Reduction of pathological retinal neovascularization, vessel obliteration and artery tortuosity by PEDF protein-based therapeutic in an oxygen-induced ischemic retinopathy rat model

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Research Article

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Additional Declarations: Competing interest reported. US has a patent.
Abstract

Retinopathy of prematurity (ROP) is a worldwide severe disease which can lead to visual impairment or even blindness. It is characterized by obliteration of retinal vessels, presence of tortuous vessels and pathological neovascularization in the retina. The current treatments, cryotherapy, laser ablation or intravitreal injection of anti-VEGF produce limited effect and ineluctable complications. There is therefore still a high medical need for alternative, efficient and safer therapies.

Pigment epithelium-derived factor (PEDF), a potent angiogenesis inhibitor, appears late in gestation and its lack may contribute to ROP. Using an ex vivo model of ischemia and an in vivo model of choroidal neovascularization, we recently discovered that PEDF protein inhibited pathological neovascularization by protecting the endothelial cells which subsequently enhanced the survival of neural retinal cells and photoreceptors respectively. Here we examined the effect of PEDF protein alone or in combination with anti-VEGF drugs and compared their efficacy after a single intravitreal injection in an oxygen-induced ischemic retinopathy (OIR) rat model. PEDF protein alone or in combination with anti-VEGFs significantly suppressed the pathological neovascularization and reduced vessel obliteration compared to anti-VEGF drugs alone demonstrating that the treatment inhibited pathological neovascularization but not physiological angiogenesis. Importantly, PEDF protein-based therapeutics significantly reduced the artery tortuosity indicating an improvement of the retinal vasculature’s quality. No functional or histological side-effects were found in rat eyes after intravitreal protein injection even at high dose.

Thus, the use of PEDF protein alone or combined with anti-VEGF is beneficial, and is a promising therapeutic for ROP.

Introduction

Retinopathy of prematurity (ROP) is a severe disease that can lead to severe visual impairment, blindness and life quality plummet in infants in developed and developing countries [1,2] [3,4]. Premature babies are placed in incubators which generate high oxygen saturation [5]. Oxygen saturation extent is a dilemma in the resuscitation and the physical development of the preterm-born infants. It is necessary for the survival of the infants however, according to the clinical trial BOOST II, increment of the oxygen saturation in the incubators of the premature infants can lead to a reduction of severe cerebral functions and can cause mortality [1,6,5].

ROP is characterized by abnormal retinal vasculature with changes in tortuosity, avascular areas or areas with pathological neovascularization which can eventually lead to retinal detachment [7]. The precise pathophysiology of ROP has not been completely elucidated. The widely accepted theory of this process is the two-phase theory [7,8]. The vascularization of retina in preterm born infant is not entirely completed. The phase I begins with the initiation of infant breathing. The oxygen saturation of the developing retina in the premature baby is relatively higher than in the intrauterine state, followed by the attenuated production of vascular endothelial growth factor (VEGF) in the retina which suspends the
vascularization of the retina. High oxygen incubation exacerbates VEGF suppression. During the phase I, the retina of the infant endures relative hyperoxia and incomplete vascularization. During the phase II, the avascular area of the retina suffers from ischemia and secretes an abundant amount of VEGF, followed by a disorganized retinal vascular growth. Immature and pathological neovascularization can cause severe damages such as vitreous hemorrhage and retinal detachment if untreated.

Treatments for ROP have been improving in the former four decades. Cryotherapy was the standard treatment for ROP in 1980s [9], it has been proved to reduce the severity of the Threshold ROP according to the CRYO ROPCG trial. In the 2000s, several clinical trials showed that the outcome of laser ablation therapy for ROP outweighs cryotherapy [10–12]. Both cryotherapy and laser ablation aim in sacrificing the avascular peripheral retina to reduce complications which may compromise the central retina [13]. However, none of these treatments preserve the function of the avascular retina, which can lead to long-term visual field loss during the development of the infant. Moreover, both cryotherapy and laser ablation can cause severe complications in eyes such as intraocular hemorrhage, cataracts, phthisis bulb i, iris atrophy, hypotony, corneal haze, conjunctival lacerations and nystagmus [14]. Most of the complications are irreversible. The investigation conducted by Iwase et al. [15] showed that cryotherapy and laser ablation triggered myopia in ROP patients, more importantly, the larger the area of retina was treated, the worse the visual acuity became over long term. Since 2007, the anti-VEGF therapy has been used in the treatment of ROP. Both RAINBOW and BEAT-ROP clinical trials showed that the efficacy of anti-VEGF transcends laser ablation [16,17]. The intravitreal injection of anti-VEGF is less traumatic to the eye and easier to perform. However, the use of anti-VEGF drugs is still off-label due to reported adverse effects [16,18–20] such as a higher possibility for infants to suffer from severe neurodevelopmental disability and abnormal body weight gain. Therefore, searching for new alternative treatments is exceedingly essential in the public health crisis in ROP patients.

