Inhibitory Effects of Plumbagin on Retinal Pigment Epithelial Cell Epithelial-Mesenchymal Transition *In Vitro* and *In Vivo*

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Background: This study aimed to explore the effects of plumbagin (PLB) on epithelial-to-mesenchymal transition in retinal pigment epithelial (RPE) cells and in proliferative vitreoretinopathy (PVR) rabbit models.

Material/Methods: Rabbit RPE cells were exposed to various concentrations (0, 5, 15, and 25 μM) of PLB. Motility, migration, and invasion of PLB-treated cells were determined *in vitro* using Transwell chamber assays and scratch wound assays. The contractile ability was evaluated by cell contraction assay. Expression of matrix metalloproteinases (MMPs) and epithelial-mesenchymal transition (EMT) markers were assessed by western blotting. Furthermore, PLB was injected in rabbit eyes along with RPE cells after gas compression of the vitreous. The presence of PVR was determined by indirect ophthalmoscopy on days 1, 7, 14, and 21 after injection. Also, optical coherence tomography (OCT), ultrasound images, electroretinograms (ERG), and histopathology were used to assess efficacy and toxicity.

Results: PLB significantly inhibited the migration and invasion of RPE cells. The agent also markedly reduced cell contractive ability. Furthermore, PLB treatment resulted in the decreased expression of MMP-1, MMP2, α-SMA, and the protection of ZO-1. In addition, the PLB-treated eyes showed lower PVR grades than the untreated eyes in rabbit models. PLB exhibited a wide safety margin, indicating no evidence of causing retinal toxicity.

Conclusions: PLB effectively inhibited the EMT of rabbit RPE cells *in vitro* and in the experimental PVR models. The results open new avenues for the use of PLB in prevention and treatment of PVR.

MeSH Keywords: Epithelial-Mesenchymal Transition • Plumbaginaceae • Retinal Pigment Epithelium

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Background

Proliferative vitreoretinopathy (PVR) is a fibrotic disease characterized by the formation and constriction of epiretinal membranes (ERMs) composed of various kinds of cells and their extracellular matrix (ECM), finally leading to tractional retinal detachments and visual impairment [1]. The most important cell type in PVR pathogenesis is retinal pigment epithelial (RPE) cells, which are believed to undergo epithelial-mesenchymal transition (EMT) to a fibroblastic phenotype, then migrate, proliferate, and at last form epi-retinal membranes [2]. Nowadays, more efforts have been made to look for prophylactic or therapeutic agents to inhibit the EMT of RPE in order to stop PVR at an early stage [3].

Plumbagin (PLB; 5-hydroxy-2-methyl-1,4-naphthoquinone) is a kind of natural naphthoquinone component isolated from the root of *Plumbago zeylanica L*, which has an extensive range of effects including anti-inflammation, anti-microbe, anti-cancer, and neuroprotection in multiple cell lines and animal models [4]. Recently, the anti-EMT effect of PLB has been a high interest research topic. It has been proven that this effect of PLB may be the main reason causing cell viability suppression [5,6].

In the present study, we aimed to investigate whether PLB could effectively inhibit the EMT of rabbit RPE cells *in vitro* and in an experimental model of PVR.

Material and Methods

In vitro study

Rabbit RPE cell culture

The tissue-cultured RPE cells in our study were taken from New Zealand albino rabbits. We enucleated the rabbit eyes, removed the anterior segment and vitreous, and trypsinized the RPE cells with 0.25% trypsin with 0.02% EDTA (Sigma-Aldrich; St. Louis, MO, USA) at 37°C for 30 minutes. Afterwards, the cells were cultured in a DMEM/F12 medium supplemented with 10% FBS and regular antibiotics (1% penicillin and streptomycin) (Gibco®; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere with 5% CO_2 with medium changes every three days, and subcultured if confluence was reached. PLB; Sigma-Aldrich; St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stocked at 100 mM, which was diluted into working concentrations with culture medium. The control group received the vehicle (0.05% DMSO) only.

Transwell chamber assays

Transwell chamber assay (Costar, Cambridge, MA, USA) was used to measure the motility of rabbit RPE cells. In brief, the wells of 24-well culture plates were separated into upper and the lower chambers with 8-μm pore size inserts. There was 600 μL DMEM/F12 containing 10% FBS in the lower chamber, while 5×10⁴ RPE cells with various concentration of PLB (0, 5, 15, or 25 μM) were cultured for 24 hours in the upper chamber. The cells which migrated through the pores were displayed by crystal violet and calculated using the microscope (Axiovert 200, Zeiss; Oberkochen, Germany).

