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# Effect of Artesunate on The Expression of ICAM-1 and MMP-9 in Vascular Endothelial Cells Under High Glucose Conditions

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## ABSTRACT

**Background:** Diabetic retinopathy (DR) is the most common cause of diabetes-induced microvascular complications and it is the leading cause of blindness in working age worldwide. At the present stage, the main treatment for neovascularization and leakage in DR is anti-VEGF therapy, however, anti-VEGF therapy has its limitation, such as single target and short half-life of anti-VEGF drugs.

Therefore, clarifying more therapeutic targets according to the molecular mechanism of neovascularization and leakage is needed, treating the disease by a drug which is multi-target and long-acting. Previous studies have shown that artesunate (ART) can inhibit retinal neovascularization and leakage through multiple targets. This study aimed to clarify the new mechanism of ART inhibiting retinal neovascularization and leakage.

**Objective:** To investigate the new mechanism of retinal neovascularization and leakage inhibited by artesunate (ART)

**Methods:** Human Umbilical Vein Endothelial Cells (HUVEC) were divided into glucose (G) group, 40mmol/L G+ART(G40+ART) group, mannitol (M) control group, dimethyl sulfoxide (DMSO)control group. The concentration gradient of G group is 5.5mmol/L G (G5.5), 25mmol/L G (G25), 40mmol/L G (G40); The concentration gradient of M control group is 5.5 mmol / L G + 19.5 mmol / L M (M25), 5.5 mmol / L G + 34.5 mmol / L M (M40), The concentration gradient of ART of G40 + ART group is G40 + 10ug /ml ART(10A), G40+20ug/ml ART(20A), G40+40ug/ml ART(40A); the volume of DMSO in the DMSO control group is the same as it is in the 40A group. Western blot, and cell Immunofluorescence technique were used to detect the protein expression of ICAM-1 and MMP-9 in each group.

**Results:** Western blot, and cell Immunofluorescence showed that the protein expression of Intercellular adhesion molecule-1（ICAM-1）and Matrix metalloproteinase-9（MMP-9）in G25 group was higher than that in G5.5 group ($P<0.01$), and it increased in G40 group compared with G25 group ($P<0.01$);The protein expression of ICAM-1 and MMP-9 in G25 group was higher than that of M25 group ($P<0.01$),and it increased in G40 group compared with M40 ($P<0.01$);the protein expression of ICAM-1 and MMP-9 of G40+ART group was lower than that of G40 group, in which, it was lower in 20A group than that of 10A group ($P<0.01$), and it was lower in 40A group compared with 20A group ($P<0.01$). The DMSO control group showed that the protein expression of ICAM-1 and MMP-9 in G40+ART was lower than that of G40+DMSO group ($P<0.01$).

**Conclusion:** The two targets of ICAM-1 and MMP-9 may act as new therapeutic targets of ART to suppress the retinal neovascularization and leakage in DR, offering assistance for ART used in DR to treat the neovascularization and leakage.

## INTRODUCTION

In diabetic microvascular complications, DR is the most common Rubsam et al. (2018) and has become the leading cause of blindness in working-age people worldwide Tan et al. (2023). The clinical features of DR include increased vascular permeability, formation of microvascular lesions, neovascularization, and macular edema Barber et al. (2003). ART is a new type of antimalarial drug that has been reported to have anti-tumor effects and has received widespread attention for its anti-angiogenesis effects in tumors He et al. (2008). ART has an effect of inhibiting the proliferation, migration, and lumen formation of vascular endothelial cells, and can effectively inhibit the formation and growth of tumor blood vessels in a dose-dependent manner Chen et al. (2004). Recent studies have shown that ART can inhibit the growth and leakage of retinal neovascularization

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**Key words:** Artesunate; Intercellular adhesion molecule-1; Matrix metalloproteinase-9; Vascular endothelial cells; Mannitol; Diabetic retinopathy.

