

## Original Research Article

# Artemisinin ameliorates diabetic retinopathy by upregulating CASC2/miR-155/SIRT1 axis

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### Abstract

**Purpose:** To explore the protective effects of artemisinin (Art) against diabetic retinopathy (DR) and the probable mechanism of action.

**Methods:** MIO-M1 cells were treated with high glucose (HG) and Art, and the cells' proliferative ability was determined using cell counting kit-8 (CCK-8) and 5-ethynyl-2'-deoxyuridine (EdU) assay. The relative levels of inflammatory factors in the culture medium of MIO-M1 cells were determined by enzyme-linked immunosorbent assay (ELISA). While the expression levels of CASC2, miR-155 and Sirtuin1 (SIRT1) in MIO-M1 cells were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). Interaction of Art with the cell target was assessed using dual-luciferase reporter assay. The role of the CASC2/miR-155/SIRT1 axis in Art-induced protection against the proliferation and inflammation of MIO-M1 cells was evaluated.

**Results:** HG induced elevated proliferation of MIO-M1 cells and production of inflammatory factors, but these effects were countered by Art treatment ( $p < 0.05$ ). CASC2 and SIRT1 were upregulated, while miR-155 was downregulated in HG-treated MIO-M1 cells; changes in their expressions remained the same following Art treatment. CASC2/miR-155/SIRT1 axis was responsible for the ameliorative effect of Art on HG-treated MIO-M1 cells.

**Conclusion:** Artemisinin treatment inhibits cell activation and production of pro-inflammatory cytokines in HG-induced MIO-M1 cells via CASC2/miR-155/SIRT1 axis. Thus, artemisinin has potentials for development into a therapeutic agent for the management of diabetic retinopathy.

**Keywords:** Diabetic retinopathy, Artemisinin, CASC2, MiR-155, SIRT1

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## INTRODUCTION

Diabetic retinopathy (DR) is a common diabetic microvascular complication which is the leading cause of blindness in working-age people in developed countries [1]. According to the prediction of the World Health Organization, the number of global diabetic people will double by

2030 when compared to 2004, and the number of DR patients will also increase [2]. The pathogenesis of DR is complex, including increased flows of the polyol pathway and hexamine pathway, increased production of advanced glycation end-products (AGEs) and activation of protein kinase C (PKC) [3]. Recently, the role of epigenetics in the

development of DR has been well documented [4].

Artemisinin (Art) is the first-line drug for the treatment of malaria because of its rapid toxicity to Plasmodium [5]. Artemisinin extract is able to obviously decrease blood sugar and blood lipid levels in diabetic rats, which protects the liver and kidney functions [6]. Moreover, the anti-inflammatory effect of Art has been reported. They exert an anti-angiogenic effect on rheumatoid arthritis by downregulating angiogenesis-associated genes, including MMP-2, MMP-9, VEGF and HIF-1 $\alpha$  [7]. Wei *et al* [8] reported that Art is of significance in the development of DR.

There are three types of glial cells in the retina: Müller cells, microglia and astrocytes. Müller cells are the most important type because of their biological functions in supporting and nourishing neurons. They are distributed in the entire retina, from the photoreceptors to the inner limiting membrane.

Müller cells are connected to cell bodies and neurites of neurons in the retina, and retinal vessels [9]. Previous evidence has shown that Müller cells are of significance in the survival and regeneration of nerve cells [10]. In this paper, human Müller cell line MIO-M1 is used as the experimental model to study the pathogenesis of DR.

In the human genome, less than 2 % of gene sequences are able to encode proteins, while more than 98 % of are non-coding sequences. Among these non-coding RNAs, long non-coding RNAs (lncRNAs) with a length of more than 200 nucleotides, can transcriptionally or post-transcriptionally regulate expressions of protein-encoding genes. LncRNAs are products of RNA polymerase and they were once considered to have no biological function. Later on, their involvement in human diseases emerged [11].

