

Artesunate Inhibits the Development of PVR by Supressing TGF- β /Smad Signaling Passway

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Research Article

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Abstract

Proliferative vitreoretinopathy (PVR) is the main reason for the failure of retinal detachment surgery. Epithelial-mesenchymal transition (EMT) induced by transforming growth factor (TGF- β 2) plays an important role in the development of PVR. Artesunate has been widely studied in the treatment of ophthalmic diseases because of its antioxidant, anti-inflammatory, anti-apoptosis and anti-proliferation effects. The purpose of this study was to investigate the effect of artesunate on EMT induced by TGF- β 2 in ARPE-19 cells and its effect on PVR process. We found that artesunate can inhibit the proliferation of ARPE-19 cells after EMT transformation, inhibit the contraction of ARPE-19 cells after EMT transformation, and inhibit the autocrine of TGF- β 2 in ARPE-19 cells. We also found that the contents of Smad3 and p-smad3 in clinical samples increased. Artesunate can inhibit the contents of Smad3 and p-smad3 in ARPE-19 cells induced by TGF- β 2. Artesunate can inhibit the occurrence and development of PVR diseases in vivo. To sum up, artesunate can inhibit the occurrence and development of PVR diseases by inhibiting the EMT process of ARPE-19 cells.

Introduction

Proliferative vitreoretinopathy (PVR) refers to the re-retinal detachment caused by the contraction and traction of extensive fibrous proliferative membranes on the retinal surface and behind the vitreous body after retinal detachment reduction and vitrectomy. PVR is a major complication after ocular penetrating injury and vitrectomy, and has become one of the main causes of blindness[1]. At present, surgery is the main treatment for PVR, but PVR recurrence is still very common after operation. Therefore, it is still an important issue to explore the mechanism of PVR and find effective alternative therapies.

The exact mechanism of PVR is not clear at present, but existing studies suggest that proliferative vitreoretinopathy is a pathological process of over-repair, and retinal pigment epithelial cells (RPE) play a key role in the occurrence of the disease[2]. Epithelial-mesenchymal transition (EMT) of RPE cells is considered as the core of PVR development. In this process, RPE cells changed from epithelial cells to fibroblasts, and took part in fibrosis, but deviated from the characteristics of epithelial cells[3]. Therefore, the study of new regulatory targets of EMT in RPE cells can establish effective intervention measures to prevent and treat PVR diseases.

The EMT regulation of RPE cells may be regulated by various molecular mechanisms. In PVR, various growth factors secreted by retinal epiretinal cells are important promoters of EMT, including platelet-derived growth factor, connective tissue growth factor and transforming growth factor β (TGF- β)[4,5]. TGF- β can promote the synthesis of type I collagen in RPE cells and lead to tissue fibrosis. At the same time, it can promote the production of extracellular matrix, tissue repair and composition change, cell proliferation, increase VEGF expression, promote angiogenesis and promote epithelial-mesenchymal transformation of RPE cells[6]. Therefore, the EMT process of RPE cells induced by TGF- β plays an important role in the occurrence, development and outcome of PVR[7]. TGF- β has been proved to activate a variety of downstream pathways Smads, phosphatidylinositol -3- kinase (PI3K), RhoA and MAPK to

induce EMT[8,9].In eye research, EMT induced by TGF- β 2 has been studied as a model system in many cell lines, including RPE cells, lens epithelial cells, corneal epithelial cells, etcBlocking TGF- β activity at the level of Smad signaling pathway can inhibit EMT[3,10].Therefore, Smad signal inhibitor is a potential therapeutic target to prevent PVR.

Artesunate is a kind of artemisinin derivatives, which is a traditional Chinese medicine widely used as anti malaria drugs.Artesunate has good water solubility and high oral bioavailability, so it has good pharmacological effect [11].Recent studies have found that,Artemisinin, a long-term anti malaria drug, has been proved to have antioxidant, anti-inflammatory, anti apoptotic and anti proliferative effects, making it a therapeutic option for eye diseases [11,12].Studies have shown that artesunate can inhibit the proliferation of human ovarian cancer cells and TGF- β [13].However, its preventive effect on RPE cells is not clear.

1. Materials And Methods:

1.1 Collection of clinical specimens

All patients were fully informed of the study and signed consent forms were provided.Patients with cancer, autoimmune diseases and other serious systemic diseases were excluded.This study followed the guidelines of Helsinki ethics committee and was approved by the ethics review committee of ophthalmic hospital affiliated to Nanchang University.Vitreous samples were collected during vitrectomy,Pvr-a (n = 3), pvr-b (n = 3), pvr-c (n = 3), pvr-a (n = 3), pvr-c (n = 3), pvr-a (n = 3), pvr-b (n = 3), pvr-c (n = 3).In the control group, the vitreous bodies of patients with corneal donation were collected (n = 3).The use of human tissue materials is in accordance with the Helsinki Declaration on the use of human body materials for research.