(NV) Zong et al. (2016). During the early stage of neovascularization, intercellular adhesion molecule-1 (ICAM1) and matrix metalloproteinase-9 (MMP-9) are crucial. Firstly, MMP-9 can degrade extracellular matrix, which is a prerequisite for the formation of neovascularization Rocca et al. (2017). Clinical evidence from fluorescein angiography of DR patients shows that the blood-retina barrier (BRB) is the main site of vascular leakage leading to macular edema, and MMP-9 can promote increased vascular permeability Kowluru et al. (2012). Secondly, ICAM-1 is involved in leukocyte adhesion to vascular endothelial cells Cronstein et al. (1993) Mackay et al. (1993), induces capillary occlusion Schroder et al. (1991), causes damage and death of endothelial cell Joussen et al. (2001), and leads to non-perfusion and neovascularization of retinal capillary Adamis et al. (1996), Aiello et al. (1995). Currently, the main treatment for neovascularization and leakage in DR is anti-VEGF therapy. However, monotherapy with anti-VEGF drugs has certain limitations such as a single target point and short half-life. Therefore, we need to clarify more therapeutic targets based on the molecular mechanisms of neovascularization and use drugs that can exert a long-term therapeutic effect through multiple targets to prevent and treat this disease. Previous studies have shown that ART can inhibit retinal neovascularization and leakage through multiple targets. In this experiment, ART was used to treat HUVEC under high glucose conditions, and the expression of ICAM-1 and MMP-9 proteins was detected using Western blot and cell immunofluorescence to explore the new mechanism of ART in inhibiting retinal neovascularization and leakage.

## MATERIALS AND METHODS

### Materials

HUVEC-C (Model: ZQ0446, Specification: 5 x 10^5 cells/vial) was obtained from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd.

Main reagents included DMEM low glucose medium (China Solarbio), DMEM high glucose medium (China Solarbio), fetal bovine serum (HyClone, USA), penicillin-streptomycin mixture (100X) (China Solarbio), 0.25% trypsin digestion solution (China Solarbio), dimethyl sulfoxide (Sigma, USA), rabbit anti-human MMP-9 antibody (Abcam, UK), rabbit anti-human ICAM-1 antibody (Abcam, UK), rabbit anti-human GAPDH antibody (Elabscience, China), goat anti-rabbit Western blot secondary antibody (Elabscience, China), and goat anti-rabbit fluorescent secondary antibody (Elabscience, China), WB-specific primary-secondary antibody dilution buffer (Proteintech Group, USA), and fluorescent antibody dilution buffer (China Solarbio).

Main equipment included a clean bench (Suzhou Antai Air Technology Co., Ltd., China), CO2 constant temperature incubator (Heraeus, Germany), inverted microscope (OLYMPUS, Japan), inverted fluorescence microscope (OLYMPUS, Japan), multifunctional imaging system (Vilber, France), low-speed tabletop centrifuge (Beijing SSBT Times Biotech Co., Ltd., China), tabletop low-temperature high-speed centrifuge (Sigma, USA), ultra-pure water purification system (Heal Force, China), electronic balance (METTLER TOLEDO, USA).

### Methods

Cell culture: The cells were recovered from the frozen tube and added to low glucose (5.5 mmol/L glucose) DMEM medium containing 10% FBS. Adjusted the cell density to 1×105/mL, then seeded 4 mL per bottle in a 25 cm2 culture flask and incubated at 37°C with 5% CO2 in a constant temperature incubator.

Western blotting was used to detect the expression of ICAM-1 and MMP-9 proteins.SDS-PAGE was used for protein immunoblot analysis. The protein samples were mixed with 5X SDS-PAGE protein loading buffer at a ratio of 4:1. According to the target protein molecular weight, 10% separation gel and 5% concentration gel were prepared for electrophoresis. The protein was transferred onto a PVDF membrane, which was then blocked with prepared milk blocking solution to prevent non-specific binding sites on the membrane. Rabbit anti-human MMP-9 antibody (Abcam, UK) and rabbit anti-human ICAM-1 antibody (Abcam, UK) were used as primary antibodies, and rabbit anti-human GAPDH antibody (Elabscience, China) was used as an internal reference control. Then, goat anti-rabbit Western blot secondary antibody (Elabscience, China) was used as a secondary antibody. ECL luminescence solution was used for gel imaging analysis. Image J software was used for quantitative analysis of band gray values. The ratio of the gray value of each target band to the gray value of the internal reference GAPDH band was used as the relative expression level of the protein. The mean of three repeated experiments was used as the final statistical value.

Immunofluorescence staining was used to detect the expression of ICAM-1 and MMP-9 proteins in cells.