A relevant study on human  $\beta$ -cell transcriptome found that lncRNAs can be dynamically regulated and abnormally expressed in T2DM cases [12]. Overexpression of lncRNA CASC2 inhibits the development of liver cancer cells [13]. CASC2 inhibits the proliferative potential and phenotype transformation of PASM in hypoxia-induced pulmonary hypertension [14]. The potential role of CASC2 in DR development remains unclear.

The purpose of the present study was to investigate the ameliorative effects of Art in diabetic retinopathy (DR), and to elucidate its mechanism of action.

## EXPERIMENTAL

### Cell culture and treatment

Human Müller cells (MIO-M1) were provided by American Type Culture Collection (ATCC) (Manassas, VA, USA), and cultivated in Dulbecco's modified eagle medium/F-12 (DMEM/F-12) (#11320082; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco, Rockville, MD, USA).

MIO-M1 cells were induced with normal level of glucose (NG) (5 mM), high level of glucose (HG), 30 mM or HG+Art ( $10^{-4}$  M) for 24 h. Mannitol (25 mM) and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) were the solvent control of NG and HG, respectively.

### Cell counting kit-8 (CCK-8) assay

Cells were inoculated in a 96-well plate with  $5 \times 10^3$  cells per well. At different time points, absorbance value at 450 nm of each sample was read using the CCK-8 kit (RIBOBIO, Guangzhou, China) for plotting the viability curves.

### 5-Ethynyl-2'- deoxyuridine (EdU) assay

The cells were pre-inoculated in a 24-well plate ( $2 \times 10^4$  cells/well). They were incubated in 4% methanol for 30 min, followed by 10-min permeabilization in 0.5% TritonX-100, and 30-min reaction in 400  $\mu$ L of 1  $\times$  ApollorR. The cells were dyed in 1  $\times$  Hoechst 33342 for another 30 min. Positive EdU-stained cells were calculated (RIBOBIO, Guangzhou, China).

### Enzyme-linked immunosorbent assay (ELISA)

After cell induction, culture medium was collected and centrifuged to harvest the supernatant. Secreted protein levels were detected using commercial ELISA kits (R & D Systems, Minneapolis, MN, USA).

### Quantitative real-time polymerase chain reaction (qRT-PCR)

Cellular RNAs were isolated using TRIzol (Invitrogen, Carlsbad, CA, USA), chloroform and isopropanol. They were reverse-transcribed to cDNAs and subjected to qRT-PCR using the SYBR Premix Ex Taq II (TaKaRa, Dalian, China) at 95°C for 30 s, then 95 °C for 5 sec and 60 °C for 34 sec, for 40 cycles. The primer sequences used are shown in Table 1.

**Table 1:** Primer sequences used in of qRT-PCR

Protein	Sequences (5'-3')
CASC2 (Forward)	GCTGATCAGAGCACATTGGA
CASC2 (Reverse)	ATAAAGGTGGCCACAACCTGC
miR-155 (Forward)	TTAATGCTAATCGTGATAGGGGT
miR-155 (Reverse)	GTGCAGGGTCCGAGGT
SIRT1 (Forward)	TAGACACGCTGGAACAGGTTGC
SIRT1 (Reverse)	CTCCTCGTACAGCTTCACAGTC
GAPDH (Forward)	AGGTCGGTGTGAACGGATTTG
GAPDH (Reverse)	GGGGTCGTTGATGGCAACA

### Transfection

Cells were seeded in 6-well plates ( $1 \times 10^6$  cells/well) and cultivated to 75% confluence. A mixture containing 7.5  $\mu$ L of Lipofectamine RNAiMAX and 25 pmol plasmid was added to each well for 24-h transfection.