Scratch wound assay

RPE cell monolayers were scratched by a 200 μL pipette tip and washed thrice. Then the cells were cultured in serum-free medium with different concentration of PLB (0, 5, 15, or 25 μM) for 24 hours. The migratory ability was assessed by the relative migration rate.

Cell contraction assay

After treatment as described previously, 1×10⁶ RPE cells from each group were suspended in a solution containing 1.5 mg/mL neutralized collagen I (INAMED; Fremont, CA, USA), and transferred into a 24-well plate which had been pre-incubated with 5 mg/mL bovine serum albumin overnight. After incubation at 37°C for 90 minutes, the collagen gel polymerized and the area was calculated to evaluate the contractile ability of RPE cells.

Western blot

The expressions of EMT-related proteins were assessed by western blotting assays. Rabbit RPE cells were incubated with various concentrations of PLB (0, 5, 1, and 25 μM) for 24 hours, or exposed to 15 μM PLB for 12, 24, and 48 hours. The treated cells were lysed with RIPA buffer (Solario; Beijing, China) and protein contents were determined by Pierce™ bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc.; MA, USA). Each protein sample at 50 ng and rainbow molecular weight markers (11–245 kDa; Solario; Beijing, China) were electrophoresed on 8–10% sodium dodecyl sulfate polyacrylamide gel electrophoresis minigel (SDS-PAGE) after thermal denaturation at 95°C for five minutes. Proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore; Billerica, MA, USA) at 100 V at 4°C for two hours, and then stopped with 5% non-fat milk. Subsequently, membranes were probed with indicated primary antibody against MMP1 (ab38929), MMP2 [EPR1184](ab92536), alpha-SMA[EPR5368] (ab124964), and ZO-1(ab96587), (Abcam, Cambridge, UK) at 4°C overnight, then marked by goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (IgG; catalog no. 7074S; Cell Signaling Technology Inc.; MA, USA) for one hour at room temperature according to the manufacturer's protocol. The protein bands were visualized by the Bio-Rad Chemi Doc™ XRS system (Bio-Rad Laboratories Inc.; CA, USA) and

the density was measured by Image ImageJ analytical software (National Institutes of Health, Bethesda, MD, USA). The protein concentrations were calculated based on the value of the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ab128915; Abcam; Cambridge, UK).

In vivo study

Animals

All animal management in the present study abided by the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and the NIH Principles of Laboratory Animal Care (revised 1985). Thirty-four New Zealand albino rabbits weighing between 2.5 kg and 3 kg were used. The animals were sedated by intravenous injection of pentobarbital (2 mg/kg). The eyes were topically anesthetized with proparacaine hydrochloride eye drops and the pupils were dilated with topical 1% tropicamide and 2.5% phenylephrine. Rabbits were sacrificed by intravenous injection of an overdose of pentobarbital.

Toxicity study

We use electroretinograms (ERG) as a criterion to assess the retinal toxicity of PLB. Twelve rabbits were divided into three groups: four eyes accepted intravitreous injections through pars plana with 25 μM PLB, four eyes with 50 μM PLB while four eyes with DMSO (0.5‰) only as the control group. Each right eye was treated with the left eye as the control one. Bilateral ERG (Neuropack-II Plus; Nihon, Kohden, Japan) were operated on the day before the injection, on days 3, 7, 14, and 21 after the injection, and maximal b-wave amplitude was used for evaluation. Three weeks later, the eyeballs were taken out instantly after the rabbits were sacrificed, and fixed with formaldehyde, dehydrated in ethanol series, sliced into epoxy resin sheets, stained with hematoxylin and eosin, then examined by light microscopy.

Efficacy study

Rabbit PVR model induction and observation

Rabbits were acclimated for one week and then 0.1 mL perfluoropropane gas (C3F8; Alcon; Fort Worth, TX, USA) was injected into the vitreous cavity of each right eye. One week later, the experimental eyes were co-injected with 2×10^5 RPE cells suspended in 0.1 mL platelet-rich plasma (PRP) and 0.1 mL balanced salt solution (BSS) of either 25 uM PLB in DMSO (11 eyes experimental), or DMSO alone (11 eyes control) under aseptic conditions. The retinal status was monitored using indirect ophthalmoscopes (Keeler Instruments, Broomall, PA, USA) on 3, 7, 14, and 21 days after injection. Extent of PVR was evaluated according to Fastenberg classification as follows:

stage 0, represented normal; 1, ERM formation; 2, vitreoretinal traction without retinal detachment; 3, localized retinal detachment of one to two quadrants; 4, extensive retinal detachment of two to four quadrants; 5, complete retinal detachment. Retinal function was assessed by ERGs of every week after injection. Intraocular pressures were monitored daily for three days after gas injection and for one day after the co-injections using a tonometer (TONO-PEN XL; Reichert; Depew, USA). Fundus images were obtained using the Spectralis optical coherence tomography (OCT) (Heidelberg, Germany), while ultrasound images were obtained using AVISO ultrasonic diagnostic instrument (Quantel Medical Ltd., France). Signs of toxicity, including intraocular inflammation and hemorrhages, were assessed during each fundus examination and by histological analysis of retinas after sacrifice of animals. Animals were sacrificed at day 21, and eyes were enucleated for later analysis. All examinations were accomplished by the same observers.

Statistical analysis

The *in vitro* experiments were performed at least three times and data were expressed as mean ± standard deviation (SD). SPSS version 22.0 (SPSS, Inc.; Chicago, USA) was used to perform statistical analyses. Differences between control and treated samples were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison procedure. A Pearson test was performed for correlation. For *in vivo* studies, the Wilcoxon signed-rank test was chosen to compare the degree of PVR between the two groups. A value of $p < 0.05$ was considered as statistically significant in each experiment.