Sterile cover glasses were placed in 12 or 10 wells of a 24-well plate according to the experimental needs. HUVECs were seeded onto the cover glasses at a density of 1×105/mL and cultured for 24 hours. After removing the treatment solution,the cells were washed with PBS, fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with 0.1% Triton X-100 for 1 minute at room temperature. The cells were blocked with 10% normal goat serum in PBS for 1 hour. Rabbit anti-human ICAM-1 fluorescence antibody (Abcam, UK) was used as the primary antibody and incubated overnight at 4°C. One well without primary antibody was used as a negative control.

Goat anti-rabbit fluorescent secondary antibody (Elabscience, China) was used as the secondary antibody and incubated at room temperature for 1 hour. DAPI was used for nuclear counterstaining for 10 minutes. Anti-fluorescence attenuation sealing agent was used for slide sealing, and results were observed and photographed under a fluorescence microscope.

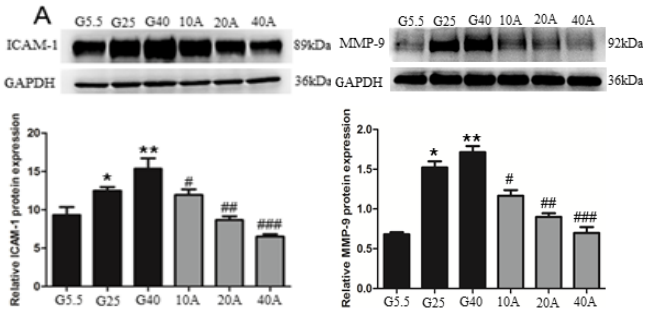
Statistical methods: SPSS 17.0 was used for statistical analysis of the data. The data from each group were evaluated with the Shapiro-Wilk test and found to be normally distributed. Comparisons between two groups were analyzed using t-tests, and a P-value less than 0.01 or 0.05 indicated statistical significance.

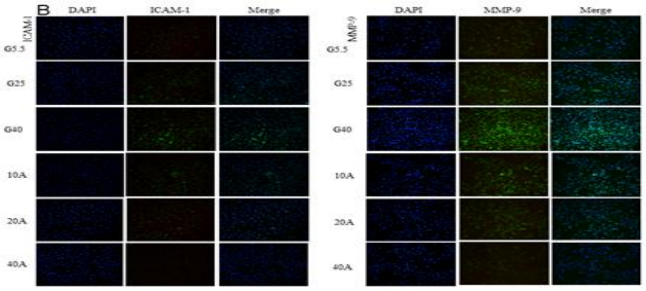
## RESULTS

Effects of high glucose conditions on the expression of ICAM-1 and MMP-9 proteins in HUVECs, as well as the effects of ART on the expression of ICAM-1 and MMP-9 proteins under high glucose conditions.

The cells were treated with G5.5, G25, and G40 culture media, and ICAM-1 and MMP-9 protein expression was detected using Western blotting and immunofluorescence staining. The results showed that the expression of ICAM-1 and MMP-9 proteins in the G25 group was higher than that in the G5.5 group (t=4.796,17.31, P<0.01), and the expression in the G40 group was higher than that in the G25 group (t=3.430,2.987, P<0.01). The cells were treated with three concentrations of ART (10A, 20A, 40A), and ICAM-1 and MMP-9 protein expression was detected using Western blotting and immunofluorescence staining. The results showed that the expression of ICAM-1 and MMP-9 proteins in the G40+ART group was lower than that in the G40 group. Specifically, the expression in the 10A group was lower than that in the G40 group (t=3.846,8.887, P<0.01); the expression in the 20A group was lower than that in the 10A group (t=6.536,5.329, P<0.01); and the expression in the 40A group was lower than that in the 20A group (t=6.169,3.947, P<0.01) (Figure 1 A, B).

**Figure 1:** A and B demonstrate the effect of high glucose conditions on the expression of ICAM-1 and MMP-9 proteins in HUVECS, as well as the effect of ART on the expression of ICAM-1 and MMP-9 proteins in HUVECS under high glucose conditions.