1.2 Cell culture and treatment

ARPE-19 was obtained from Shanghai Yubo Biotechnology Co., Ltdand cultured in DMEM / F12 medium (01-172-1acs, biological industries) containing 10% FBS (04-001-1acs) and 1% PS, and incubated in thermo (USA) at 37 ° C and 5% CO₂ saturation humidity.24 hours later, the cells adhered to the wall completely.The cells were cultured to 80% – 90% fusion degree, starved overnight in serum-free medium, and replaced with DMEM / F12 containing 1% FBS,Different concentrations of artesunate (Solarbio) (0,50,100,150,200 μ Cell proliferation and migration were detected at different time points (0, 24, 48 and 72 h).TGF was used- β The degree of EMT was detected after 48 hours of pretreatment with artesunate.

1.3 Cell cycle was detected by flow cytometry

The cells were digested with trypsin (EDTA) and centrifuged at 350 g for 5 minthe supernatant was discarded.Cells were collected, washed with cold PBS, resuspended with 1 ml cold PBS, centrifuged for 5

min with 350 g, and the supernatant was discarded. Add 1 ml ice cold 75% ethanol, mix, incubate overnight at 4 °C. 500 g, centrifuged for 5 min, and the supernatant was discarded; The cells were then resuspended with 1 ml cold PBS and centrifuged at 500 g

5min, discard the supernatant. The sample was completely resuspended, added with 0.5 mL PI solution, and incubated for 25min at room temperature in the dark for flow cytometry analysis (in Excitation wavelength and emission wavelength of 615 nm detect red fluorescence signal). Cell fragments and aggregated cells were removed by flow cytometry, and DNA content of single living cells was analyzed by Modifit LT5 software.

1.4 Cell contraction assay

A cell contraction detection kit (cell Biolabs, San Diego, CA, USA) was used to evaluate the contractility of ARPE-19ARPE-19 (2×10⁶ cells/ml) was suspended in basic medium, mixed with collagen gel, put in 24-well plate, and placed in incubator at 37 C and 5% CO₂ saturation humidity for 1 h. After gel polymerization, 1ml mixture of cells and collagen was added. After 48 h of cell culture, collagen gel was gently released from the side. At last, Image-J(NIH, Bethesda, MD, USA) to image and quantify the size change of collagen gel (relative to the initial collagen gel size).

1.5 ELISAs

ARPE cells were treated with different concentrations of artesunate (0,75,150,200 μ mol/L) for 48 hours. Collecting cell culture solution, centrifuging to remove impurities. ELISA (□) Pre-prepared reagents were used: diluted human TGF-β2 reference substance, biotin-labeled anti-TGF-β2 antibody working solution, avidin peroxidase complex (ABC) working solution. Add the standard substance and each group of samples into the ELISA plate in a volume of 100 μL/ well, seal the plate with membrane, and incubate at 37°C for 90min. Shake off the liquid in the ELISA plate, do not wash it, add biotin-coupled anti-human TGF-β2 antibody, the workload is 100 μL/ well, and incubate at 37°C for 60min after sealing the membrane. Then, 300 μ L of 1× washing solution was added to each hole, and washed for 3 times, each time for 1min. Add ABC working solution into 100 μL/ well, The plates were sealed with membrane and incubated at 37°C for 90min. Add 1× washing solution (300 μL/ hole) and wash for 5 times, each time for 1min. Add TMB chromogenic solution 90 μL/ well, and incubate at 37°C in the dark for 15min. We added TMB termination solution at 100 μL/ mouth (in the same order as TMB). With enzyme-labeled analyzer, the OD value was measured immediately at 450nm wavelength, and the sample concentration was calculated by using the OD value as a standard curve.

1.6 Western blotting assays

The cells and tissues were washed twice with ice-cold phosphate buffer (PBS, 02-024-1acs, Biological Industries, Israel), added with protease inhibitor (PMSF, AR1178, Boster) and centrifuged at 12000 rpm for 15 minutesCollect supernatant lysate for BCA protein detection (BCA protein quantification kit, e112-01/02, Nanjing nuoweizan biotechnology co., ltd)Equal amount of protein was loaded on 10% SDS-PAGE gel (SDS-PAGE gel preparation kit, AR0138,Boster) and separated.Transfer to PVDF membrane (Millipore, Billerica, MA, USA)The membrane was sealed with 5% skimmed milk (skimmed milk powder, 23210, BD Company, USA) in TBST at room temperature for 1 hour, and mixed with primary antibody alpha-smooth muscle actin (1: 1000, D4K9N, XP, Rabbit mAb, # 19245, CST), vimentin (1: 1000, D21H3, CST) # 5741, CST), phospho-pi3 kinase p85, GAPDH antibody (6c5) loading control (1: 5000, ab8245, abcam) stayed overnight at 4 C .The membrane was washed with TBST and incubated with the second antibody Goat Anti-Rabbit IgG(BA1054, Wuhan boside bioengineering co., ltd.) for 1 hour at room temperature.And then scanned on the exposure machineThen, the comprehensive intensity of each detection band is determined by using image jThese experiments were repeated at least three times.