### Dual-luciferase reporter assay

The cells were cultivated in the 96-well plate ( $5 \times 10^3$  cells/well). Luciferase vectors were constructed based on binding sequences predicted by bioinformatic database, and they were co-transfected into cells with miR-155 mimics or miR-NC for 24 h. Luciferase activity was measured using the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

### Statistical analysis

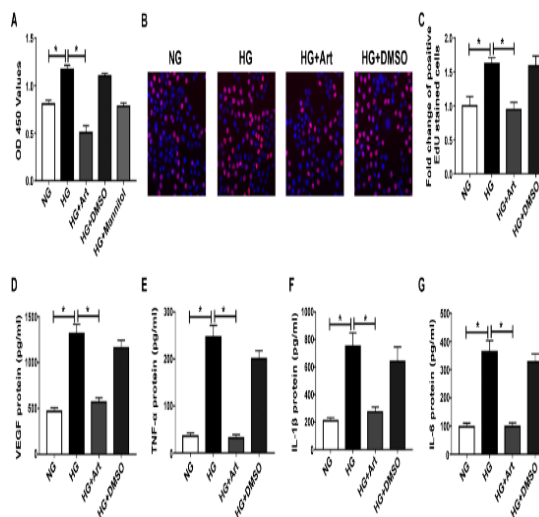
Statistical analysis was conducted using SPSS 19.0 software (IBM, Armonk, NY, USA). Differences between two groups were analyzed by using Student's t-test. Comparison between multiple groups was done using one-way ANOVA test, followed by post hoc test (least significant difference). Significant difference was assumed at  $p < 0.05$ .

## RESULTS

### Art treatment inhibited cell activation and production of pro-inflammatory cytokines in HG-induced MIO-M1 cells

MIO-M1 cells were induced with NG, HG or HG + Art, with mannitol and DMSO as the solvent control for NG and HG, respectively. HG induction markedly enhanced cell viability and EdU-positive percentage in MIO-M1 cells, which were reduced after Art treatment (Figure 1 A and

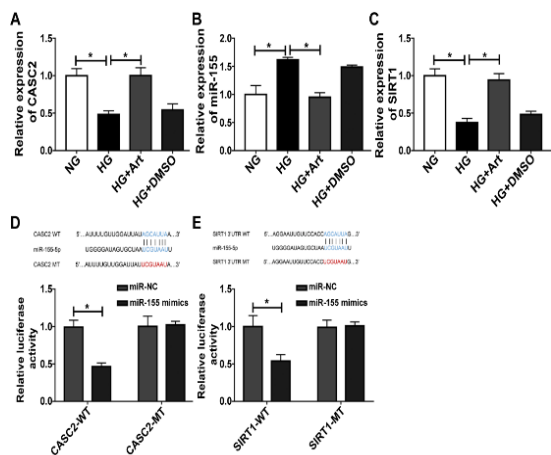
B). Moreover, relative expression levels of VEGF, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in culture medium of HG-induced MIO-M1 cells were enhanced in comparison to those with NG induction, and their increased levels were downregulated by Art treatment (Figure 1 C - F). Thus, Art exerted inhibitory effects on HG-induced cell activation and production of pro-inflammatory factors in MIO-M1 cells.



**Figure 1:** Art treatment inhibited cell activation and production of pro-inflammatory cytokines in HG-treated MIO-M1 cells. MIO-M1 cells were induced with NG (5 mM), HG (30 mM) or HG + Art ( $10^{-4}$  M) for 24 h, with mannitol (25 mM) and DMSO as the solvent control of NG and HG, respectively. (A) Cell viability; (B) EdU-positive percentage; (C - F) Relative expression levels of VEGF, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in culture medium

### Art treatment inhibited cell activation and production of pro-inflammatory cytokines in HG-induced MIO-M1 cells

A previous study showed that CASC2 is downregulated in diabetic patients [15]. Here, CASC2 was downregulated in HG-induced MIO-M1 cells, which was reversed by Art treatment (Figure 2 A). The level of miR-155 was upregulated in HG-induced MIO-M1 cells, and it was further downregulated by treatment with Art (Figure 2 B). SIRT1 was downregulated following HG induction in MIO-M1 cells, and its downregulated level was elevated by Art treatment (Figure 2 C). Dual-luciferase reporter assay verified that CASC2 exerted miRNA sponge effect on miR-155, thereby upregulating SIRT1, the target gene of miR-155 (Figure 2 D and E). Taken together, the above results have demonstrated the regulatory interaction in the CASC2/miR-155/SIRT1 axis.