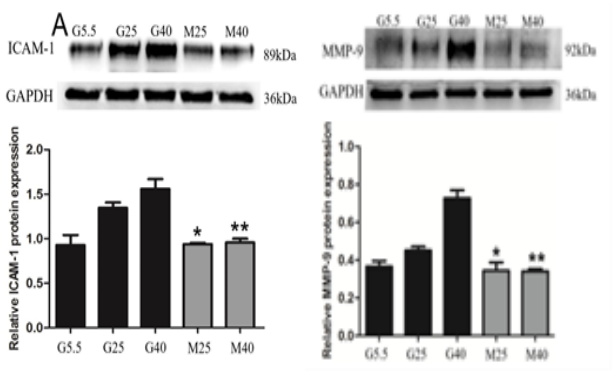

Western blotting and cellular immunofluorescence results showed that the expression of ICAM-1 and MMP-9 increased under high glucose conditions, with concentration dependence; ART can inhibit the expression of ICAM1 and MMP-9 proteins under high glucose conditions,with concentration dependence (* represents t=4.796, 17.31, P<0.01 compared to the G5.5 group; * * represents t=3.430, 2.987, P<0.01 compared to the G25 group; # represents t=3.846, 8.887, P<0.01 compared to the G40 group; # # represents t=6.536, 5.329, P<0.01 compared to the 10A group; # # # represents t=6.169, 3.947, P<0.01 compared to the 20A group).

Investigating the effect of high osmotic pressure on the expression of ICAM-1 and MMP-9 proteins in HUVECS.

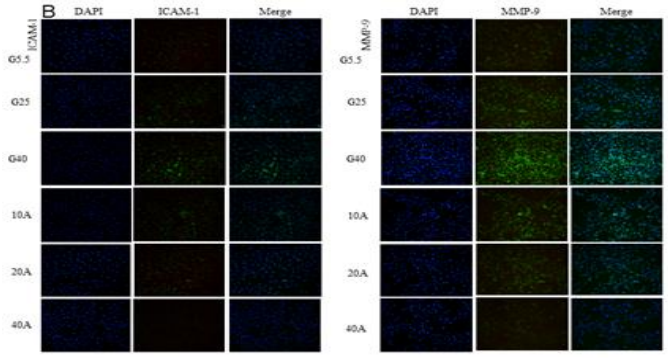
In the control group (M group), the expression of ICAM-1 and MMP-9 proteins was detected using Western blotting and immunofluorescence staining.

The results showed that the expression of ICAM-1 and MMP-9 proteins in the G25 group was higher than that in the M25 group (t=11.84,3.845, P<0.01) and the expression in the G40 group was higher than that in the M40 group (t=8.803,15.30, P<0.01) (Figure 2 A, B).

**Figure 2:** A and B show the effect of high osmotic pressure on ICAM-1 and MMP-9 protein expression in vascular endothelial cells. Western blot and cellular immunofluorescence showed that high osmotic pressure had no significant effect on ICAM-1 and MMP-9 protein expression. (* indicates t=11.84, 3.845, P<0.01 compared to G25, * * indicates t=8.803, 15.30, P<0.01 compared to G40).



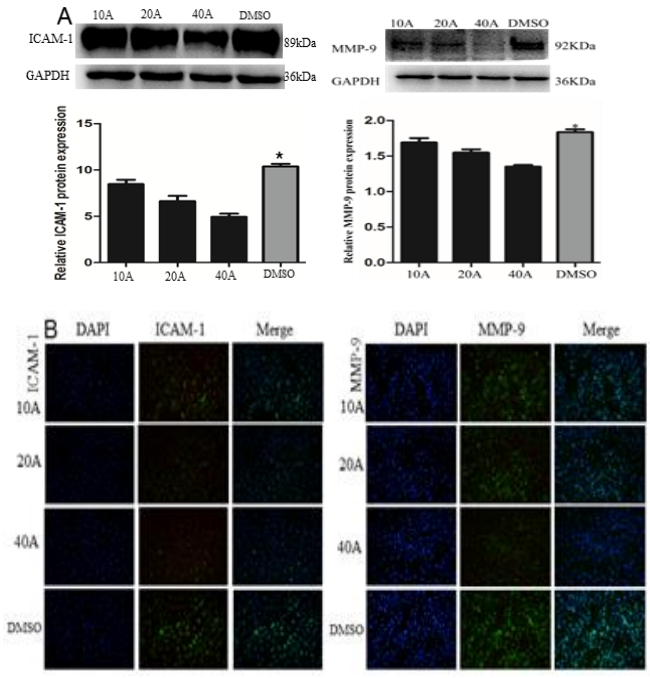



To further confirm the effects of ART on the expression of ICAM-1 and MMP-9 proteins in HUVECs under high glucose conditions, a DMSO control group was set up.