1.7 Animal experiment

Twelve adult pigmented rabbits were selected, each weighing about 2-2.5kg, After extracted 0.2ml vitreous,12 traumatic PVR model rabbits were prepared by intravitreal injection of 0.1ml platelet rich plasma and 0.1mlPBS[14,15]. They were randomly divided into blank group, control group (0.1mlprp + 0.1mlpbs) and experimental group: (0.1mlPRP + 0.1ml 20ug/ml artesunate). In the control group, 0.1mL platelet-rich plasma and 0.1mL PBS were injected into the vitreous cavity at the same time. In the experimental group, 0.1mL platelet-rich plasma and 0.1ml artesunate (20ug/ml) were injected into the vitreous cavity at the same time. Fundus photography and B-ultrasound machine were used to observe the proliferation of the vitreous and retina at different periods. The expression of Vim, Smad3 and P-Smad3 in vitreous retinal hyperplasia was detected by Western blotting 28 days after surgery, and the retinal tissue structure of each group was observed by frozen section.

1.8 Statistical analysis

All experiments were repeated at least three times with representative results. All experimental data were processed and analyzed using Microsoft Excel and GraphPad Prism 7, and one-way analysis of variance (ANOVA) was used for comparison between groups. Data were expressed as mean ± standard deviation, p < 0.05 was considered statistically significant.

2. Results

2.1 Artesunate inhibits the proliferation of ARPE-19 cells

ARPE-19 cells were treated with different concentrations of artesunate for 48 hours.The DNA content of cells was analyzed by flow cytometry, and the cell cycle distribution was detectedThe cell cycle showed

dose dependenceAfter 48 hours of treatment, the number of cells in G1 phase increased and the number of cells in S phase decreased (Fig. 1)This finding indicated that artesunate inhibited the proliferation of ARPE-19 cells by blocking G1 phase.TGF- β 2 was used to induce the EMT transformation of ARPE-19 cellsafter 48 hours intervention with artesunate, it was found that the number of cells in G1 phase increased and the number of cells in s phase decreased (Fig. 2), which indicated that artesunate also inhibited the proliferation of ARPE-19 cells after EMT by blocking the progress of G1 phase.

2.2 The effect of artesunate on collagen gel contraction mediated by TGF- β 2 in ARPE-19 cells

In this study, we tested the effect of artesunate on collagen gel contraction of ARPE-19 cells induced by TGF- β 2We used a mixture of freshly polymerized collagen and ARPE-19 cells to measure collagen gel contraction (Fig. 3)TGF- β 2 incubated cells for 48 h, and artesunate with different concentrations (0,50,150,200uM) incubated for 48 hCompared with the control group,Using artesunate alone did not reduce the percentage of original cell areaHowever, compared with the control group, TGF- β 2 significantly reduced the proportion of the original area of cell pairs ($P < 0.0001$)However, the proportion of ARPE-19 cells induced by TGF- β 2 in the original region decreased significantly after artesunate treatment ($P < 0.0001$)All in all, these results show that,Artesunate inhibits the contractile function of ARPE-19 cells induced by TGF- β 2.

2.3 Artesunate inhibits the secretion of TGF- β 2 in ARPE-19 cells

In this study, we used the TGF- β 2 detection kit to detect the changes of TGF- β 2 content in ARPE-19 cells treated with artesunate at different concentrationsThe results showed that artesunate inhibited the secretion of TGF- β 2 by ARPE-19 cells in a concentration-dependent manner compared with the control group, and the effect was statistically significant ($p < 0.0001$) (Fig. 4)

2.4 Smad signaling pathway is activated in proliferative vitreoretinopathy

We detected WB in vitreous samples of clinical PVR patients, and compared them with the control groupThe results showed that smad3 and p-smad3 in patients with grade C and B lesions increased with statistical significance ($p < 0.0001$) (Fig. 5)Smad3 and p-smad3 in patients with grade A increased compared with the control group, but the results were not statistically significantTherefore, we guess the progress of EMT and the expression of Smad3 pathway.

