repisalud
Repositorio Institucional en Salud

This is the peer reviewed version of the following article:

Peral A, Mateo J, Domínguez-Godínez CO, Carracedo G, Gómez JA, Crooke A, Pintor J. Therapeutic potential of topical administration of siRNAs against HIF-1α for corneal neovascularization. Exp Eye Res. 2022 Jun;219:109036. doi: 10.1016/j.exer.2022.109036. Epub 2022 Mar 31. PMID: 35367249.

which has been published in final form at: https://doi.org/10.1016/j.exer.2022.109036

Title

THERAPEUTIC POTENTIAL OF TOPICAL ADMINISTRATION OF siRNAs AGAINST HIF-1α FOR CORNEAL NEOVASCULARIZATION

Assumpta Peral[a], Jesús Mateo[b], Carmen O. Domínguez-Godínez[a], Gonzalo Carracedo[a], Jose Antonio Gómez[c], Almudena Crooke[d], Jesús Pintor[d]

[a] Department of Optometry and Vision, Faculty of Optics and Optometry, University Complutense of Madrid, C/Arcos de Jalon 118, 28037, Madrid, Spain
[b] Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), C/ de Melchor Fernández Almagro, 3, 28029, Madrid, Spain
[c] Department of Optics, Faculty of Optics and Optometry, University Complutense of Madrid, C/Arcos de Jalon 118, 28037, Madrid, Spain
[d] Department of Biochemistry and Molecular Biology, Faculty of Optics and Optometry, University Complutense of Madrid, C/Arcos de Jalon 118, 28037, Madrid, Spain

Corresponding author:
Assumpta Peral
Department of Optometry and Vision
Faculty of Optics and Optometry
University Complutense of Madrid
C/ Arcos de Jalón, 118
28037 Madrid, Spain
Phone: +34 654 953 234
E-mail: assumpta@ucm.es

Disclosure section:
No author has a financial or proprietary interest in any material or method mentioned.

Funding:

This work was supported by a grant from Ministerio de Ciencia e Innovación (SAF2010-16024 and SAF2013-44416-R) and RETICS (RD12/0034/0003), and a grant from the Instituto de Salud Carlos III of Spain and Fondo Europeo de Desarrollo Regional (FEDER, "Una manera de hacer Europa") (FIS-FEDER PI07-1168 to J. Mateo)

http://doi.org/10.1016/j.exer.2022.109036
Received 5 November 2021; Received in revised form 19 February 2022; Accepted 12 March 2022

ABSTRACT

Given the implications of the problem of neovascularization on ocular health, as well as the growth in the number of cases, the purpose of the present study has been testing the efficacy of siRNAs (small interfering RNA) designed to silence Hypoxia Inducible Factor -1α (HIF-1α) and to demonstrate that their use stops neovascularization in a model of corneal burn.

Corneal wounds in the limbic zone were made in the eyes of New Zealand white rabbits. Topical applications of siRNAs were done the next day to the wound for four consecutive days and eyes were examined with a slit lamp. Evaluation of neovascularization progress was done by analyzing images by ImageJTM and to determine the neovascular area in Matlab ® was used. At the same time, a rabbit corneal cell line was used for in vitro study of hypoxia exposure and western blot analysis of the cell's extracts were done.

Under normal cell culture oxygenation, the expression of HIF-1α was lower than that observed under hypoxic conditions. After 2 hours of hypoxia, there was a significant increase in the HIF-1α expression, effect that was maintained up to six hours. The increased in HIF-1α was mimicked by a cell permeable prolyl-4-hydroxylase inhibitor. Cobalt chloride showed no capacity to increase HIF-1α in vitro. The effect of three different siRNA on HIF-1α was tested after 4 hours of hypoxia. siRNA#1 was able to silence 80% of HIF-1α expression, siRNA#2 and siRNA#3 reduce the expression in 45% and 40% respectively.

In addition, the three siRNA were tested in a corneal model of neovascularization. scrambledsiRNA#2 was the most effective inhibitor of blood vessel production, followed by siRNA#3 and siRNA#1. Compared to the scrambled siRNA (100% of blood vessel generation), siRNA#2 blocked the presence of blood vessels by 83±2%, siRNA#3 inhibited 45±7% and siRNA#1 only inhibited 18±5%. The necessary time to observe the 50% of effect showed values of NV50 of 10.2±2.4 days for the scrambled siRNA, 9.1±1.4 for siRNA#1, 6.5±1.85 for siRNA#2 and

4.8±1.8 days for siRNA#3. In conclusion, the topical application of siRNA towards HIF-1α seems to be an effective and reliable method to stop neovascularization.