Pigment epithelium-derived factor (PEDF) is a promising candidate for the treatment of ROP because it appears late in gestation and it has been suggested that its absence may contribute to ROP upon transition from high-to-ambient oxygen environment or with intermittent hypoxia [21]. PEDF is naturally expressed in eye tissues [22]. In the human eye, PEDF protein is localized in rods and cones, the inner nuclear layer, ganglion cell layer and inner plexiform layer, the choroid, corneal epithelia and endothelia, ciliary body non-pigmented epithelia and muscle and the RPE cells [23]. In rats, PEDF protein is found in the same tissues [23]. It is the natural antagonist of VEGF [24] and therefore it is a potent inhibitor of angiogenesis. Michalczyk et al. [25] have shown that PEDF decreased scar formation and promoted tissue regeneration in wound healing. We recently discovered that PEDF protein can suppress pathological changes in vessels and enhance the survival of neural retinal cells in an ex vivo ischemia model [26] and in a choroidal neovascularization (CNV) rat model (Tschulakow et al., ARVO 2023) [27]. Here, we investigated the effect of PEDF protein alone or in combination with anti-VEGF drugs (bevacizumab, an anti-human VEGF antibody and an anti-rat VEGF164 antibody) and compared their efficacy in our established oxygen-induced ischemic retinopathy (OIR) rat model [28]. In our model, an infant’s situation was simulated by first putting rat pups under high oxygen conditions. The rat retinas
adapted to the given situation. Then the pups were returned to room air, which lead to hypoxia in the developing retinal tissues and resulted in a disturbed VEGF/PEDF balance. Typical pathological changes in the vascularization of the retina were observed. In the present study, we examined whether the disturbed ratio between VEGF and PEDF may be balanced by a single intravitreal injection of different treatments and focused on angiogenesis parameters and on the quality of the retinal vasculature using in vivo imaging (scanning laser ophthalmoscopy/fluorescein angiography/optical coherence tomography (SLO/FA/OCT)) and flatmount analysis.

**Results**

**Safety study in rat eyes**

*Light microscopical findings*

The HE stained images of the retina did not show any alterations even at the highest dose of PEDF used (100 µg/eye) (Fig. 1a-b). However, independently of the PEDF dose used, infiltrating cells were detected within the vitreous three days after PEDF injection. Seven and 14 days after PEDF injection, infiltrating cells were rarely observed (data not shown).

*Mueller and microglia cell/macrophage activation*

GFAP is a Mueller cell marker, which is up regulated in ageing and when the retina is damaged or stressed. In retina of untreated control eyes, GFAP was only expressed close to the vireo-retinal interface within the end feet of the Müller cells (Fig. 1c-e). Examples for the different types of scores for activation are shown in the Fig. 1c-e. Mild GFAP activation was observed one and three days after PEDF injection. After three days, it was more prominent at the lowest concentration (4 µg PEDF). After treatment with 20 and 100 µg PEDF, there was an increase in GFAP expression throughout the observation period with a peak at day 14. At a dose of 4 µg PEDF there was a permanent decrease of GFAP expression from 3 to 14 days and the Mueller cells returned to their not activated state 14 days after treatment (Fig. 1f). No change of the Mueller cells shape or activation of astrocytes was seen.

In untreated control eyes, resident microglia cells were located within the inner plexiform layer and within the ganglion cell layer (Fig. 1g). After injection of PEDF, microglia cells became activated which became apparent due to the enhanced development of dendrites (Fig. 1h), called hyper-ramification. There was a high intra-individual variation in the number of activated cells in all treatment groups and no significant differences were observed. The number of cells with elongated ramified processes in the sections was slightly, enhanced after PEDF treatment. After immunocytochemical staining it became obvious that nearly all infiltrating cells within the vitreous were positive for Iba-1, indicating that infiltrating cells are microglia cells or macrophages (Fig. 1i). One and three days after PEDF injection, there was no difference in the number of retinal microglia cells with respect to the different concentrations (Fig. 1j). Fourteen days after treatment with 20 and 100 µg PEDF, the number of elongated ramified processes was already reduced and similar to the untreated retinæ.
**Quantification of apoptosis**

Apoptotic cells were completely absent in retinal cells of untreated animals and within the inner nuclear layer as well as the outer nuclear layer of all treated rats. Occasionally, single apoptotic cells were detected in the ganglion cell layer of treated rats. However, these findings could not be related to a dose-effect of PEDF and were not statistically significant (data not shown).

**ERG measurements**

No functional changes were observed after injection of 4, 20 or 100 µg PEDF at days 1, 3, 7 and 14 after injection compared to the corresponding controls (Fig. 1 k-l).

**PEDF protein dose range finding study in the rat OIR model**

Representative *in vivo* results of the PEDF protein dose range finding study are shown in Fig. 2. The analysis showed that the hyaloid vessels (Fig. 2a) and the vessel density and tortuosity (Fig. 2b) after treatment with 6.125 µg PEDF were less pronounced than in the other groups and showed the closest resemblance to that in animals growing under normoxic conditions as shown in [29]. The analysis of the flatmounts showed that all three PEDF doses significantly suppressed neovascularization (Fig. 2c), but only PEDF at a dose of 6.125 µg/eye significantly reduced the avascular area compared to the untreated OIR control (Fig. 2d). Taken together, the dose of 6.125 µg PEDF protein/eye showed the most beneficial effects on the eyes after OIR and was used in the main experiment.

**Analysis of the pathological retinal neovascularization and vessel obliteration in the rat OIR model**

As indicated in Fig. 3a-d, the percentage change of the neovascularization area of each of the treatment groups: PEDF, PEDF+bevacizumab, PEDF+anti-rat VEGF, bevacizumab and anti-rat VEGF was significantly reduced compared to the neovascularization area of the untreated OIR control which was set at 100 %. The PBS treated group showed no significant change.