2.5 The effect of artesunate on smad3 signaling pathway in ARPE-19 cells induced by TGF

The results showed that artesunate decreased the expression of smad3 and p-smad3 in ARPE-19 cells induced by TGF in a dose-dependent manner. The ARPE-19 cells were pretreated with TGF- β 2(20ng/ml) for 48 hours compared with the control group, TGF- β 2 significantly increased the expression of smad3 and p-smad3. The results were statistically significant ($p < 0.01$ or 0.0001) after artesunate treatment, the contents of smad3 and p-smad3 decreased. Compared with TGF-treated group, the results were statistically significant ($p < 0.0001$) (Fig. 6).

2.6 Effect of artesunate on PVR process in rabbits

By establishing animal PVR model, we found that after 4 weeks of injection of autogenous plasma and PBS, B-ultrasound showed vitreous opacity accompanied by posterior detachment; fundus photography showed the formation of fixed retinal fold, and the myeloid line was distorted and elevated; tissue section showed the retinal fundiform detachment; cytoskeletal protein VIM, The expression levels of Smad3 and P-Smad3 increased compared with the blank control group, and the results were statistically significant ($P < 0.01$ or 0.0001). After the addition of artesunate intervention during the model, the degree of vitreous opacity was reduced compared with the control group, and the formation of fibrotic membrane in the vitreous was not observed. Fundus photography showed that the retina was only focal swelling, and the tissue section showed that the arrangement of each layer of the retina was slightly distorted, and the formation of preretinal membrane was not observed. The expression levels of Smad3 and p-Smad3 were decreased compared with the control group, and the results were statistically significant ($P < 0.05$, 0.001 or 0.0001) (Fig. 7). Overall, the PVR process was significantly inhibited in eyes injected with artesunate compared with eyes injected with PBS.

3. Discussion

The purpose of this study was to explore the inhibitory effect of artesunate on the progression of PVR disease and its mechanism. The experiment confirmed that artesunate inhibited the proliferation, contraction and secretion of TGF- β 2 in ARPE-19 cells, and inhibited the progression of emt in RPE cells through smad3 signaling pathway. We verified that artesunate can inhibit PVR disease in vivo and in vitro. It is further considered that artesunate can be used as a potential target for treating pvr.

The EMT transformation of RPE cells becomes a crucial step in the development of PVR. During this process, RPE cells transform into mesenchymal cell phenotype, recombine the cytoskeleton, lose cell adhesion and polarity, increase the ability to migrate, invade, and contract [16,30], and form the preretinal membrane with other cells and extracellular matrix. Traction retinal detachment. Therefore, inhibiting the proliferation and migration of RPE cells and EMT transformation is an important way to treat PVR disease. In our in vitro model, RPE cells underwent EMT transformation via TGF- β 2, during which RPE

cells obtained the interstitial marker protein Vimentin and gained the ability to proliferate and contract. Our model simulated the early process of PVR. After the addition of artesunate intervention, the proliferation and contraction of the in vitro model were inhibited, the expression of interstitial cell marker protein was reduced, and the ability of cells to secrete TGF- β 2 was inhibited. Therefore, we verified the inhibitory effect of artesunate on EMT in PVR in vitro.

TGF- β 2 is abundant in vitreous humor of pvr patients, and its concentration is significantly related to the severity of PVR[17,18], in addition, it is the main promoter of PVR diseasesPrevious studies have shown that TGF- β 2 intervention can induce emt transformation of RPE cells, and transformed RPE cells play an important role in excessive healing of pvr diseasesIn[19,20].In tumor cells, the combination of TGF- β and its receptor is out of control,Activation of several downstream pathways, including Smad, Ras and MAP kinases, PI3k/ Akt and so on, leads to cell dysfunction[21,22,23].Smads family proteins play a key role, and different Smad proteins can mediate the signal transduction of different TGF- β family members[24,25].Smad3 is a key signal transduction intermediate downstream of TGF- β 2 activin receptor, and platelet-derived growth factor receptor can be induced by TGF- β signal.Furthermore, it can promote the EMT process of RPE cells, and tissue fibrosis and tissue contraction also depend on the activation of TGF- β /smad pathway[26,27,28].Studies have shown that after retinal detachment and traumatic rupture, pigment epithelial cells leave their normal positions, enter vitreous cavity and subretinal space, and autocrine TGF- β activates smad signaling pathway, thus promoting emt transformation of RPE cells[29].Through experiments,We found that in clinical vitreous samples, the expression of smad in class B and class C PVR samples was up-regulated compared with that in control group and class A PVR samples, and the content of activated smad expression also increased, thus further verifying the role of smad signaling pathway in the occurrence and development of PVRCOMPARED with the control group, artesunate group can reduce the content of TGF- β 2 secreted by ARPE-19 cells,Thereby reducing the activation degree of TGF- β /smad pathwayIn vitro cells induced by TGF- β , smad signaling pathway and phosphorylated smad increased, while smad3 and phosphorylated smad3 decreased after art treatmentIn animal experiments, we found that after injecting artesunate into vitreous cavity while modeling, the degree of vitreous opacity was reduced compared with the control group, and no retinal membrane was formed.The expression levels of cytoskeletal proteins VIM,smad3 and p-smad3 were lower than those of the control group.