Keywords

Blood vessel, cornea, HIF-1α, hypoxia, neovascularization, siRNA

1. Introduction

Neovascularization or angiogenesis is the process of formation of new vessels derived from other existing ones (Auerbach and Auerbach, 1994). For the formation of new vessels an angiogenic stimulus must be given, since under normal conditions there is a balance between angiogenic and anti-angiogenic factors. Neovascularization is a complex process that involves numerous genes expressed by different cell types, and that together contribute to a well-integrated sequence of events, whose purpose is to adapt the supply of oxygen and nutrients to changes in the mass of a tissue or in its metabolic activity (Kather and Kroll, 2014). Even so, a deregulated angiogenesis is the common element in the development of different diseases that have a high incidence, such as the growth of tumours, vascular insufficiency, diabetic retinopathy, neovascular AMD (Chappelow and Kaiser, 2008), among others (Folkman, 1995).

According to the fundamental role that hypoxia plays in the neovascular process, it is now known that many genes involved in the different stages of this process respond independently to hypoxia. Hypoxia-sensitive genes include: the inducible nitric oxide synthetase (NOS2), which governs the vascular tone, growth factors such as vascular endothelial growth factor -VEGF-, fibroblast growth factor (bFGF) as well as several of the receptors for these factors and genes involved in the metabolism of the extracellular matrix and several others (Krock et al., 2011; Pugh and Ratcliffe, 2003). Similarly, many of the individual processes that occur in neovascularization, such as cell migration, or the proliferation and organization of endothelial cells to form tubular structures (Auerbach et al., 2003) can induce hypoxia.

Most of the genes involved in the neovascular response contain in their promoter region specific sequences called "hypoxia response elements" to which the transcription factor HIF (Hypoxia Inducible Factor) binds, the main transcription factor responsible for the adaptive response to hypoxia and, therefore, main responsible for the angiogenic response (Pugh and Ratcliffe, 2003). The HIF-1 protein is composed of two subunits HIF-1α and HIF-1β. In a situation of normoxia, HIF-1α is hydroxylated by specific prolyl-hydroxylases and destroyed, whereas in a situation of lack of oxygen, as it is the case of hypoxia, HIF-1α cannot be hydroxylated and accumulates, which allows dimerization with HIF-1β gives rise

to HIF-1 and this results in the activation and stimulation of different genes (Huang et al., 2010).

Angiogenesis plays a central role in various physiopathological processes within human body, not only during foetal development but also in tissue repair after surgery or trauma, or during inflammation and infections, which lead to tissue hypoxia (Chang et al., 2001). Angiogenesis can be a hallmark of wound healing, the menstrual cycle, cancer, and various ischemic and inflammatory diseases.

Recognition that control of angiogenesis could have therapeutic importance has stimulated great interest during the last decades. Stimulation of angiogenesis can be therapeutic in ischemic heart disease, peripheral arterial disease, and wound healing. Decreasing or inhibiting angiogenesis can be therapeutic in cancer, ophthalmic conditions, rheumatoid arthritis, and other diseases.

A study carried out in the USA estimated that 1.4 million patients per year could have corneal neovascularization and that 4% of the US population was already enrolled with it. In addition, 20% of tissue samples obtained from corneal transplants had histological evidence of neovascularization. According to this study, there was no other work that had analyzed the prevalence of neovascularization in the general population (Lee et al., 1998).

The cornea is a transparent tissue, avascular, richly innervated, and highly differentiated that allows the refraction and transmission of light. This ocular tissue has high oxygen demand for the development of its metabolism, which is mainly obtained from the tear film (Edelhauser and Ubels, 2004). When the quantity of oxygen does not meet their needs a state of hypoxia is produced that can lead to a process of neovascularization, extending the vessels of the superficial arcade of the limbus, and invading the cornea, with the consequent loss of transparency. This loss will lead to a decrease in vision and in the most severe cases, to blindness (Epstein et al., 1987).

A variety of circumstances can lead to a corneal neovascularization. Among them are: traumatisms and wounds (Lin et al., 2011); infections by bacteria (staphylococci, streptococci, pseudomonas) (Manire, 1997; Tabbara and Ross-

Degnan, 1986), viruses (herpes simplex, herpes zoster) (Liesegang, 2001), and protozoa (onchocerca volvulus, *Leishmania brasilensis*) (Taylor and M., 1996); burns by chemical agents (Lin et al., 2011); immune system pathologies (Steven-Johnson syndrome, Graft rejection, Cicatricial pemphigoid) (Bachmann et al., 2010); degenerative problems such as Pterigium or Terrien's marginal degeneration (Chan et al., 2015).; anterior pole eye surgeries or use of contact lenses and dry eye (Guillon et al., 1985; Holden et al., 1986; Papas, 1998; Papas, 2003).