In the DMSO control group, the amount of DMSO was the same as the volume of DMSO used to dissolve ART in the 40A group.

The expression of ICAM-1 and MMP-9 proteins was detected using Western blotting and immunofluorescence staining. The results showed that the expression of ICAM-1 and MMP-9 proteins in the G40+ART group was lower than that in the DMSO group ($t=5.997,10.31,21.43,3.378,8.229,18.19$, $P<0.01$). (Figure3 A、B).

**Figure 3:** A and B further demonstrate the effect of ART on the expression of ICAM-1 and MMP-9 proteins under high glucose conditions, with DMSO control group set up. Western blot and cell immunofluorescence results showed that the expression of ICAM-1 and MMP-9 proteins in the ART group was significantly lower than that in the DMSO group (* represents $t=5.997$, 10.31, 21.43, 3.378, 8.229, 18.19, $P<0.01$ compared to the 10A, 20A, and 40A groups).




## DISCUSSION

This study demonstrated at the cellular level that high glucose concentration can promote the expression of ICAM-1 and MMP-9 proteins in HUVECs in a concentration-dependent manner. ART can inhibit the expression of ICAM-1 and MMP-9 proteins under high glucose conditions, and the inhibitory effects are also concentration-dependent.

There is increasing evidence that inflammation plays an important role in the pathogenesis of DR. DR begins as a low-grade chronic inflammatory disease Adamis et al. (2002). During the development of DR, pro-inflammatory factors, chemokines, and leukocyte adhesion increase Joussen et al. (2004), and leukocyte stasis is a major component of the inflammatory process El-Asrar et al. (2012). ICAM-1 expressed on endothelial cells can not only regulate leukocyte adhesion to endothelial cells but also play an important role in regulating blood-retinal barrier disruption and vascular permeability Campbell et al. (1998), Sumagin et al. (2011), Poulaki et al. (2003), which can induce retinal capillary non-perfusion and neovascularization Adamis et al. (1996), Aiello et al. (1995). MMP-9 can promote leukocyte stasis Mcguire et al. (2012), and its mechanism is related to basement membrane degradation and leukocyte aggregation at the site of tissue damage Sherer et al. (2006).

In addition, the stages of neovascularization in DR include basement membrane degradation, endothelial cell migration and proliferation, followed by capillary formation. The migration and remodeling of this tissue are regulated by matrix metalloproteinases (MMPs) El-Asrar et al. (2012). MMP-9 is the largest member of the MMP family Malemud et al. (2006), which can promote increased vascular permeability Giebel et al. (2005), degrade the capillary basement membrane, a part of extracellular matrix Rocca et al. (2017), and is a necessary condition for endothelial cells to infiltrate and form new lumens in the subendothelial matrix Kowluru et al. (2012). Moreover, MMP-9 can degrade the tight junction protein components of the BRB endothelial cells, increasing vascular leakage Giebel et al. (2005).

Previous studies have shown that the expression of ICAM-1 and MMP-9 is increased under high glucose conditions in endothelial cells and DR patients Kowluru et al. (2012) Matsumoto et al. (2012), Limb et al. (1999), Das et al. (1999), and the increase in ICAM-1 expression on endothelial cells is glucose concentration-dependent Lee et al. (2014). The results of this study are consistent with previous research. In addition, this study further demonstrated that with the increase of glucose concentration, the expression of MMP-9 in endothelial cells will also increase, indicating that higher blood

glucose levels may worsen the severity of diabetic retinopathy. The results showed that ART can inhibit the expression of ICAM-1 and MMP-9 proteins in endothelial cells under high glucose conditions, and the inhibitory effects were concentration-dependent. ART can suppress iris and retinal neovascularization in rabbits and alleviate macular edema in monkeys by downregulating the expression of VEGFR2, PKCα, and PDGFR Zong et al. (2016).