In animal experiments, we found that after injection of PRP and PBS, severe vitreous opacity could be seen, focal traction and bleeding could be seen in fundus photography, and the staining of tissue sections showed retinal detachment. After the injection of artesunate into the vitreous cavity during the modeling, the degree of vitreous opacity was reduced compared with the control group, no preretinal membrane formation was observed, no retinal detachment occurred, and the expression levels of cytoskeletal proteins VIM, Smad3 and P-Smad3 were reduced compared with the control group. We hypothesized that artesunate affected the amount of smad3 phosphorylation by inhibiting total Smad3 protein. Therefore, we conclude that artesunate inhibits emT development in RPE cells by inhibiting smad3 pathway expression

To sum up, this study confirmed the inhibitory effect of artesunate on the occurrence and development of EMT and PVR in ARPE-19 cells in vitro and in vivo, and found a new therapeutic target for treating PVR diseases.

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Figures

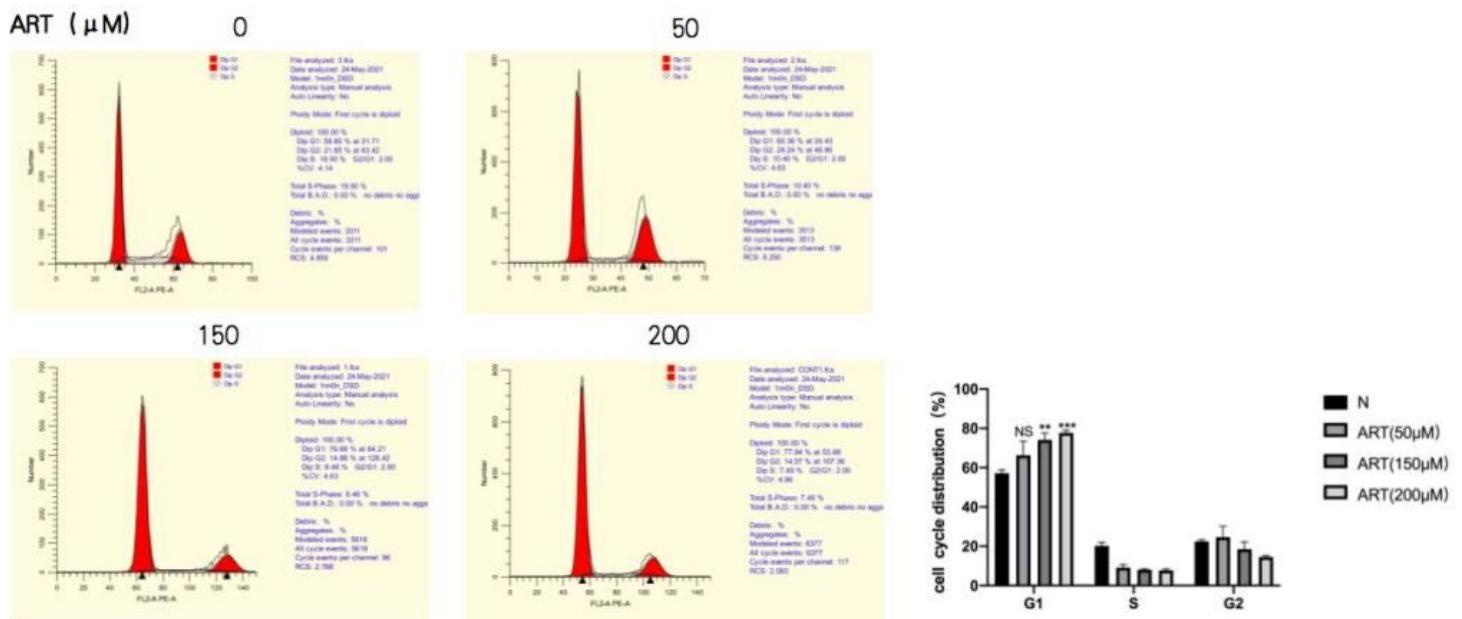


Fig 1. **Artesunate induces cell cycle arrest.** Cells were treatment with or without 50 , 150 and 200 μM artesunate for 48 h. DNA was stained with PI for flow cytometric analysis. The number of cells in G1 phase was significantly increased in the artesunate-treated group compared with that in the untreated group. NS($P>0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The data are presented as the mean \pm S.D. ($n = 3/\text{group}$).