Blocking blood vessel development has been always a matter of interest since neovascularization has been related to pathological situations such as cancer as well as several ocular diseases as previously commented. Searching for new strategies to stop corneal neovascularization, approaches such as RNA interference (siRNA) become interesting and attractive since its mechanism of action lacks the problems of conventional pharmacology (Supe et al., 2021). The use of siRNA has already been used to treat several ocular diseases such as some types of age-related macular degeneration, glaucoma, dry eye, or diabetic retinopathy (Kaiser et al., 2010; Martinez et al., 2014; Nguyen et al., 2012a; Nguyen et al., 2012b). There are few reports indicating the inhibition of VEGF using siRNA to treat corneal neovascularization (Chen et al., 2012; Fu and Xin, 2019). Therefore, the purpose of the present study is to test the efficacy of siRNAs designed to silence HIF-1α and to demonstrate that their use stops neovascularization in a model of corneal burn.

2. Materials and Methods

2.1 Animals

Eighteen adult New Zealand white rabbits (males, 2–3 kg) were used. The animals were kept in individual cages with free access to food and water, under controlled cycles (12h light: 12h dark). Experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and the statement of the Association for Research in Vision and Ophthalmology on the Use of Animals in Ophthalmic and Vision Research.

2.2 Cell culture

SIRC (Statens Seruminstitut Rabbit Cornea, ATCC® CCL60™), a rabbit corneal cell line, was purchased from ATCC (LGC Promochem SL., Barcelona, Spain). Cells were maintained in minimum essential medium (MEM) with Earle's salts, L-glutamine, and non-essential amino acids (Invitrogen, Paisley, UK) supplemented with 10% inactivated Foetal Bovine Serum (Invitrogen) and incubated at 37° C in 5% CO_2 and 95% humidity until confluence. For hypoxia exposure, cells were incubated at 37°C in a humidified hypoxic chamber equipped with an O_2-regulator (COY Labs., Grasslake, MI, USA) and flushed with a blend of 5% CO_2, the desired percentage of O_2 (i.e. 1% O_2) and N_2 to total 100%.

2.3 Wounding of the corneal epithelium

Corneal wounds were made in both eyes after topical application of anaesthesia with oxibuprocaine and tetracaine (0.4 and 1% respectively) (Alcon Cusi, Barcelona, Spain). Corneal wounds were made by applying a 1/6 sector of circle of radius 1 cm of Whatman no. 1 paper soaked in NaOH, following the protocol described by Medicute et al. 1997. Whatman paper sectors were placed in the eye with the apex towards the pupil and the base to the top of the eye covering the limbic zone and left there for 1 minute [31]. After that, Whatman papers were removed, and eyes were washed repeatedly with isotonic saline solution. Eyes were examined with a Topcon SL-8Z slit lamp (Topcon Spain, Madrid, Spain) and images were taken at 0, 7, 12, 15, 22, 25 and 31 days after the injury with the IMAGEnet 2000 system (Topcon). Topical applications of the siRNAs were done the next day to the wound in the right eye leaving the left eye as the control. siRNAS were applied for four consecutive days and those days slit lamp images were no taken.

Images were analysed by ImageJ™ to measure the neovascularization progress.

2.4 siRNAs

Three siRNAs against rabbit HIF-1α (Accession No AY273790) were designed and purchased from Gene Link (Hawthorne, NY, USA). siRNA#1: 5'-AGUCACCACAGGACAGUAUTT-3'; siRNA#2: 5'-CAAGCAACGGUCAUAUAUATT-3'; siRNA#3: 5'-CAUUGUGUGUGUCAAUUAUTT-3'. A scrambled siRNA (sc-37007) was purchased from Santa Cruz (Hercules, CA, USA) and used as a negative control. For *in vitro* experiments, the siRNAs were transfected into cells at a final concentration of 50 nM in 60-mm dishes using 20 µL *Trans* IT-TKO transfection

reagent (Mirus, Madison, WI, USA) according to the manufacturer's instructions, and the experiments were carried out 48 h after transfection.