There was also a statistically significant reduction of the percentage change of the avascular area meaning a significant reduction of vessel obliteration for all treatment groups, but the PBS treated group compared to the untreated OIR control (Fig. 3e).

**Analysis of the vessel tortuosity on flatmount images**

As shown in Fig. 4a-c, the overall artery tortuosity was significantly higher compared to the overall vein tortuosity in the untreated control group. None of the used treatment modalities influenced the vein tortuosity (Fig. 4d). However, the PEDF treatment as well as each of the PEDF combination treatments (PEDF+bevacizumab and PEDF+anti-rat VEGF) significantly reduced the artery tortuosity (Fig. 4e).

**Analysis of the vessel diameter on flatmounts and on OCT images**
Flatmounts (Fig. 7a-c) and OCT images (Fig. 7d-f) analysis revealed that none of the used treatments affected the artery or vein diameters compared to the control.

**Discussion**

In the present study, a PEDF protein safety study was performed in rat eyes by testing three doses (4, 20 and 100 µg/eye) at different time points (day 1, 3, 7 and 14) after intravitreal injection. The eyes were investigated using histology and electroretinography for functional retinal testing. Cellular infiltration into the vitreous has been observed predominately three days after PEDF injection and may be caused by PEDF directly or by bacterial contamination which cannot completely be excluded. Also the fact that the protein is human may have stimulated the microglia cells. Similar results were already found after intravitreal injection of Fc-fragments into the vitreous of rats [30]. Because cellular infiltrates disappeared nearly completely after seven and 14 days these were not regarded as an adverse effect. The results of GFAP staining showed that the treatment with PEDF activated the Mueller glial cells/macrophages throughout the observation period. Because the Mueller cells did not change their shape and always extend longitudinally from inner limiting membrane to the outer limiting membrane, this was considered as a mild activation. In a monkey study using bevacizumab at the same dose that is used in patients, the expression of GFAP was more prominent and even seen in astrocytes [31]. Nevertheless, bevacizumab is used to treat numerous diseases in ophthalmology since many years. Therefore and because astrocytes did not express GFAP in this study the slightly enhanced expression of GFAP was also not considered as an adverse effect.

Microglia cells are resident macrophages and show a regular distribution [32]. Microglia cells have several physiological functions such as the control of neuronal cell production, neural migration, axonal growth, synaptogenesis and angiogenesis. They also play a role under pathological conditions by defending the retina against infection, inflammation, trauma, ischemia, tumor, and neurodegeneration. Microglia cells have two modes of action - in the healthy retina, they are inactive (resting) and become active during an immune response. The resting and activated microglia cells have a distinct morphology and cell surface markers. Resting microglia cells have a small soma and elongated ramified processes extending and retracting continuously, surveying their microenvironment. During the first activation step the elongated ramified processes become more prominent in the cells. When microglia cells recognize a pathogen or other inflammatory stimulus, they rapidly reach an active state, retract their processes and become efficient mobile effector cells. The results of this study revealed that the microglia cells were activated already one and three days after PEDF injection which was clearly indicated by the enhanced number of cells with elongated ramified processes in the sections. However, it returned nearly to that of untreated retinas in both high dosages groups (20 and 100 µg PEDF) after 14 days. This indicates that the activation is restricted to a short period, even at high PEDF dose, and is therefore also not regarded as an adverse effect.

The OIR model has been developed first in the kitten by Ashton et al. [33], then extended among others to the rat [34] and the mouse [35]. Due to the similar features in the retina of OIR animals and in the ROP
patients, the OIR model has been widely used in the research involving ROP. In the OIR rat model, the retinal angiogenesis is more similar to the pathology observed in ROP patients [29,36]. Indeed, the avascular area (meaning vaso-oblation) is more peripheral in the rat OIR model and in human ROP while it occurs primarily in the central retina in the murine OIR model. In the initial publication from Penn et al. in 1993, the authors used albino rats and compared the occurrence of pre-retinal neovascularization in animals exposed to cyclic high oxygen periods followed by a room air periods vs. constant oxygen followed by room air. Here, the animals were treated immediately after birth and they found that the alternating hyperoxia-hypoxia cycles simulated better the ROP conditions. Later, the alternating oxygen cyclic protocol was revised. The new protocol reached a higher incidence and severity of neovascularization [37]. Although our group [38] and others have reported that the lack of melanin causes an impaired visual acuity, lower number of ipsilaterally projecting RGCs, defects in the optokinetic nystagmus, and a lower functional response [39], the vast majority of eye researchers is still using albino rat strain. In this study, we improved our previously established rat OIR model [28] by using pigmented rats. They were exposed to 75% oxygen from postnatal day 7 (P7) to P12, then moved to room air followed immediately by intravitreal injections of the different therapeutics. The read-out was performed at P17. Our OIR model showed typical ROP characteristics such as vessel tortuosity and peripheral vessel obliteration and neovascularization (Fig. 3). This model was used in the present study to evaluate the effects of PEDF protein alone or combined with anti-VEGF and anti-VEGF alone on pathological neovascularization but also non-neovascular remodeling characterized by tortuosity and dilation of the retinal vessels in the OIR model. Indeed, abnormalities of the retinal vessels are a clinically important finding of ischemic retinopathy in addition to pathological angiogenesis. Bevacizumab, an anti-human VEGF drug, was used as positive control for countering neovascularization as it is widely used in retinal vessel diseases [17,40,41]. In addition, an anti-rat VEGF drug was also used as species-specific positive control. Human PEDF protein was used in study, the amino acid homology between human PEDF and rat PEDF is about 83% [42]. A dose-ranging study of human PEDF protein (24.5, 12.25 and 6.125 µg/eye) was conducted in the OIR rat model in order to find the dose with the highest efficacy. The dose of 6.125 µg PEDF protein/eye was shown to significantly reduce the pathological neovascularization and vessel obliteration. Moreover the hyaloid vessels and the overall retinal vessel density and tortuosity were also reduced (Fig. 2a-b) and showed the closest resemblance to that in animals growing under normoxic conditions as published by Vahatupa et al [29]. Thus, the dose of 6.125 µg PEDF protein/eye showed the most beneficial effects on the eyes after OIR and was used in the present study.