Figure 1

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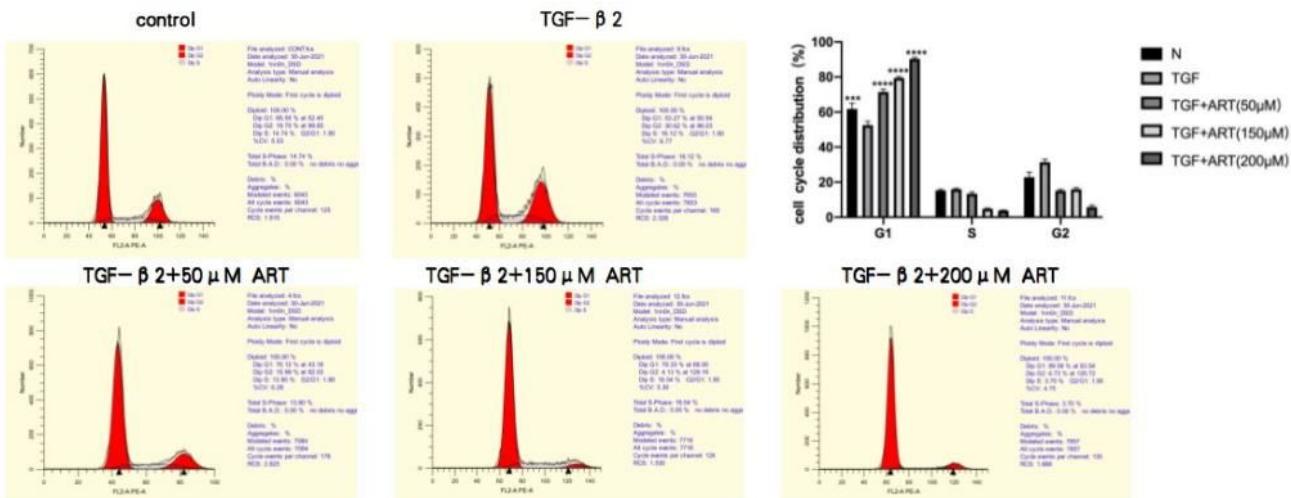


Fig 2. Artesunate induces TGF- β 2 pretreated cell cycle arrest. Cells were pretreatment with TGF- β 2 and then treatment with or without 50 , 150 and 200 μ M artesunate for 48 h. DNA was stained with PI for flow cytometric analysis. The number of cells in G1 phase was significantly increased in the artesunate-treated group compared with that in the only TGF- β 2 treatment group. ***P < 0.001, ****P < 0.0001. The data are presented as the mean \pm S.D. (n = 3/group).

Figure 2

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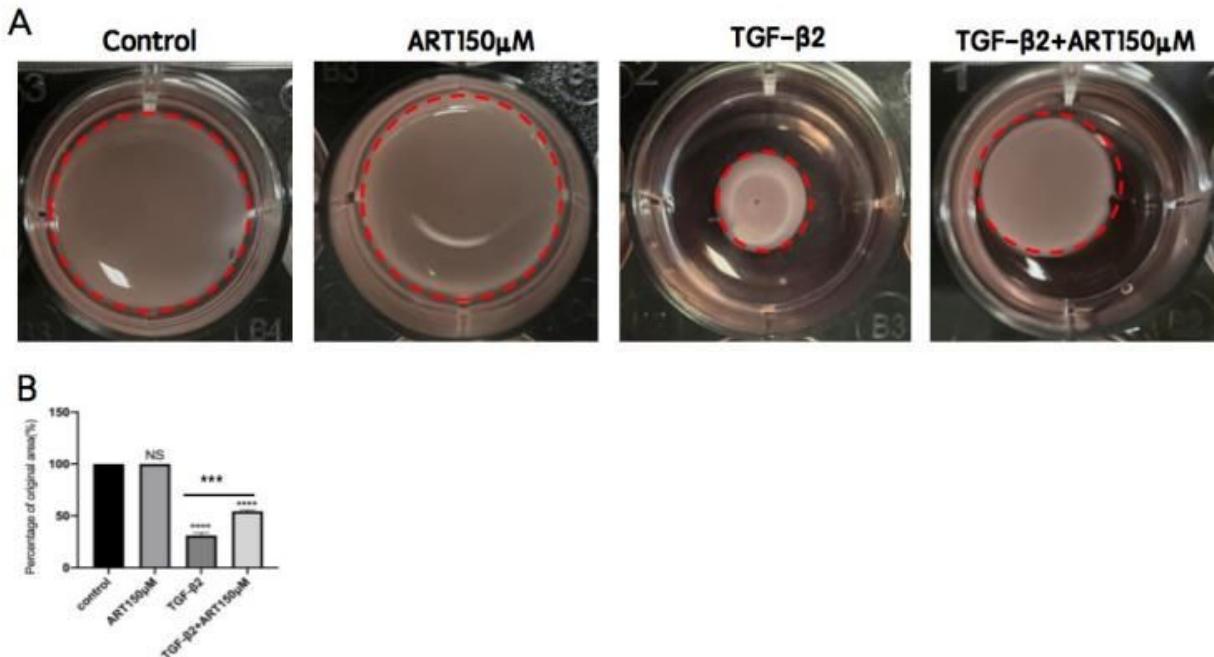