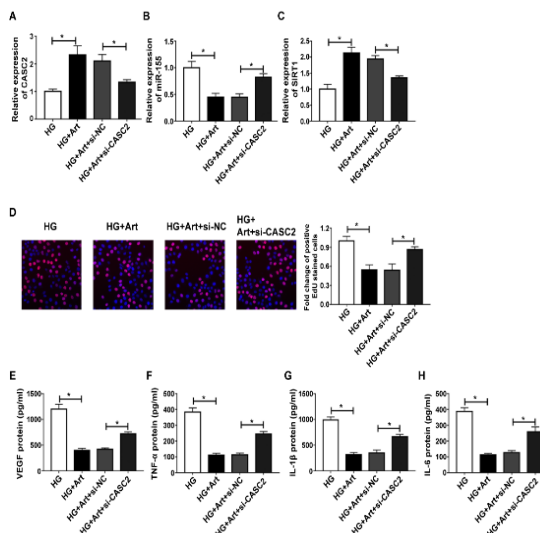
**Figure 2:** Art treatment inhibited cell activation and production of pro-inflammatory cytokines in HG-induced MIO-M1 cells via the CASC2 / miR-155 / SIRT1 axis. MIO-M1 cells were induced with NG (5 mM), HG (30 mM) or HG + Art ( $10^{-4}$  M) for 24 h, with mannitol (25 mM) and DMSO as the solvent control of NG and HG, respectively. (A-C) Relative levels of CASC2, miR-155 and SIRT1 in MIO-M1 cells; (D) Direct binding between CASC2 and miR-155; (E) Direct binding between miR-155 and SIRT1

**Knockdown of CASC2 eliminated the protective effect of Art on HG-induced MIO-M1 cells**

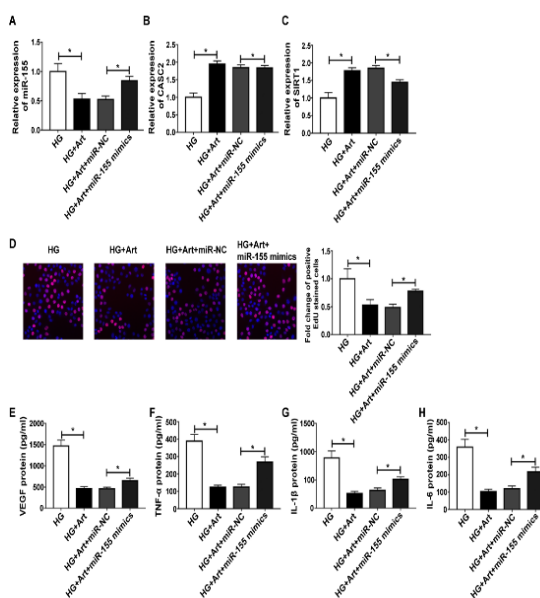
To unravel the biological role of CASC2, si-CASC2 was constructed (Figure 3 A). Knockdown of CASC2 upregulated miR-155, but downregulated SIRT1 in MIO-M1 cells induced with HG and Art (Figure 3 B and C). The above data showed that Art exerted inhibitory effects on HG-induced cell activation and production of pro-inflammatory factors in MIO-M1 cells. Interestingly, they were abolished by transfection of si-CASC2 (Figure 3 D - H). It is concluded that CASC2 was responsible for the biological effects of Art on HG-induced MIO-M1 cells.

**Overexpression of miR-155 damaged the protective effect of Art on HG-induced MIO-M1 cells**

The involvement of miR-155 was analyzed. Transfection efficacy of miR-155 mimics was first examined in MIO-M1 cells induced with HG and Art (Figure 4 A). Overexpression of miR-155 did not affect CASC2 level, suggesting that CASC2 was the upstream gene of miR-155 (Figure 4 B). Transfection of miR-155 mimics markedly downregulated SIRT1 (Figure 4 C). The inhibitory effects of Art on HG-induced cell activation and production of pro-inflammatory factors in MIO-M1 cells were abolished by overexpressed miR-155 (Figure 4 D - H).