We showed that PEDF protein alone or in combination with anti-VEGF drugs significantly suppressed the pathological neovascularization and reduced vessel obliteration as observed when using anti-VEGF drugs alone. Consistently, the decrease of the avascular area in addition to inhibition of the formation of neovascular tufts in the retina of the OIR model suggest that the PEDF protein-based therapy could lead to a recovery from ischemia. Therefore, PEDF showed similar effects as the established anti-VEGF agents having the advantages of being a natural VEGF antagonist and potentially lacking the side effects of anti-VEGF injections such as unfavorable neurodevelopment in the long term [43].
Our results corroborate some effects of PEDF derived-peptides investigated by Sheibani et al. [21] in a murine OIR model. They found that PEDF 336, a nonapeptide, can reduce the avascular area and the pathological neovascularization in the OIR mouse retina.

However, and in contrast to our study performed in rats, Sheibani et al. [21] have found that bevacizumab, the anti-human VEGF drug, did not reduce the avascular area nor the neovascularization in their murine OIR model. Stemming from the aforementioned comparison, although mice and rats both belong to rodent, murine and rat VEGF depicts different response to bevacizumab which challenge the finding from Yu et al. [44] in which they emphasized that bevacizumab has poor affinity to murine VEGF. Therefore, bevacizumab seems to have a higher affinity to rat VEGF than to the murine VEGF.

In addition to the avascular and neovascular areas, artery and vein tortuosities are also important parameters which illustrate retinal vasculature in the OIR rat model [45]. The tortuosity has also been proved to be the early readout for neovascularization in OIR mice model [46] which also simulate the characteristics of the intraretinal microvascular abnormality [47] in diabetic retinopathy.

For the quantification of the vessel tortuosity, veins were not differentiated from the arteries by some researchers [48], whereas other do quantify the artery and vein tortuosities separately [49]. Hartnett et al. [49] attested that only the artery tortuosity increased in the OIR model while the vein tortuosity remained statistically unchanged. In order to find the appropriate way, vein and artery tortuosities were quantified separately in the untreated group in the present study (Fig. 4). We found that the artery tortuosity was significantly higher than the vein tortuosity in the untreated OIR group, therefore vein and artery tortuosities were compared independently in the different treated groups. Interestingly, only PEDF protein-based treatments (i.e. PEDF protein alone or combined with an anti-VEGF drug) were able to reduce the artery tortuosity in our OIR rat retina. Ergo, only the PEDF protein-based therapy improved the quality of the retinal vasculature in OIR rats which differs from the study of Hartnett et al. [49] in which the artery tortuosity was reduced after treatment with an anti-rat VEGF. As we measured the artery tortuosity three days later than Hartnett et al., one possible explanation for this discrepancy could be that the anti-rat VEGF may reduce the artery tortuosity in the early stage in the OIR rat model, but this reduction does not last.

Another clinical hallmark of acute ROP is dilation of the retinal vasculature [50]. Therefore, the effect of the different treatments on vessel diameter was investigated as well. Considering that the perfusion alteration may influence the vessel diameter, this was measured in vivo using SLO/OCT and ex vivo in flatmounts. In addition, as the thicknesses of the veins and arteries are statistically different in rats [51], their diameters were measured independently both in vivo and ex vivo. Neither PEDF protein alone nor anti-VEGF agents nor their combination affected the vessel diameters with or without perfusion in our study. This finding contrasts with the result from Hartnett et al. suggesting that treatment with antibody to VEGF reduces venous dilation [49]. However, in the study of Hartnett et al., the authors used a rat neutralizing antibody against VEGF\(^{164}\) at a concentration (50 ng in 1 µL) that they had found to reduce intravitreous neovascularization and intraretinal VEGFR2 signaling. The different dose of rat anti-VEGF
antibody used in their study and the fact that their experimental protocol differed from our protocol may explain this discrepancy.

Although PEDF has been found to be the most potent natural, endogenous inhibitor of neovascularization, its application is until now restricted because of its instability and short half-life [52]. It is known that the major challenge of using proteins or peptides is their poor \textit{in vivo} stability, retention, and inactivation by the immune system or by the action of proteolytic enzymes [53]. Rapid elimination leads to frequent and excessive administration, which is improvident and causes non-specific toxicity. Thus, controlled release, which will make it accumulate to effective levels and being metabolized with minimum toxicity and without intolerable adverse effects, becomes particularly important. PEDF gene was loaded in poly (D,L-lactide-coglycolide acid) nanoparticles for PEDF delivery, but the critical drawback of such a microsphere delivery system for proteins is the activity loss during formulation [54]. A crucial strategy for controlled release is the use of polymers [55]. Conjugation of proteins with polymers reduces recognition by the immune system and decreases the clearance rate [53]. Currently, polyethylene glycol (PEG) is one of the most widely used polymers for the modification of protein therapeutics, with many applications from industrial manufacturing to medicine [54]. Because it is inert, inexpensive, and has low toxicity and increased solubility, PEG has been approved by the FDA for drug modification for several years [56]. It has been shown that the half-life of recombinant PEDF is 4.5 h, whereas the half-life of modified PEGylated-PEDF is 78 h [52]. However, it has also been demonstrated that PEG can induce choroidal neovascularization [57], such a side-effect is obviously not desirable for the treatment of ROP. Therefore, the short half-life time of PEDF protein could be overcome by using rather an implant such as the FDA approved port delivery system with ranibizumab (Susvimo, Roche, Genentech) that allows as few as two treatments per year instead of a monthly injection.

