development of drugs.¹³ In their study, Dismuke et al. isolated 10 mature miRNAs from human aqueous humor exosomes, one of which was miR-181a.¹⁴ miR-181a is a family member of miR-181, and has been reported to act as a regulator in cell proliferation, migration and invasion.¹⁵ Interestingly, miR-181a has different functions in different types of cells. For instance, miR-181a improved colorectal carcinoma cells and cartilage cell proliferation, while it inhibited the proliferation of glioma.^{16–18} The levels of miR-181a were most decreased with aging.¹⁹ The exact underlying mechanism of miRNAs regulate retinal apoptosis or degeneration is largely unknown. In a study by Parikh et al., miR-181a was found to promote transdifferentiation towards mesenchymal-like cells.²⁰ The activation of these pathways is thought to contribute to the dysfunction of normal outflow facility.²¹

Also, a study by Kozloski et al. demonstrated that miR-181a negatively regulated NF- κ B activity, leading to a significant change in the proliferation and survival phenotype of the B-cell-like diffuse large B-cell lymphoma malignancy.²² However, the role of miR-181a in the pathogenesis of glaucoma remains elusive.

In this study, we investigated the role of miR-181a in apoptosis in human trabecular meshwork cells induced by H₂O₂ through the NF- κ B and JNK pathways.

Material and methods

Cell culture and H₂O₂ treatment

Human primary explant-derived trabecular meshwork (TM) cells (TM cell line) were obtained from ScienCell Research Labs (Catalog No. 6590; Carlsbad, USA). Primary human TM cells were grown in the Fibroblast Medium (Catalog No. 2301; ScienCell Research Labs, Carlsbad, USA) wherein the cells from the 3rd to 6th passage were used. For maintenance, the cells were incubated at 37°C in a 5% CO₂ environment. The TM cells were seeded in a 6-well plate or a 96-well plate and treated with different concentrations of H₂O₂ for 2 h.

Small interfering RNA transfection

Mature miR-181a mimic and si-miR-181a were designed and synthesized by GenePharma (Shanghai, China). The insertion fragment was confirmed by DNA sequencing. Cell transfection and co-transfection were performed using the Lipofectamine 3000 reagent (Invitrogen Life Technologies, Carlsbad, USA) according to the manufacturer's instructions.

MTT assay

The viability of the cells was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay according to standard methods described previously. Each experiment was performed 3 times.

Apoptosis assay

Flow cytometry analysis was performed to identify and quantify the apoptotic cells by using an Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). The cells (100,000 cells/well) were seeded in a 6-well plate. The treated cells were washed twice with cold PBS and resuspended in buffer solution. The adherent and floating cells were combined and treated according to the manufacturer's instructions and measured with a flow cytometer (Beckman Coulter, Fullerton, USA) to differentiate apoptotic cells (Annexin-V-positive and PI-negative) from necrotic cells (Annexin-V-positive and PI-positive).

Quantitative reverse transcriptase-polymerase chain reaction

Total RNA was isolated from transfected cells by using TRIzol reagent (Invitrogen, Carlsbad, USA) and treated with DNase I (Promega, Southampton, UK). Reverse transcription was performed by using a Multiscribe RT kit (Applied Biosystems, Foster City, USA) and random hexamers or oligo(dT). The reverse transcription conditions were 10 min at 25°C, 30 min at 48°C, and a final step of 5 min at 95°C.

Western blot

The protein used for western blotting was extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using a BCA™ Protein Assay Kit (Pierce, Appleton, USA). The western blotting system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Primary antibodies were incubated with the membrane at 4°C overnight, followed by washing and incubation with secondary antibodies marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the polyvinylidene difluoride (PVDF) membrane carrying blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 µL Immobilon Western Chemiluminescent HRP Substrate (Millipore, Bedford, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

Statistical analysis

All data were shown as mean ±SD. Statistical analyses were performed using SPSS 19.0 software (SPSS Science, Chicago, USA). Differences between groups were analyzed using one-way analysis of variance (ANOVA) and/or Student's t-test. A p-value of <0.05 was considered statistically significant. All experiments were conducted in triplicate.