**Figure 3:** Knockdown of CASC2 damaged the protective effect of Art on HG-induced MIO-M1 cells. MIO-M1 cells were induced with HG (30 mM) or HG+Art ( $10^{-4}$  M) for 24 h, and the latter treated cells were further transfected with si-NC or si-CASC2, respectively. (A-C) Relative expression levels of CASC2, miR-155 and SIRT1 in MIO-M1 cells; (D) EdU-positive rate; (magnification: 200×) (E-H) Relative expression levels of VEGF, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in culture medium



**Figure 4:** Overexpression of miR-155 damaged the protective effect of Art on HG-induced MIO-M1 cells. MIO-M1 cells were induced with HG (30 mM) or HG+Art ( $10^{-4}$  M) for 24 h, and the latter treated cells were further transfected with miR-NC or miR-155 mimics, respectively. (A - C) Relative expression levels of CASC2, miR-155 and SIRT1 in MIO-M1 cells; (D) EdU-positive percentage (200×) (E - H) Relative levels of VEGF, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in culture medium

## DISCUSSION

Diabetic retinal microangiopathy is a major complication of blindness in diabetic patients in recent years. At present, effective therapies for DR are lacking. During the pathological process of DR, chronic glutamate toxicity, activation of inflammatory glial and increased expressions of other neurotrophic factors cause apoptosis of ganglion cells, Müller cells and other neuronal cells, as well as the activation of microglia [16]. In the meantime, positive expression of GFAP in Müller cells increases, which is a symbol of neurodegeneration. It is generally considered that oxidative stress, increased levels of AGEs and activated renin-angiotensin system attributable to DR-induced neurodegenerative changes [17].

Artemisinin is isolated from the plant *Artemisia annua*, sweet wormwood, a sesquiterpene lactone, containing an unusual peroxide bridge. In addition to the well-known antimalarial effect, Art has been confirmed to have neuroprotective functions [18]. Art is a small lipophilic drug that can easily cross the blood-brain barrier and provides neuroprotection against oxidative stress damage. Therefore, Art is beneficial to neuronutrition. These findings discovered that HG induction strengthened proliferative ability, and drove the production of pro-inflammatory cytokines of MIO-M1 cells, which were reversed by Art treatment.

With the development and application of high-throughput sequencing, a large number of lncRNAs have been discovered. Functionally, lncRNAs act as miRNA inhibitors by competitively binding to mRNAs [19]. Previous studies have shown that lncRNA CASC2 is downregulated in diabetic patients [15]. Art is able to mediate lncRNA expressions. In the present study, CASC2 was downregulated in HG-induced MIO-M1 cells, which was then reversed to the baseline level (NG induction), owing to Art treatment. Through dual-luciferase reporter assay, CASC2 was identified to competitively bind SIRT1 as the miR-155 inhibitor.

Sirtuin1 (SIRT1) was initially discovered in 1999 by Roy Frye. It is distributed in the cytoplasm and nucleus of mammalian cells. SIRT1 regulates various metabolisms, including insulin sensitivity improvement, anti-inflammation, inhibition of tumor growth, and diabetes complications [20]. SIRT1 was upregulated in MIO-M1 cells induced by HG and Art as the downstream target binding miR-155. In addition, rescue experiments uncovered that the CASC2 / miR-155 / SIRT1

axis was responsible for the protective effect of Art on HG-induced MIO-M1 cells.

This study has some limitations. Firstly, our findings are not validated in an *in vivo* model. Secondly, clinical samples are lacking for the detection of expressions of CASC2, miR-155 and SIRT1.

## CONCLUSION

Artemisinin treatment inhibits cell activation and the production of pro-inflammatory cytokines in HG-induced MIO-M1 cells via CASC2/miR-155/SIRT1 axis, thus impeding the development of diabetic retinopathy.

## DECLARATIONS

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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