Fig 3. Effects of artesunate on TGF- β 2-induced collagen gel contraction in ARPE-19 cells. (A) Cells were pretreated with or without TGF- β 2 and then treated with artesunate in the presence or absence of 150 μ M resveratrol. (B) Contraction or expansion was determined as a percentage of the original area. The results are the mean values SEM of three independent experiments. ***P<0.001; ****P<0.0001; NS, not significant. The data are presented as the mean \pm S.D. (n = 3/group).

Figure 3

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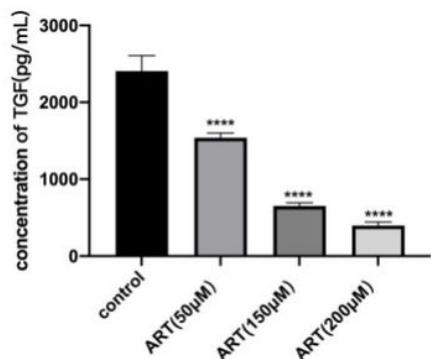


Fig 4. Artesunate inhibited TGF- β 2 secretion in ARPE-19 cells. TGF- β 2 secretion in ARPE-19 cells treated with Artesunate at different concentrations for 48 hours was detected with ELISAs. Values are presented as the mean \pm standard deviation from three independent experiments. **** P < 0.0001. vs the 0 μ mol/L luteolin group.

Figure 4

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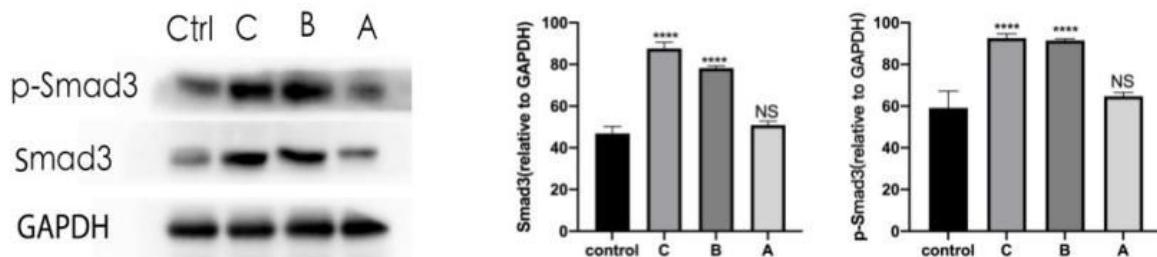
A

Fig 5. smad3 signaling pathway is activated in proliferative vitreoretinopathy. Vitreous tissue were collected from clinical patients . Western blot analysis levels of smad3, p-smad3 and the housekeeping protein GAPDH in the lysates of clinical tissue. NS($P>0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The data are presented as the mean \pm S.D. ($n = 3/group$).

Figure 5

Please See image above for figure legend.

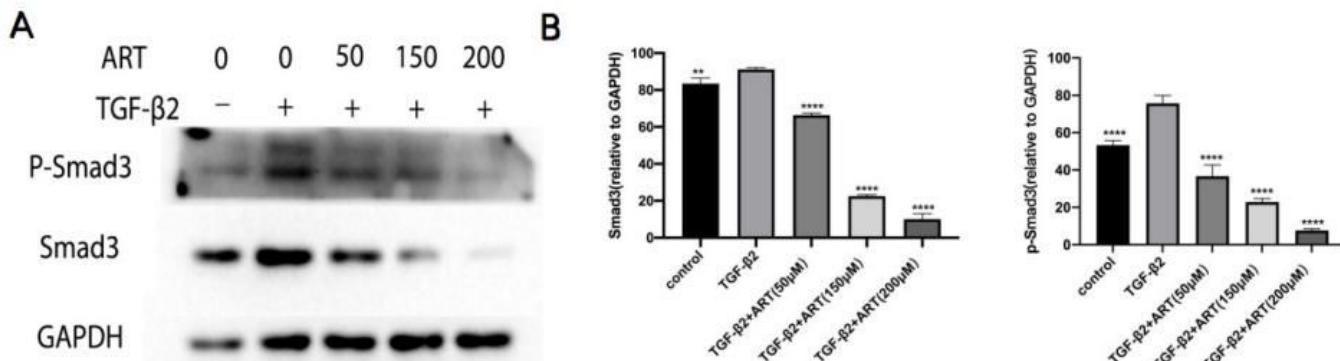


Fig 6. Artesunate reduce smad3 signaling pathway in transformed ARPE-19 cells. After 48 h of pretreatment with TGF, ARPE-19 cells stimulated with or without artesunate for 48 h. Western blot analysis levels of smad3, p-smad3 and the housekeeping protein GAPDH in the lysates of ARPE-19 cells after treatment for 48 h. NS($P>0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The data are presented as the mean \pm S.D. ($n = 3/group$).