Results

In vitro studies

PLB inhibited migration and invasion of rabbit RPE cells

To investigate whether PLB could inhibit migratory and invasive properties of rabbit cells, scratch wound assay and Transwell chamber assay were used. When cells were incubated with 0, 5, 15, and 25 μM PLB, the total cell numbers in the scratch area were (98.7±12.2), (72.3±5.1), (37.7±4.0) and (7.3±3.5) respectively (Figure 1A). As shown in Figure 1B, the relative cell migration rate were (0.74±0.09), (0.39±0.08) and (0.08±0.04) respectively, compared to the control group (p=0.005, 0.000, 0.000 respectively). Furthermore, Transwell chamber assay showed that cell numbers in the lower chamber were (184.0±11.1), (127.7±8.6), (92.7±13.8) and (51.3±9.1) respectively (Figure 2A). And the relative cell invasion rate were (0.69±0.05), (0.50±0.07) and (0.28±0.05) respectively, compared to the control group (p=0.001, 0.000, 0.000; Figure 2B). The results demonstrated that PLB could significantly inhibit
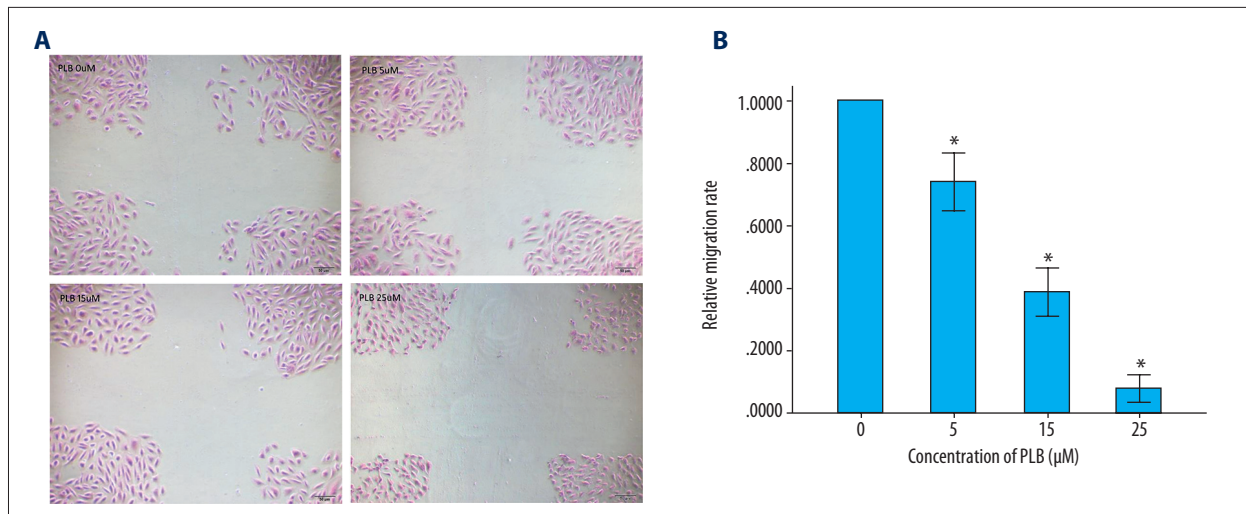
Figure 1. Plumbagin (PLB) inhibited migration of rabbit retinal pigment epithelial (RPE) cells. (**A**) Rabbit RPE cells were treated with indicated concentrations (0, 5, 15, and 25 μM) of PLB. (**B**) Bar graphs show the relative migration rate of the cells treated with PLB at 0, 5, 15, and 25 μM for 24 hours; * $p<0.05$ by one-way ANOVA, n=3.
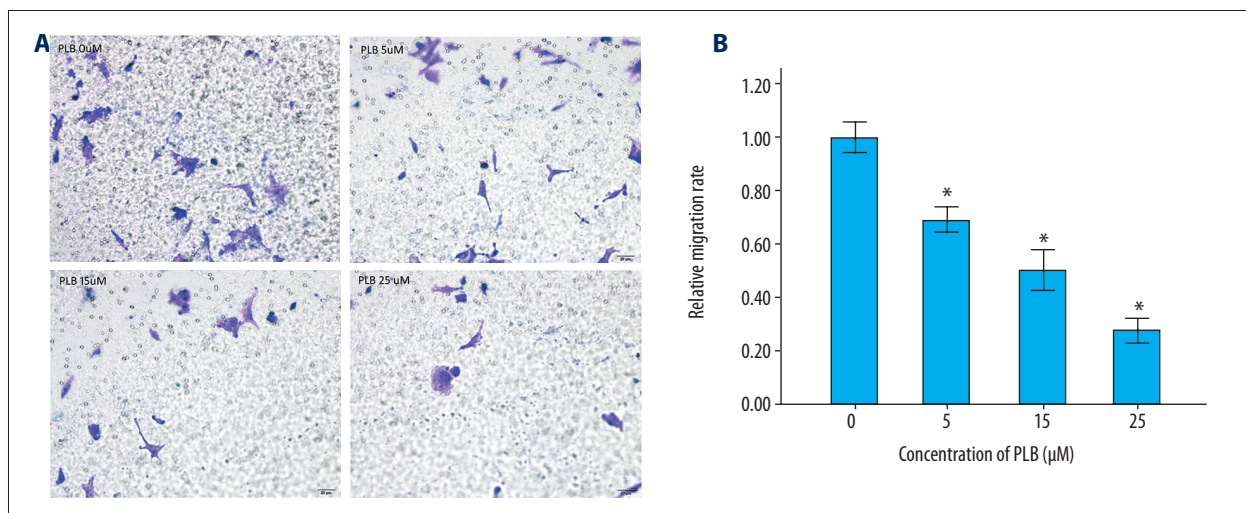