To conclude, a single intravitreal injection of PEDF protein is safe and does not induce adverse effects in rat eyes even at high dose (100 µg). PEDF protein alone or in combination with the anti-VEGF agents significantly reduced the pathological neovascularization and the vessel obliteration in the rat OIR model as did the anti-VEGF agents. Moreover, our PEDF protein alone or combined with anti-VEGF drugs was able to reduce the artery tortuosity indicating an improvement of the retinal vasculature's quality. Since pathological neovascularization is the trigger for severe complications such as retinal detachment in diabetic retinopathy [47], retinal vein occlusion [58] and ROP [7] patients, a PEDF protein-based therapy may be very promising for the treatment of these diseases. According to the results of this study and the latest international classification of ROP in 2021 [59], a PEDF protein-based therapy could keep the ROP below stage 3 by suppressing the pathological neovascularization and by reducing the avascular area as well as the artery tortuosity.

\section*{Materials and Methods}

\section*{Animals}
For the ocular safety study, 52 adult Long Evans rats from Janvier Labs (Le Genest-Saint-Isle, France) were used.

For the oxygen-induced ischemic retinopathy (OIR) study, three pregnant Long Evans rats (Janvier Labs) were used, which delivered in total 31 pups which were used for the experiment. Each mother rat and her pups were kept in an extra cage.

The animal experiments were performed after approval by the local authorities (Regierungspraesidium Tuebingen (AK 01/21G)). All animals were handled according to the German Animal Welfare Act and were under control of the animal protection agency and supervision of veterinarians of the Eberhard-Karls University of Tuebingen.

**Safety study in rat eyes**

Both eyes of adult Long Evans rats were injected intravitreally with 5 µl of PEDF protein solutions. The ocular safety/toxicity of three doses of PEDF protein (4 µg, 20 µg and 100 µg per eye) at 4 different time points (day 1, day 3, day 7 and day 14) after intravitreal injection was investigated using histology and functional assessment using electroretinography (ERG).

**Intravitreal injections**

A small incision was made into the conjunctiva at the outer corner of the eyes. The eyeball was rotated by grasping the conjunctiva with a pair of fine tweezers and gentle pulling. A volume of 5 µl was injected intravitreally through the hole using a 10 µl NanoFil syringe with a NanoFil 34 gauge beveled needle (World Precision Instruments). After the injection, the needle remained in the eye for an additional 3 or 4 seconds to reduce reflux and was then drawn back. The eyeball was brought back into its normal position, and the antibiotic ointment was applied to the eye. The whole procedure was performed using a surgical microscope equipped with illumination.

**Processing of the eyes**

On days 0, 1, 3, 7 and 14, the animals were sacrificed, the eyes were fixed in 4.5% formalin (Roti Histofix, Carl Roth, Karlsruhe, Germany) and embedded in paraffin according to standard procedures.

**Histopathological and inflammation assessments**

Hematoxylin eosin (HE) stained section were investigated for changes in the morphology of retina and vitreous. The number of infiltrating cells was counted within the vitreous in paraffin sections under the light microscope at a magnification of 20 fold which corresponds to an area of 0.89 mm$^2$. If there were more than 100 infiltrating cells in this area the number was set 100.

Mueller cell activation was estimated by using an anti-glial fibrillary acidic protein (GFAP) mouse monoclonal antibody (Cell Signaling Technology, Inc. Los Angeles, USA) on sagittal retinal sections. Three neighboring section were stained from the left eyes of all rats. To estimate the activation of Mueller cell, the following score was used:
1 = GFAP staining only around retinal ganglion cells (Fig. 1c)

2 = Mueller cells contained GFAP from inner limiting membrane (ILM) to the outer limiting membrane (OLM) locally (Fig. 1d)

3 = Mueller cells contained GFAP from ILM to OLM throughout the section (Fig. 1e).

Microglia cells/macrophages were stained using anti-ionized calcium binding adaptor molecule (Iba-1) rabbit antibody (Wako Chemicals USA).

Glia cells within the retina were counted under the light microscope in sections from left eyes of each rat at a magnification of 40 fold which corresponds to the retinal length of 40 µm. If several dendrites were cut in an area about 50 µm in diameter, they were regarded to originate from only one MC.

**Quantification of apoptosis**

Cell death was assessed using the TUNEL assay (*in situ* cell death detection TMR red TUNEL kit, Roche Diagnostics, Mannheim, Germany) according to manufacturer’s instructions. The slides were photographed with an Imager Z2 ApoTome microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany).

The number of apoptotic cells per 1000µm length of retina was calculated for each eye.