This study provides new targets and theoretical support for ART in inhibiting the formation and leakage of retinal neovascularization. In addition to high levels of ICAM-1, the levels of its ligands CD11a/CD18 and CD11b/CD18 are also increased in diabetic patients Song et al. (2007). Blocking the expression of ICAM-1 or CD18 reduces leukocyte stasis, endothelial cell death, and vascular leakage in the retina of diabetic animals Kociok et al. (2009). In DR mouse models with ICAM-1 and CD18 genes knocked out, the number of leukocytes adhering to retinal vessels decreases, the number of endothelial cell injuries decreases, the breakdown of the blood-retinal barrier is reduced, and the histopathological changes in retinal vessels are alleviated Joussen et al. (2004). Inhibiting MMP-9 activity can suppress corneal neovascularization and protect the integrity of BRB function, reducing retinal vascular leakage Bhatt et al. (2010), Samtani et al. (2009). Therefore, based on the results of this study, ICAM-1 and MMP-9 may become new targets for treatment of DR by ART and play a therapeutic role.

In the pathogenesis of DR, vascular endothelial growth factor (VEGF) is the main factor that promotes the formation of neovascularization and macular edema VEGF Inhibitors for AMD and Diabetic Macular Edema et al. (2015). However, other factors such as ICAM-1, MMP-9, HMGB-1 Klaassen et al. (2013), Yu et al. (2015), Santos et al. (2014), TNFα Joussen et al. (2002), Clauss et al. (2001) also play similar roles. Therefore, VEGF is not the only factor for neovascularization and leakage. Currently, the treatment for neovascularization and macular edema mainly involves the use of anti-VEGF drugs. Anti-VEGF drugs can inhibit neovascularization and leakage and improve visual acuity in patients with macular edema VEGF Inhibitors for AMD and Diabetic Macular Edema et al. (2015), Klaassen et al. (2013), Yu et al. (2015), Santos et al. (2014), Joussen et al. (2002), Clauss et al. (2001), Jampol et al. (2014). However, anti-VEGF drugs have certain limitations. For example, they have a short half-life Lu et al. (2015), and the duration of drug action is short. For instance, widely used drugs such as ranibizumab require monthly intravitreal injections to maintain therapeutic levels of the drug in eye tissues Wong et al. (2007). They can only target some subtypes of VEGF. Aflibercept and conbercept are able to target placental growth factor Lu et al. (2015). The most important limitation is that less than 50% of patients receiving anti-VEGF treatment achieve an improvement in vision Nguyen et al. (2012), Rajendram et al. (2012), Brown et al. (2015), Michael et al. (2008). Studies suggest that strict control of blood glucose can reduce the incidence of blindness caused by DR Ebneter et al. (2016), but it is difficult to maintain normal blood glucose levels in clinical practice, and sometimes even impossible. Therefore, more treatment targets need to be elucidated based on the molecular mechanisms of this disease to prevent and treat it effectively. As ART can inhibit the occurrence, development, and leakage of retinal neovascularization by acting on three targets, VEGFR2, PKCα, and PDGFR, the drug has a long duration of action, lasting up to 6 months Zong et al. (2016). In addition, this study demonstrated that ICAM-1 and MMP-9 can serve as two new targets for ART at the cellular level. Furthermore, ART has good tolerance Zang et al. (2014). The concentration of artemisinin and its derivatives required for anti-neovascularization effects is only one-thousandth of the clinical dose used for malaria treatment Efferth et al. (2003). Under long-term systemic administration conditions of low doses (10mg/L), there are no additional side effects Ebneter et al. (2016), Berger et al. (2005), White et al. (2006), Crespo-Ortiz et al. (2012), Ba et al. (2013), D'alessandro et al. (2007). Therefore, ART may be used for early and late-stage treatments of DR.

## CONCLUSION

In conclusion, this study demonstrated that the increase in ICAM-1 and MMP-9 is glucose concentration-dependent in cell models, and based on previous research, it suggests that DR patients should strictly control their blood glucose level to some extent. Furthermore, ICAM-1 and MMP-9 may serve as new targets for ART in the treatment of DR, providing information for further research on its potential therapeutic value as a new probable type of drug for DR. However, this experiment is limited to the effect of ART on the expression of ICAM-1 and MMP-9 at the cellular level. Further research is needed to investigate the changes in ICAM-1 and MMP-9 expression involved in the mechanism of inhibition of ART of retinal neovascularization and leakage.

## DECLARATIONS

### Data Availability

Raw Datasets is provided within the supplementary information files, and it can be published, for further needs contact corresponding author.

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