Figure 2. Plumbagin (PLB) inhibited invasion of rabbit retinal pigment epithelial (RPE) cells. (**A**) Rabbit RPE cells stained with crystal violet. (**B**) Bar graphs show the relative invasion rate of the cells treated with PLB at 0, 5, 15, and 25 μM for 24 hours; * $p<0.05$ by one-way ANOVA, n=3.

the migration and invasion of rabbit RPE cells dose-dependently (Pearson test; $p=0.000$).

PLB suppressed contractile ability of RPE cells

The formation and contraction of the epiretinal membrane is the hallmark of PVR and plays an important role in tractional retinal detachment during PVR. We have developed an assay to examine the contractile ability *in vitro* using rabbit RPE cells. As shown in Figure 3, the matrices mixed with RPE cells treated under 0, 5, 15, 25 μM PLB shrank to 26.0%, 40.1%, 56.9% and 77.1% respectively compared to initial area respectively. The collagen matrix contraction was reduced significantly when

cells were cultured with 15 and 25 μM PLB ($p=0.003$, 0.000 respectively). Moreover, PLB inhibited the contractile ability of RPE cells in a dose-dependent manner (Pearson test; $p=0.000$).

PLB modulated expression of EMT-related proteins and MMPs in RPE cells

In order to further explore the anti-motive mechanism of PLB on RPE cells, we evaluated the expression of proteins associated with this phenomenon. It was well known that MMPs induced epithelial to mesenchymal transition and thus increased the invasive potential of cells. To determine their involvement in RPE cells, the expression levels of protein MMP-1, MMP-2 were
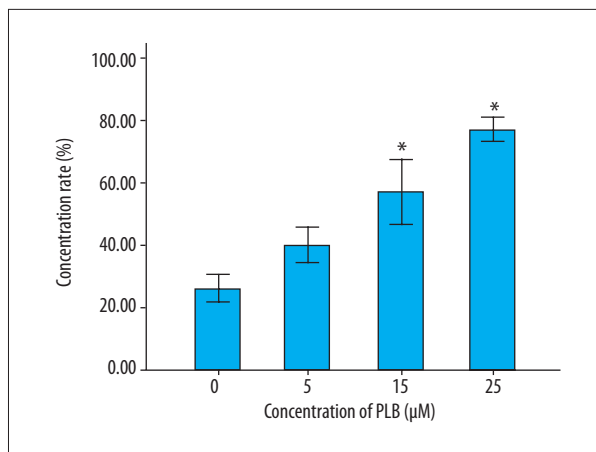
Figure 3. Plumbagin suppressed contractile ability of retinal pigment epithelial cells; * $p < 0.05$ by one-way ANOVA, n=3.

assessed by western blotting analysis (Figure 4A). When ARPE cells were incubated with PLB at 5, 15, and 25 μM for 24 hours, the level of MMP-1 decreased to 69.4%, 39.7% and 26.7% respectively compared to control group (each $p=0.000$). Similarly, the content of MMP-2 reduced to 83.5%, 65.2% and 49.9% respectively ($p=0.013$, 0.000, 0.000; Figure 4B). Furthermore, the differences between adjacent groups also had statistical significance ($p<0.05$) and the changes was dependent on the concentration of PLB (Pearson test; $p=0.000$). In parallel experiments, we found that PLB regulated these MMP proteins in a time-dependent manner as well (Pearson test; $p=0.000$). Briefly, RPE cells were exposed to 15 μM PLB for 12, 24, or 48 hours, the expression of MMP-1 decreased to 87.3%, 49.7%, and 48.8% respectively ($p=0.033$, 0.000, 0.000) and the level of MMP-2 was reduced to 93.8%, 76.3% and 64.4% respectively compared to control cells, respectively ($P=0.450$, 0.001, 0.000; Figure 4C). Together with the data from our previous study, these results demonstrated a downregulating role in the MMPs expression of PLB in ARPE cells.

As is known to all, cells that have undergone EMT usually have reduced epithelial features such as zonula occludens-1 (ZO-1), whereas increase mesenchymal features such as α-smooth muscle actin (α-SMA). Herein, we observed the expression of α-SMA and ZO-1 as EMT symbolic factors using western blotting (Figure 4A). After treatment with 5, 15, and 25 μM PLB for 24 hours, the content of α-SMA in RPE cells decreased significantly from 0.98 at base level to 0.88, 0.75 and 0.63 respectively, dose-dependently ($p=0.104$, 0.002, 0.000; Pearson test, $p=0.000$). On contrast, the level of ZO-1 increased slightly from 0.39 at the basal line to 0.42, 0.44, and 0.40 respectively ($p=0.067$, 0.001, 0.918; Pearson test, $p=0.672$; Figure 4D). Furthermore, when RPE cells were exposed to 15 μM PLB for 12, 24, and 48 hours, there was a 11.5%, 24.4%, and 27.4% respective reduction in the level of α-SMA ($p=0.009$, 0.000, 0.000;

Pearson test, $p=0.000$), while 8.2%, 11.4%, and 4.1% respective augment in the level of ZO-1 ($p=0.027$, 0.004, 0.324; Pearson test, $p=0.512$), in comparison to the control group(Figure 4E).

In vivo studies

Toxicity study

Bilateral ERGs were obtained to evaluate the toxicity of PLB and the ratios of scotopic b-wave amplitudes from right eyes to left eyes were used to lessen variability. Figure 5A demonstrates the mean b-wave ratio was 1.03 at base line, changed to 1.05 on day 3, then mildly decreased on day 7, 14, and at last to 1.00 on day 21 in the 25 uM group. At the same time, in the 50 uM group, the mean b-wave ratio declined from 1.07 to 1.01 on day 3, transiently increased to 1.03 on day 7, thereafter declined to 0.94 on day 14 and at last 0.95 on day 21. These numbers were not significantly different compared to control group (DMSO group), confirming no electrophysiologically detectable injury in the PLB group.

Furthermore, indirect ophthalmoscopy indicated no signs of retinal damage on either day in any group. Histologic observations using light microscopy in Figures 5B showed well preserved nuclear layers and photoreceptor segments 21 days after injection. Based on the above-mentioned events, a concentration of 25 uM PLB was determined for subsequent experiments.