**Electroretinography (ERG)**

The animals were dark adapted overnight before ERG measurements. The ERGs were measured in 27 animals. The rats were anaesthetized by an intraperitoneal injection of three-component narcosis (0.005 mg fentanyl, 2 mg midazolam and 0.15 mg of medetomidine/kg body weight). The pupils were dilated with 1 to 2 drops of a Mydriaticum (Pharmacy of the University of Tuebingen, Germany). Gold ring Electrodes (1.5mm diameter), (Roland Consult, Stasche & Finger GmbH, Brandenburg, Germany) were placed on the cornea of both eyes. Methocel (OmniVision, Puchheim, Germany) eye drops were used for coupling and to avoid drying of the eyes. Subdermal platinum (27 gauge) needles (Technomed Europe, Maastricht, The Netherlands) in the forehead between the eyes and at the base of the tail served as reference and ground electrodes, respectively. The light stimuli were delivered in a Ganzfeld dome (Roland Consult, Brandenburg, Germany). The ERG-response amplitudes were measured using two automated standard protocols.

For the first protocol dark-adapted, responses were elicited by brief flashes of white light on a dark background. Two stimulus intensities were used, a low one (0.001 cd.s/m²) to analyse the rod function and a high one (10 cd.s/m²) to analyse the mixed rod-cone function (Fig. 1k). After that the eyes were bleached (20 s, 10 cd.s/m²) and reanalysed (10 cd.s/m²). For the second protocol measurements after increasing stimulus intensities (0.01, 0.1, 3, 10 cd.s/m²) were performed (Fig. 1l).
The RETI system software (Roland Consult, Brandenburg, Germany) was used for recording and analysis of the ERG data. The electroretinograms were corrected by a system-intern 50 Hz filter for background suppression. After that the a-wave and b-wave amplitudes and implicit times were measured on the electroretinograms.

**Statistics and graphing for the safety study**

For the analysis Excel, JMP (Version 16.0) and Prism (Version 8.0) software were used. The error probability was 5% (p < 0.05 statistical significant). The data from histology were analyzed using Students t-test. The data from TUNEL assay were analyzed by using the Wilcoxon test with Kruskal-Wallis test and those form the ERG measurements by ANOVA with a Dunnett’s post-test. The results of the ERG analyses were graphed using the ORIGIN PRO software (Version 2024 10.1).

**Oxygen-induced ischemic retinopathy (OIR) rat model**

From postnatal day 7 (P7) to P12 the mother rats with their pups were exposed to 75% oxygen in an incubator (Biospherix, Ltd., Parish, NY, USA), then returned to room air. After a short adaptation time of two hours, they received intravitreal injections of the different treatments in both eyes (Fig. 6, Table 1) under isoflurane anesthesia [28]. After that the animals were kept at normal air conditions for another five days to the time point of the *in vivo* and final analyses.

**Table 1** Summary of the rat and eye number and the different treatment groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>untreated OIR control</th>
<th>PBS</th>
<th>PEDF</th>
<th>PEDF-bevacizumab</th>
<th>PEDF-anti-rat VEGF</th>
<th>bevacizumab</th>
<th>anti-rat VEGF</th>
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<td>number of rats</td>
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<td>6</td>
<td>4</td>
<td>5</td>
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<td>12</td>
<td>8</td>
<td>20</td>
<td>6</td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

PBS: Phosphate buffered saline.

**Intravitreal injection (IVI)**

The injections were performed under isoflurane narcosis (3%). Additionally, a local anaesthetic, Novesine (OmniVision, Puchheim, Germany) was applied on the eyes. For the intravitreal injections (IVI) NANOFIL Syringes (WPI, Sarasota, USA) with NanoFil 34-gauge beveled needles were used. 2 µl of the corresponding substance solution were delivered through the *pars plana* into the vitreous cavity in each eye [60]. After the injections the eyes were treated with an antibiotic ointment, Bepanthen (Bayer Leverkusen, Germany) to prevent ocular infection.

**Substances**

PBS (Gibco, Life Technologies limited, UK); bevacizumab, an anti- human VEGF monoclonal antibody (25 mg/ml, Roche, Basel, Switzerland) and an anti-rat VEGF (0.2 mg/mL, AF564, Bio-Techne © R&D)
Systems, MN, USA) were used.

Recombinant human PEDF protein was produced in CHO cells using an expression vector encoding a full length human PEDF protein. The expressed protein was secreted into the culture supernatant and was purified using chromatographic techniques. The purified human PEDF protein was used alone or in combination with anti-rat or anti-human VEGF drug.

**PEDF protein dose range finding study**

In order to find the optimal dose of human PEDF protein that inhibited significantly the retinal pathological neovascularization as well as the vessel obliteration, four eyes per dose of PEDF protein were intravitreally injected on P12 i.e. on the day of transition from high-to-normal oxygen with 24.5 µg, 12.25 µg and with 6.125 µg PEDF in 2 µl PBS. The effects of the different doses on neovascularization, vessel obliteration as well as on hyaloid vessels were assessed at P17.