For the *in vivo* experiments, 6 rabbits were used for each SiRNA, being the experiment repeated 3 times. An amount of 4 nmol of siRNA in a total volume of 40 μL of RNAse-free saline solution was instilled in the right eye of the rabbits for four consecutive days. Left eye acted as a control and 40 μL of saline solution 0.9% RNAsa free containing the scrambled siRNA was instilled the same three consecutive days. The development of the corneal blood vessels was followed for 31 days measuring blood vessel extension at the indicated days (0, 7, 12, 15, 22, 25 and 31 days).

2.5 Western blot analysis

Cells for *in vitro* analysis were harvested and homogenised in ice-cold RIPA buffer supplemented with protease and phosphate inhibitor cocktails (Thermo Fisher Scientific). The lysates were subsequently submitted to centrifugation at 15,000× g for 15 min at 4°C. The supernatants were stored at -80°C until processed. Protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Cell extracts (40–50 μg) were resolved by SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and western blotting carried out as described earlier (Mendicute et al., 1997) using monoclonal antibodies against HIF-1α (1:100, Ab-4; clone H1alpha67, Thermo Fisher Scientific) and α-actin (1:10,000, Sigma). The intensity of the bands was quantified with the Quantity One software (Bio-Rad, Madrid, Spain) and normalized to respective densitometric actin values.

2.6 Image analysis

To determine the vascularization area of the corneal images, an image processing algorithm in a software written in MatLab® as a GUI (guided user interface) has been developed. Images analysis was performed by a blinded operator (J.A.G) who did not participate in the experiments and did not know the treatment used in each eye. Figure 1 shows an example of the processing software. More information about the images analysis has been added like supplementary data and a video of the procedure is able in the following link:

https://youtu.be/t3obg4Y1qxE. The algorithm employed can be summarized in the following steps:

1. The user loads an image and determines whether it is a standard or a control image. If the image is a control one, the user selects a region of interest (ROI), which will be used in successive steps to account for the total area where the blood vessels will grow.
2. If the user classifies the image as a standard, he/she must also select a ROI to compute the vascularization area. After the ROI selection the image is processed as follows:
3. First a high image contrast is obtained by dividing the image by the background obtained by filtering the original image with a median filter defined in a 15x15 neighborhood. Then the contrast is adjusted to get the final high contrast image as shown in figure 1.
4. Once the high contrast image is obtained, the algorithm performs a binarization to segment the dark blood vessels from the clear background. In our implementation, the threshold is computing automatically using the technique reported by Otsu et al. 1979 (Otsu, 1979) It is also possible to change manually the selected threshold if deemed necessary. From the binary mask, the vascularization area is computed and compared with the total area of the injury defined for the control image. In figure 1, the vascularization mask (in gold) superposed over the high contrast image is shown.
5. To improve the accuracy of the algorithm, the user can add manually vessels undetected by the automatic technique. This is done simply by defining a polygonal path, which describes the trajectory of the blood vessel, and by selecting the vessel width as it can be seen in figure 1. Once the automatic and manual processing is finished, the user must validate the measurement by introducing the day after injury and pressing the “validation” bottom.
6. Once validated, the program stores the vascularization area and the percentage of vascularization together with additional user supplied data such as the magnification and the day after the injury. All these data are stored in a TXT file which can be processed either with MatLab® or with any spreadsheet program such as Excel®. The software computes also an evolution plot showing the variation of the vascularized area with the time after injury.

2.7 Data analysis

Data are presented as mean ± S.E.M. Significant differences were determined by two-tailed Student's t-test. P-values below 0.05 were considered significant. All the plots and data analysis were performed using GraphPad Prism v7.2 (GraphPad Software Inc.)

3. Results

3.1 HIF-1α *silencing in corneal epithelial cells*

The ability of a rabbit corneal epithelial cell line (SIRC cells) to express HIF-1α under different experimental conditions was investigated. As expected, under normal cell culture oxygenation conditions (21% O_2) the expression level of HIF-1α was lower than that observed under hypoxic conditions (1% O_2) for different times (figure 2A and 2B). There was a significant increase in the expression of HIF-1α after 2 hours of hypoxia, which was maintained at longer exposure times up to 6 h. The increase in HIF-1α was mimicked by DMOG, a cell permeable prolyl-4-hydroxylase inhibitor, which upregulates HIF-1α by preventing its degradation. Cobalt chloride (100 μM), an inducer of chemical hypoxia, showed no capacity to increase HIF-1α in SIRC cells. Since 2-4 h of hypoxia was enough to strongly trigger the expression of HIF-1α, the effect of three different siRNA towards HIF-1α were tested after 4 h of hypoxia. SIRC cells were challenged for 48 h with 50 nM of the siRNAs and their ability to modify the expression of HIF-1α was studied. As shown in figure 3A and 3B, siRNA#1 was able to silence roughly 79% of HIF-1α expression, while siRNA#2 and siRNA#3, although reduced HIF-1α protein expression, the silencing capacity was 43% and 46% respectively. It is important to note that the efficacy of the siRNAs was totally dependent on the transfection reagent, since in the absence of TransIT-TKO transfection reagent the most effective siRNA (siRNA#1) was unable to produce any silencing effect on HIF-1α expression.