Efficacy study

Twenty-two rabbits were involved in this efficacy study. One rabbit died of overdose anesthesia and another rabbit suffered severe vitreous hemorrhage which were excluded during the study period. No signs of intraocular infection such as iris synechiae or hypopyon was observed.

The PVR grades of treated group were markedly lower than those of control group on days 3, 7, 14, and 21 after injection of RPE cells ($p=0.043$, 0.009, 0.023, 0.035 respectively; Figure 6A). Ultrasound and OCT examinations showed that drug boluses and cell clusters could be visualized in rabbit vitreous cavity approximately in the first week then dissolved and dispersed gradually. The incidence of tractional retinal detachments in PLB group versus the control group was: 20% versus 60% on day 14, and 30% versus 70% on day 21. The PVR was prominently less severe in the PLB treated eyes during the whole experimental period (Figure 6B).

Discussion

Epithelial-mesenchymal transition (EMT) is a cellular metamorphosis procedure in which epithelial cells abandon their
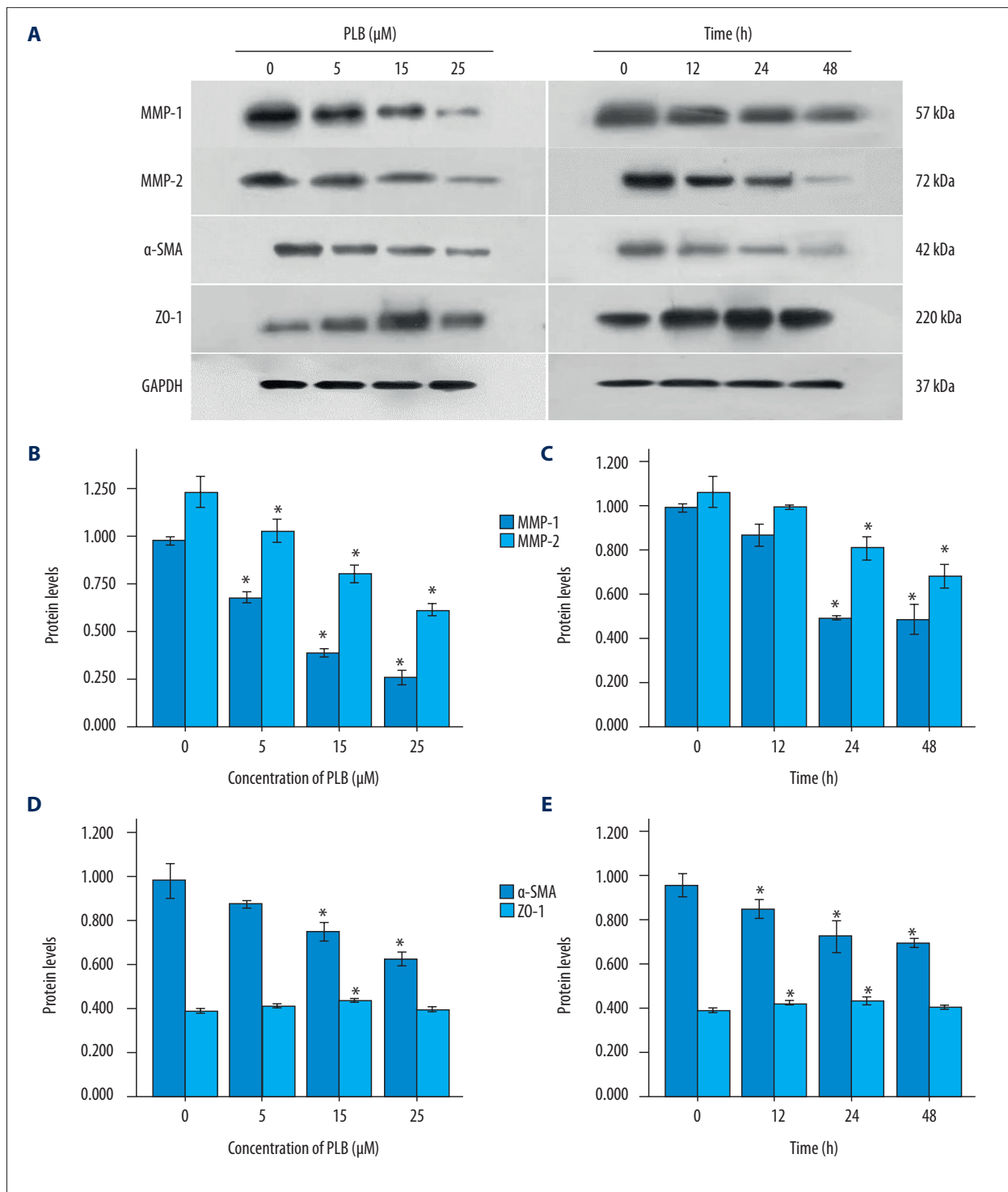
Figure 4. Effect of plumbagin (PLB) on the expression of EMT-related proteins in ARPE cells. (* $p<0.05$ by one-way ANOVA, n=3). (**A**) The expression levels of MMP-1, MMP-2, α-SMA and ZO-1 determined by western blotting assay. GAPDH was used as the internal control. (**B**) Bar graphs show the expression levels of MMP-1 and MMP-2 when ARPE cells were treated with PLB at 0, 5, 15, and 25 μM for 24 hours. (**C**) Bar graphs show the expression levels of MMP-1 and MMP-2 when ARPE cells were treated with PLB at 15 μM for 0, 12, 24 and 48 hours. (**D**) Bar graphs show the expression levels of α-SMA and ZO-1 when ARPE cells were treated with PLB at 0, 5, 15, and 25 μM for 24 hours. (**E**) Bar graphs show the expression levels of α-SMA and ZO-1 when ARPE cells were treated with PLB at 15 μM for 0, 12, 24, and 48 hours.