**In vivo SLO/OCT imaging and measurement of the vessel diameters on OCT images**

The scanning laser ophthalmoscopy (SLO), fluorescein angiography (FA), and optical coherence tomography (OCT) were performed using a Spectralis™ HRA + OCT (Heidelberg Engineering, Heidelberg, Germany) device. To make it usable for the analyses in rats, a + 78D double aspheric lens (Volk Optical, Inc., Mentor, OH 44060, U.S.A.) was placed directly on the outlet of the device and an additional custom-made contact lens with + 7 diopter (dpt) was placed directly onto the cornea of the animals directly before analysis. On P17, the pup rats were intraperitoneally injected with a three-component narcosis (fentanyl 0.005mg/kg, midazolam 2mg/kg and medetomidine 0.15 mg/kg) and their pupils were fully dilated using 1 to 2 drops of Mydriatikum (Pharmacy of the University of Tuebingen, Germany). After that Methocel (Omni Vision GmbH, Germany) was applied to avoid drying of the cornea and for better adherence of the + 7 dpt lens on the eye. For the proper positioning during the *in vivo* examination, the rats were put on an adjustable platform directly in front of the device. Then 0.15 ml of 10% Fluorescein (Fluorescein Alcon; 1/10 dilution in isotonic 0.9% NaCl solution (Fresenius, Germany)) were subcutaneously injected. Immediately after that the fluorescein angiography of each eye was performed. The device was centered on the optic nerve head (ONH) and the images were acquired (Fig. 7a). After that an OCT analysis of each eye was performed. OCT-scans were taken manually in the area of the first bifurcations of the vessels (Fig. 7b). On these images the diameters of the veins and arteries were measured using the “Heidelberg Eye Explorer” software provided by the manufacturer (Heidelberg Engineering, Heidelberg, Germany) as indicated in (Fig. 7c).

**Flatmount preparation and staining of the vessels**

After *in vivo* examinations, the pups were euthanized, and their eyes were enucleated. Flatmounts were prepared according to the protocol described by Tual-Chalot *et al* [61] with some slight adjustment i.e. the retina and the choroid were not separated to reduce the risk of tearing the retina.
The vessels were stained with Griffonia Simplicifolia Lectin I (GSLI) isolectin B4-Fluorescein (Vector Laboratories, CA, USA) following standard protocols provided by the manufacturer. The flatmounts were mounted using Dako Fluorescence Mounting Medium (Agilent Technologies, Inc. CA, USA). The scan images covering the whole retina were generated from each flatmount using an Axio Imager Z1 microscope (ZEISS, Germany).

The protocol published by Vähätupa and colleagues [29] was followed. In brief, using the Image J (Java 13.0.6, http://imagej.nih.gov/ij) free hand drawing tool the whole area of each flatmount (WA) as well as the avascular areas (AV) and the neovascular areas (NV) were selected manually (Fig. 3). Areas on flatmounts or whole flatmounts which were not analyzable due to preparation artefacts were excluded. The ratio of NV versus WA and the ratio of AV versus WA were calculated. For the statistical analyses, we calculated the percentage changes of the NV/WA and AV/WA for each of the treatment groups compared to the untreated OIR control, which was set at 100%.

**Quantification of the vessel tortuosity on flatmounts**

The veins were differentiated from the arteries as indicated in Pannarale et al. [51]. The arteries in proximity to the optic nerve head (ONH) overlie the central veins. The length of all veins and arteries from the ONH to their first bifurcation point as well as the distance between these two points were measured using Image J (Fig. 4a-b). The tortuosity was defined as the ratio of the vessel length from the ONH to their first bifurcation point and the distance between the ONH and the first bifurcating point.

\[
\text{Tortuosity} = \frac{\text{length of the vessel from the ONH to their first bifurcation point}}{\text{distance between ONH and the first bifurcation point}}
\]

The diameters of the veins and arteries were measured at the point of the first bifurcation. The measurements were performed perpendicular to the vessel walls, as indicated in Fig. 5.

**Statistics for the OIR study**

Prism 8.0 (GraphPad Software, San Diego, California USA, www.graphpad.com) was used for statistical quantification. The results are presented as means ± SD (standard deviation). The statistical analyses were performed using a Student’s t-test with Bonferroni correction. Differences were considered statistically significant at a P level < 0.05.

**Abbreviations**

AV
avascular area

CNV
choroidal neovascularization

FA
fluorescence angiography
Declarations

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Authors Contribution
US, SK and SJS conceived the study. US, SSK and SJS designed the experiments. SZ, AT, KW, US, SSK and SJS conducted the experiments. SZ, AT, US and SJS collected and analysed the data. SZ, AT, SK, SSK and SJS wrote the manuscript. All authors have read and approved the manuscript.

Data Availability

All data in support of the findings of this work can be found within the article, and from the corresponding author on reasonable request.

Competing Interests

SZ, AT, SSK, KW, SK and SJS have no competing interests to declare that are relevant to the content of this article. US has a patent.