Results

Different concentrations of H₂O₂ suppressed viability of TM cells in a dose-dependent manner

In our study, we cultured TM cells in a 6-well plate or a 96-well plate and treated them with different concentrations of H₂O₂ (50, 100, 200, and 300 µM) for 2 h. Cell viability was assessed by MTT assay. Our results showed

that H₂O₂ at a concentration of 200 µM was significantly suppressed. These results suggested that different concentrations of H₂O₂ suppressed the viability of TM cells in a dose-dependent manner and that H₂O₂ in increasing concentrations (100, 200, and 300 µM) suppressed cell viability significantly (Fig. 1, $p < 0.05$ or $p < 0.01$). For the analyses below 200 µM was selected.

Fig. 1. TM cell viability was significantly suppressed by H₂O₂ in a dose-dependent manner, and 200 µM was selected as a lethal concentration of 50% used in the following analyses

* $p < 0.05$; ** $p < 0.01$.

H₂O₂-treated TM cells revealed a significant fall in miR-181a expression level

The TM cells after treatment with H₂O₂ showed a significant decrease ($p < 0.01$) in the expression level of miR-181a (Fig. 2).

Fig. 2. After H₂O₂ intervention, the miR-181a levels in TM cells were significantly decreased

** $p < 0.01$.

Transfection efficiency of TM cells

TM cells were transfected and then divided into mature miR-181a mimic, si-miR-181a and negative control (siNC) which were then evaluated by qRT-PCR. The results showed a significant decrease ($p < 0.01$) in the mRNA expression level of si-miR-181a compared to the control. Furthermore, they showed a significantly increased ($p < 0.01$) mRNA expression level of miR-181a mimic compared to the control. This suggested that transfection efficiency was observed when miR-181a was overexpressed or suppressed in TM cells (Fig. 3).

H₂O₂ treatment and overexpression of miR-181a increased TM cell viability

Cell viability was measured in the TM cells after treatment with H₂O₂, and the effect of miR-181a on cell viability was measured after transfection. The results showed that cell viability was promoted with increasing time in the miR-181a mimic group compared to si-miR-181a and to control groups. These results suggest that the overexpression of miR-181a enhanced TM cell viability, while the knockdown of miR-181a inhibited cell viability (Fig. 4).

H₂O₂ treatment and overexpression of miR-181a decreased TM cell apoptosis

Cell apoptosis was measured in the TM cells after treatment with H₂O₂, and the effect of miR-181a on cell apoptosis was measured after transfection. The results showed an increased percentage of cell apoptosis ($p < 0.01$) in the si-miR-181a group (mean: 35.7) compared to control groups. Treatment with H₂O₂ caused nuclear condensation, which is an indicator of apoptosis. Also, the miR-181a mimic group (mean: 10.7) showed a significant reduction on cell apoptosis rate ($p < 0.05$), but not as significant as si-miR-181a. These results suggested that the overexpression of miR-181a suppressed TM cell apoptosis, while the knockdown of miR-181a induced apoptosis (Fig. 5).

miR-181a suppressed TM cell apoptosis via blocking the NF- κ B and JNK pathways

Generally, H₂O₂ activates the NF- κ B and JNK pathways and induces cell apoptosis. The results showed that the si-miR-181a group has a decreased expression of miR-181a and treatment with H₂O₂ suppresses the expression of miR-181a ($p < 0.05$). Thus, in the si-miR-181a + H₂O₂ group, the expression of miR-181a is low and it cannot block the NF- κ B and JNK pathways – it will rather produce just the opposite effect (Fig. 6). This suggested that the overexpression of miR-181a could suppress both the pathways, thereby decreasing the rate of cell apoptosis.

Fig. 3. Overexpressed or suppressed miR-181a in TM cells showed transfection efficiency

** $p < 0.01$.

Fig. 4. The overexpression of miR-181a enhanced TM cell viability, while the knockdown of miR-181a inhibited cell viability

Fig. 5. The overexpression of miR-181a suppressed TM cell apoptosis, while the knockdown of miR-181a induced apoptosis

* $p < 0.05$; ** $p < 0.01$.