Figure 5. Electroretinography and histology of rabbits treated with Plumbagin (PLB). (**A**) The mean ratio of b-wave amplitudes of dark adaptation electroretinograms of rabbit eyes treated with 25 µM PLB, 50 µM PLB, or DMSO only, on the day before the infection, day 3, 7, 14 and 21; n=4 at each dose. (**B**) Light microscopy of the rabbit retina 21 days after intravitreo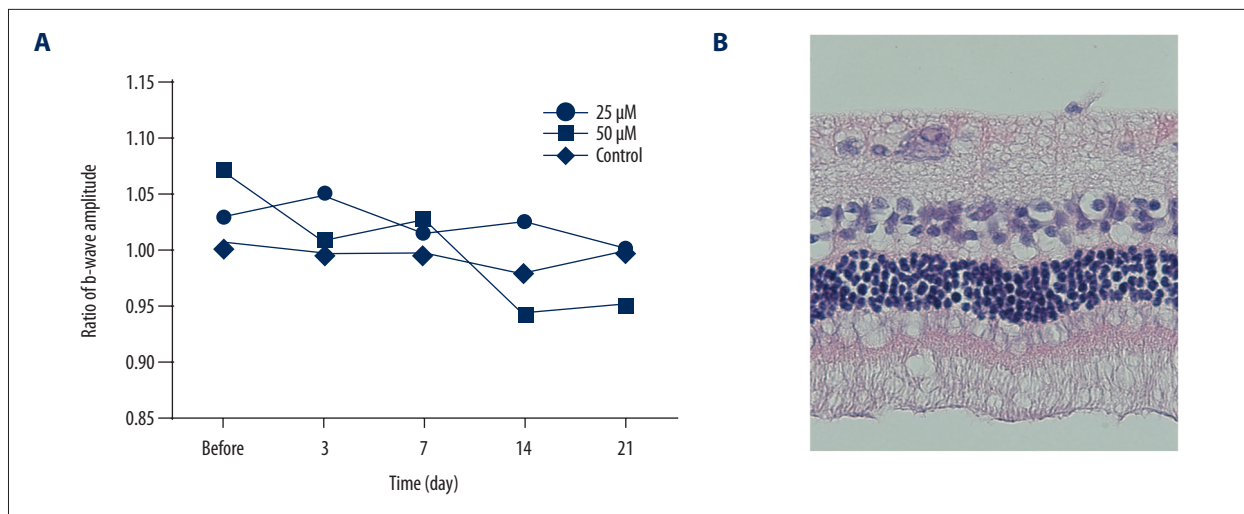us injection of PLB (50 µM). Every layer appeared to be normal with well-preserved photoreceptor outer segments; hematoxylin and eosin staining; magnification 400×.

differentiated phenotypes and turn into mesenchymal-like cells. Meanwhile, a series of cellular functions, including cell motility, proliferation, protein expression, and extracellular matrix have changed [7]. EMT mediates physiologic courses of embryonic development, organ generation or wound healing. But EMT also takes part in pathophysiological processes, for example, fibrosis [8], cancer metastasis [9], or PVR [10].