Figure 6

Please See image above for figure legend.

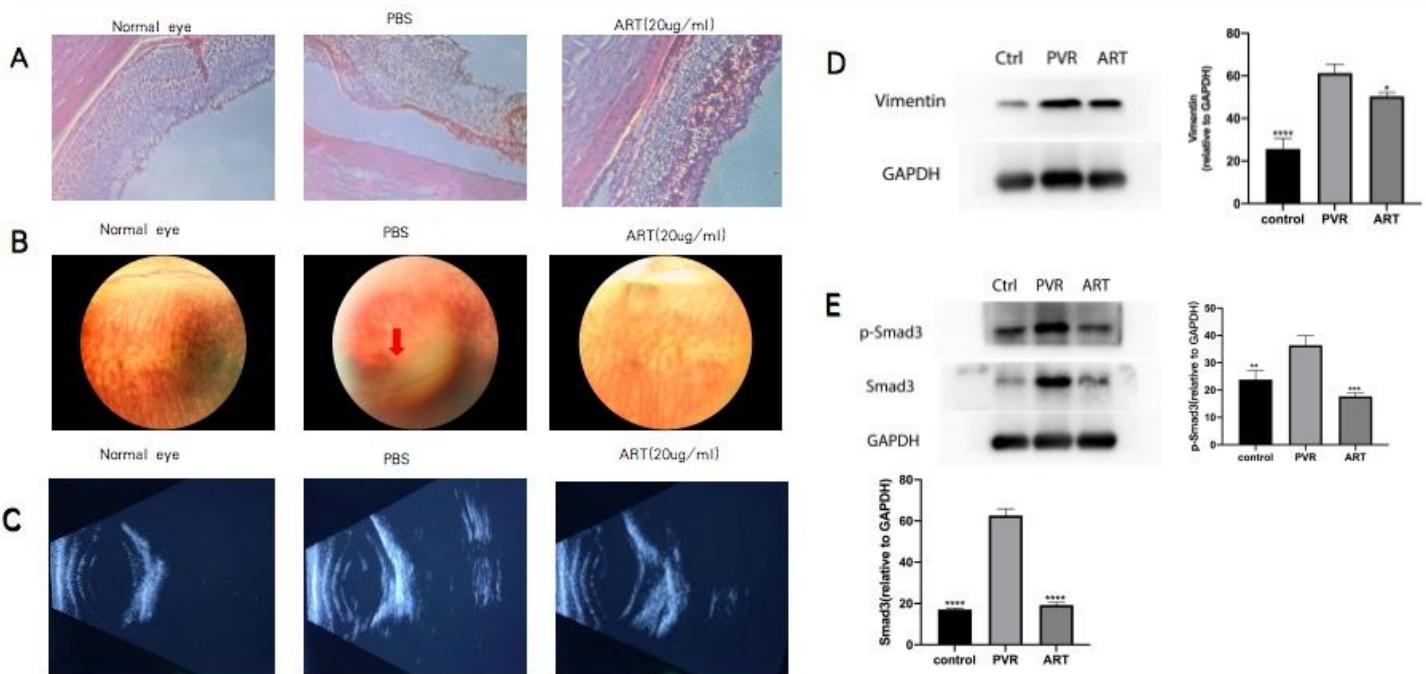


Fig 7. Artesunate inhibits progression of experimental PVR in rabbits. (A)H&E staining of rabbit retinal. (B) Ocular fundus photographs of normal eye and eyes with PVR after treatment with PBS and ART at 20ug/ml. (C)Ultrasonic photograph of normal eye and eyes with PVR after treatment with PBS and ART at 20ug/ml.(D)Western blot analysis levels of Vimentin and the housekeeping protein GAPDH in the lysates of rabbit vitreous (E) Western blot analysis levels of smad3, p-smad3 and the housekeeping protein GAPDH in the lysates of rabbit vitreous.**P < 0.01, ***P < 0.001, ****P < 0.0001 The data are presented as the mean \pm S.D. (n = 3/group).

Figure 7

Please See image above for figure legend.