3.2 Inhibition of neovascularization *in vivo*

Since all three siRNA showed a significant degree of HIF-1☐ silencing in vitro, we decided to test them also in vivo. When the three siRNA were tested in a model of corneal neovascularization, it was possible to observe noteworthy effects in the extension of the blood vessels if they extended through the cornea. As it is shown in figure 4, control experiments carried out in the presence of the scrambled siRNA, showed a wide vessel extension starting from the wound limit (figure 4A, see

arrow). Interestingly, the effect of siRNA#2, clearly produced an inhibition of the blood vessel dissemination when compared to the images obtained for the scrambled siRNA 31 days after starting the treatment (figure 4A). When the three siRNA were compared and their degree of neovascularization inhibition was plotted, we observed that as happened with the "in vitro" experiments, not all the siRNAs showed the same silencing effect. In this sense, and as it is shown in figure 4C, siRNA#2 was the most effective inhibitor of blood vessel production, followed by siRNA#3 and siRNA#1. siRNA#2 was able to block the presence of blood vessels by 83±2% (n=6) compared to the scrambled siRNA (100% of blood vessel generation). siRNA#3, was able to inhibit 45±7%, while siRNA#1 only inhibited 18±5% (n=6).

The hyperbolic behaviour observed for all the tested siRNA (including the scrambled) invited us to fit the obtained plots (figure 4B). In this sense, the mathematical fit permitted us to obtain a parameter NV_{50}, which is the necessary time to observe 50% of neovascularization reduction. The obtained analysis showed NV_{50} values of 10.2 ± 2.4 days for the scrambled siRNA, 9.1 ± 1.4 days for siRNA#1, 6.5 ± 1.85 days for siRNA#2 and 4.8 ± 1.8 days for siRNA#3.

4. Discussion

Corneal neovascularization can happen because of several pathological processes which include corneal infection, corneal inflammation, viral keratitis or even derived from the misuse of contact lenses (Ahn et al., 2014; Gupta and Illingworth, 2011; Lee et al., 1998). The present experimental work was aimed at blocking corneal neovascularization with a potential approach based on the ability of certain siRNA designed against HIF-1α. *In vitro* experiments carried out with corneal cells to silence HIF-1α using three different siRNAs showed a clear reduction in the expression of the target protein. In this case, not all the siRNAs that were assayed exhibited the same efficacy abolishing the expression of HIF-1α. Among the three tested siRNAs, siRNA#1 was significantly stronger than the other two. It is important to note that apart from the scrambled siRNA, siRNA#2 and siRNA#3 were also able to reduce the expression of the protein under study.

Interestingly, the application of the same siRNAs on a corneal neovascularization model showed that siRNA#2 was able to prevent the occurrence of the blood

vessels that conversely were observed in the presence of the scrambled siRNA. The other two siRNAs also reduced the spreading of blood vessels but to a lesser extend as it can be observed in figure 4. However, we acknowledge the big translational gap existing between the in vitro model used here (SIRC) and the in vivo model (rabbit eye), which could justify a lack of correlation in the results obtained.

There are several studies published showing the lack of correlation between the in vitro and in vivo siRNA effect. (Basiri et al., 2020; Jansson-Löfmark et al., 2020) The lack of correlation between the silencing capacity of siRNAs in vitro and in vivo may be due to different factors related to the complexity of the target tissue. In this way, the presence of extracellular nucleases, the efficiency in the delivery and uptake of siRNAs or their ability to stimulate the immune system can alter their effect in vivo. Another reason that could explain the difference between the in vitro and in vivo results refers to the uptake of the siRNA by the cells. In this study, there might be differences between the in vitro administration of siRNAs using TransIT-TKO transfection reagent and the "naked" administration of siRNAs in vivo (direct instillation in saline). This is an empirical variable that needs to be tested for each experimental approach. Thus, under our experimental conditions, siRNA#2 seems to be more efficient in vivo for this route of administration and without use of delivery vehicles. In summary, the noted difference between *in vivo* and *in vitro* experiments could be due to several reasons including off target effects of the siRNA. More experiments in this sense should be designed and performed.