As everyone knows, RPE is a monolayer of pigmented cells tightly connected and is crucial for the preservation of photoreceptor function. Normally, RPE cells remain quiescent, maintaining original characteristic morphology and action. Under pathological circumstances such as retinal break, the outer blood-retinal barrier (BRB) breaks down, RPE cells disseminate into the vitreous cavity, where they lose RPE cell-cell contact and initiate EMT [11]. During EMT, RPE cells express less epithelial symbols such as zonula occludens-1 (ZO-1), E-cadherin but more mesenchymal symbols such as α-smooth muscle actin (α-SMA), vimentin, and fibronectin [12]. These dedifferentiated RPE cells can then migrate, proliferate, change into fibroblasts, and contract, at last resulting in PVR. Previous studies showed inhibition of proliferative ability of cells may reduce the severity of the illness, but could not completely block the progress of PVR [13]. It has been suggested that perhaps the EMT of RPE deserves more attention since it plays a more crucial role in the development of PVR [10,11]. Therefore, it is imperative to develop drugs for blocking EMT, thus providing another pharmacological target to prevent PVR.

In a previous study, PLB was identified as a proliferative suppressor because of its role on cell apoptosis [14]. Here, we further defined its inhibitory effect on EMT of RPE cells. Our study proved that PLB could downregulate mesenchymal-associated molecules such as α-SMA. At the same time, PLB protected the epithelial junctional protein ZO-1, which is considered to maintained cell-cell adhesion to prevent EMT [11]. Furthermore, PLB is thought to also repress the expression of MMP-1 and MMP-2, which may cleave extracellular matrix components, and thus increase the motility of cells [15–17]. Indeed, the results of our scratch wound assay and Transwell chamber assay indicated that PLB could significantly inhibit the migratory and invasive ability of RPE cells. Herein, we elucidated these suppressive effects of PLB on EMT of PRE cells, leading to weakened motility and contraction. Moreover, these declined abilities were more than the decreased cell numbers as marker ZO-1 were elevated or maintained during the whole experiment.

In the present study, rabbit eyes were chosen as animal models to test the possibility that PLB could prevent and treat of PVR *in vivo*, because they imitated the human disorder closely, with collapsed vitreous and epiretinal membranes [18,19]. Under control conditions, a dense fibrotic membrane derived from RPE cells formed on the collagen matrix, resulting in retinal detachment. In PLB treatment group, these membranes were not as dense as in the control group and were significantly less contractile, similarly like the cell contraction assay *in vitro*. Our animal results demonstrated that intravitreous injection of 25 uM PLB could effectively reduce the morbidity and severity of experimental PVR. Moreover, no evidence of toxicity has been observed in rabbit eyes given either 25 or 50 uM PLB injection, suggesting PLB had a wide safety margin.

Figure 6. Efficacy study of Plumbagin (PLB) for rabbit experimental proliferative vitreoretinopathy (PVR). (**A**) The PVR grades of the treatment eyes versus those of the control eyes. (**B**) The optical coherence tomography and B-ultrasound observations of the treatment eyes versus those of the control eyes.

The present experiment has some limitations: first, RPE cell may be the most important cells involved in the process of PVR but they are not the only factor. The pathogenesis of PVR is extremely complicated involving many cells, such as glial cells or macrophages [1]. We only used RPE cells in this experiment, excluding the action and interference of other cells. Second, in a real retina detached eye, the retina and cells are already impaired and may be more susceptible, which could affect the efficacy and toxicity of PLB. Third, some aspects of toxicity may take longer to present. Further studies on pharmacokinetics of PLB and long-term observation of animal model are needed to perform next. Fourth, since many cell proliferation inhibitor could suppress the development of PVR [18,20], next we may choose some of them as control groups to compare the efficacy of the two therapies or discover a proper combination of drugs to prevent PVR better.

Conclusions

In the present study, PLB was proven to have inhibitory effects on migration, invasion, as well as the expression of EMT-related proteins and MMPs in RPE cells. Besides, PLB could attenuate the severity degrees of PVR in rabbit experimental models. These findings *in vitro* and *in vivo* suggest that PLB may possess potential efficacy for prophylaxis and treatment of PVR and needs further investigation regarding pharmacokinetics and comparison with analogous drugs.

Acknowledgments

Conflicts of interests

None.

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