References


Figures

Figure 1

Safety/toxicity study in rat eyes. **a-b** HE staining one day (**a**) and 14 days (**b**) after intravitreal injection of 100 µg PEDF protein. No alterations were observed. **c-f** GFAP staining (red), **c** Mueller cell activation's score 1: GFAP staining is present only in the Mueller cell end feet. **d** Mueller cell activation's score 2: GFAP is expressed locally by Mueller cells from the inner limiting membrane to the outer limiting membrane (arrowhead). **e** Mueller cell activation's score 3: increased expression of GFAP by Mueller cells throughout the section **f** Quantification of Mueller cell activation by scoring of GFAP expression. **g-j** Iba-1 staining
Untreated control eye with resident microglia cells located within the inner plexiform layer and the ganglion cell layer (arrowheads). Retina of a rat three days after injection of 20 µg PEDF with numerous activated microglia cells indicated by the development of dendrites (arrowheads) and infiltrating Iba-1 positive cells in the vitreous (arrow). Retina of a rat three days after injection of 100 µg PEDF showing less activated Iba-1 positive cells within the retina compared to (h) but numerous infiltrating Iba1 cells in the vitreous (arrow). Quantification of the number of Iba-1 positive cells with elongated ramified processes of per 40 µm retinal length. ERG results, lower left panel: representative electroretinogram for protocol 1 after dark adaptation [ad]: with stimuli of 0.001 cd.s/m² and 10 cd.s/m² and after bleaching (20 s, 10 cd.s/m²) [ab] with a 10 cd.s/m² stimulus. On the right panel the results for all a-wave and b-wave amplitude and implicit time measurements for the doses 4, 20 and 100 µg PEDF/eye at the time points 1, 3, 7 and 14 days after injection and their corresponding controls are shown as 3D mean and SD plots. No significant changes after any of the treatments at any of the analyzed time points compared to the corresponding controls were found. Lower left panel: representative electroretinogram for protocol 2, ERGs for increasing stimulus intensities of 0.01, 0.1, 3, and 10 cd.s/m². On the right panel the results for all a-wave and b-wave amplitude and implicit time measurements for the doses 4, 20 and 100 µg PEDF/eye at the time points 1, 3, 7 and 14 days after injection and their corresponding controls are shown as 3D mean and SD plots. No significant changes after any of the treatments at any of the analyzed time points compared to the corresponding controls were found.
Figure 2

PEDF protein dose range finding study. a FA/SLO images of hyaloid vessels and b of the retinal vessels, of P17 rat eyes with OIR. Images of an untreated eye and eyes treated with 24.5 µg, 12.25 µg, 6.125 µg PEDF respectively are shown. c-d Statistical analysis of the areas with neovascularization (c) and avascular areas (d) on flatmounts as percentage of the corresponding untreated OIR control. The
principle of the analysis is described under material and methods and indicated in Fig. 3c. Mean and standard deviation are shown, * P<0.05 ** P<0.001, *** P<0.0001.

Figure 3

Analysis of the neovascularization and vessel obliteration on flatmount images. a Representative image of a flatmount of a P 17 rat eye with OIR. An area with pronounced vessel obliteration and neovascularization is framed in red. b A magnified image of the red framed area in (a). c Principle of the analysis: The avascular (red) and neovascularization (yellow) areas were manually selected on the
flatmount images. d Statistical analysis of the areas with neovascularization and e avascular areas, as percentage of the corresponding untreated OIR control. AV: Avascular area; NV: Neovascularization area. Untreated OIR control (n=6), PBS (n=9), bevacizumab (n=8), PEDF+bevacizumab (n=4), PEDF (n=6), anti-rat VEGF (n=6), PEDF+anti-rat VEGF (n=5). Mean and standard deviation are shown, ** P<0.001, *** P<0.0001.

Figure 4

Vessel tortuosity analysis on flatmount images. a The red line represents the length of a retinal vessel from the optic nerve head (ONH) to the point of its first bifurcation. b The yellow line indicates the distance from the ONH to the first bifurcation point of the vessel analyzed in (a). The tortuosity of a vessel is defined as the ratio of the length (a) and distance (b) of the vessel. c Direct statistical comparison of the tortuosity of the veins and arteries in the untreated OIR control animal eyes (n=6). d Statistical analysis of the vein tortuosity and e artery tortuosity. Untreated OIR control (n=6), PBS (n=9),
bevacizumab (n=8), PEDF+bevacizumab (n=4), PEDF (n=6), anti-rat VEGF (n=6), PEDF+anti-rat VEGF (n=5). Mean and standard deviation are shown, *** P<0.0001.

Figure 5

Vessel diameter analysis on flatmount and OCT images. a The diameters (red line) were measured at the first bifurcation point of the vessels perpendicular to the vessel walls (black lines) on flatmount images. b Statistical analysis of the vein diameter and c artery diameter measurements.

Untreated OIR control (n=6), PBS (n=9), bevacizumab (n=8), PEDF+bevacizumab (n=4), PEDF (n=6), anti-rat VEGF (n=6), PEDF+anti-rat VEGF (n=5). Mean and standard deviation are shown. d Principle of the vessel diameter measurement on OCT images (red line). e Statistical analysis of the vein diameter and f artery diameter measurements. Untreated OIR control (n=8), PBS (n=8), bevacizumab (n=8), PEDF+bevacizumab (n=8), PEDF (n=7), anti-rat VEGF (n=5), PEDF+anti-rat VEGF (n=5). Mean and standard deviation are shown.
Figure 6

OIR study design. The rat pups were exposed to 75% oxygen from postnatal day 7 (P7) to P12, then returned to room air. Immediately thereafter they received an intravitreal injection of the different treatments in both eyes under isoflurane anesthesia. On postnatal day 17 (P17), the rats were investigated in vivo using SLO OCT. Then the rats were euthanized and enucleated. Flatmounts were prepared and stained with Griffonia Simplicifolia Lectin I (GSLI) isolectin B4-Fluorescein. IVI: intravitreal injection.
Figure 7

SLO/FA/OCT imaging and vessel diameter analysis on OCT images. **a** SLO image of a P17 rat eye with OIR. The green arrow indicates the area where the OCT scan, shown in **(b)** was generated. **b** OCT scan in the area of a bifurcation of the central vessel (red framed). A magnified image of this vessel is shown in **(c)**. **c** Principle of the vessel diameter measurement (indicated in red).