Fig. 6. H₂O₂ activated NF- κ B and JNK pathways and induced cell apoptosis, while the overexpression of miR-181a suppressed both pathways and decreased the apoptotic rate

* $p < 0.05$.

in the literature that an aberrant expression of miR-181a has been associated with many types of human cancers and acts as a key oncogenic regulator.²³ The underlying mechanism of miR-181a protects the TM cells is not quite clear. In the present study, we not only demonstrated an active change in the expression profile of miR-181a upon H₂O₂-induced apoptosis, but also a protective role of the miR-181a overexpression on TM cell. Thus, these results highlight the functional role of miR-181a in glaucoma.

This study explored the mechanisms of the regulation of miR-181a in TM cell injury. We found that the expression of miR-181a was downregulated in TM cells pretreated with H₂O₂. MTT results showed that the overexpression of miR-181a could improve cell viability, while the knockdown of miR-181a suppressed cell viability. Flow cytometry results demonstrated that miR-181a inhibited cell apoptosis, while the knockdown of miR-181a increased apoptosis. Western blotting assay showed a suppression of miR-181a cell apoptosis via blocking the NF- κ B and JNK signaling pathways.

In this study, we also showed that during H₂O₂-induced apoptosis downregulating miR-181a blocks the NF- κ B and JNK signaling pathways. Our results were similar to one

Discussion

Oxidative stress has been established as an important contributing factor in TM cell degeneration.⁵ The contributory role of H₂O₂ in TM cell degeneration is also known.²³ In the present study, we induced TM cell apoptosis through H₂O₂ and found that miR-181a was downregulated by H₂O₂ in a dose-dependent manner. It has been commonly shown

of the studies conducted by Kozloski et al. in B-cell-like diffuse large B-cell lymphoma.²² Thus, it is likely that miR-181a upregulation may serve a protective role in glaucoma by exerting a protective effect on H₂O₂-induced apoptosis in TM cells. This hypothesis was further supported by our results, which showed that miR-181a overexpression promoted TM cell apoptosis, which possibly induced the trans-differentiation of mesenchymal-like cells in the aqueous humor of TM cells.²⁰ NF- κ B is considered to be an important nuclear transcription factor that helps in mediating the inflammatory response caused by oxidative stress.²⁴ In endothelial cells, NF- κ B is strongly activated by treatment with H₂O₂ for 4 h.²⁵ However, to elucidate the exact molecular interaction between miR-181a and the NF- κ B and JNK pathways in protecting trabecular meshwork cells, future experiments that require blocking of these pathways or its upstream pathways upon the inhibition of miR-18a would provide solid evidence about the direct targeting of miR-181a on anti-apoptotic pathways in TM cells.

Many miRNAs are involved in the molecular mechanisms of glaucoma; some miRNAs were up-regulated in cells, while others were down-regulated.¹² These miRNAs facilitate the evaluation of specific pathophysiology and offer diagnosis. Further in-depth understanding will help in diagnosing the subtypes of glaucoma, the pathophysiological stages, and the prescription of appropriate drugs.¹² miR-183 was involved in the alteration of integrin- β 1 expression, thereby effecting the physiology of TM, resulting in glaucoma.²⁶ The miR-29 family regulates ECM synthesis in the TM.²⁷ The changes associated with ECM synthesis result in alterations in the IOP. miR-29b negatively regulates the ECM proteins, and its regulators – under chronic oxidative stress conditions – affect ECM homeostasis in human TM cells. The expression of miR-29b decreased under chronic oxidative stress conditions, resulting in ECM gene upregulation. Similarly, miR-24 plays an important role in the flow pathways.²⁸ One study identified the involvement of microRNAs in glaucoma with *hsa-let-7b-3p* as the most prevalent factor in the aqueous humor.¹³

In conclusion, this study indicated that miR-181a could improve the survival rate of TM cells after H₂O₂ treatment by blocking the NF- κ B and JNK signaling pathways. These findings might provide novel therapeutic opportunities in the treatment of glaucoma.

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