A variety of works provide interesting approaches to stop corneal neovascularization. Among them, the use of monoclonal antibodies, mimicking the efforts already done on AMD, provide interesting results. In this sense, the application of Ranibizumab by means of intrastromal and subconjunctival injections significantly reduced corneal neovascularization 6 months after treatment (Ahn et al., 2014; Ferrari et al., 2013). Comparisons between intrastromal and topical administration of Ranibizumab, Bevacizumab and Aflibercept as a therapy for corneal neovascularization, have shown that both application routes are effective although the intrastromal injection produced earlier effects (Al-Debasi et al., 2017).

The use of siRNA to treat the abnormal development of blood vessels has been investigated in the retina by using RNAi against VEGF or even against VEGFR1. The use of this technology has permitted to stop the progress of blood vessel formation. In this sense, choroidal neovascularization where Bruch's membrane was disrupted resulted in a reduced presence of vessels when those areas were treated with siRNA compared to those treated with vehicle (Campochiaro, 2006). These results, although controversial (Maguire et al., 2005), established the potential role of siRNA to treat neovascularization (Tolentino et al., 2004).

Returning to the cornea, some authors have tried to use siRNA against VEGF or its receptors to ameliorate corneal neovascularization. These authors were able to obtain good results when topically applying siRNA against VEGF-A, as well as against VEGFR1 and VEGFR2 (Kim et al., 2004). Similarly, the use of siRNA against VEGF under hypoxia-induced conditions demonstrated a clear reduction in VEGF mRNA synthesis and protein production and a concomitant reduction in corneal neovascularization (Singh et al., 2007). Also, siRNAs against NFκB have been tested, both, instilled alone or within nanoparticles (Han et al., 2016). In these studies, it was possible to observe a significant gene knock-down of angiogenesis together with a good distribution of the siRNA in the affected area. Targets different from the previous ones such as cytochrome CYP4B1 have also been assayed. A siRNA against the mentioned cytochrome was able to reduce corneal neovascularization and this effect was associated with a 75% reduction in corneal VEGF mRNA levels, suggesting a correlation between CYP4B1 and VEGF-induced vascularization (Seta et al., 2007). The use of siRNA against HIF-1alpha may have greater effect on the inhibition of angiogenesis than siRNA against a single use gene such as VEGF, since HIF-1 regulates the expression of several angiogenic genes, including VEGF (Hirota et al., 2006; Pugh and Ratcliffe, 2003).

The main limitation of this study is that no specific experiments to demonstrate that the anti-angiogenic effect is specific of HIF-1α levels in the cornea. The results obtained with siRNA#2, in terms of anti-angiogenic effect, and siRNA control (scrambled), without effect, would indicate that effect is through the HIF-1α. However, it would be interesting to performed future studies to analyse the HIF-1α expression in the cornea after siRNA treatment for neovascularization. In

addition, to evaluate changes of other factors as VEGF-α, nitric oxide synthase (NOS) or Erythropoietin (EPO) could be useful in future experiments.

5. Conclusions

In the present experimental work, three siRNAs against HIF-1α by topically applying them using saline solution as vehicle have been tested. HIF-1 antagonists have been already tested as agents to inhibit blood vessel progression in the eye (Iwase et al., 2013), but to our knowledge this is the first time siRNA against HIF-1α have been tested for corneal neovascularization. In our hands, siRNAs were able to produce 50% of their anti-angiogenic properties within the first 10 days after the treatment, while for other strategies it takes months to see the effect (Ahn et al., 2014). Moreover, we were able to measure 83% of blood vessel inhibition on the ocular surface. Other works silencing VEGF abolished blood vessel generation to 50% (Kim et al., 2004), 73 % (Singh et al., 2007) and 49 % (Zuo et al., 2010). This difference could be due to the fact of selecting a target protein that is upstream in the pathway of synthesis of VEGF, some steps before the growth factor (Adams et al., 2009; Balamurugan, 2016; Bhattarai et al., 2018). Since HIF-1 triggers the expression of VEGF (among other proteins) it seems more effective silencing the transcription factor HIF-1 than its product VEGF, obtaining therefore, similar results than those targeting VEGF directly.

In summary, the topical application of siRNA towards HIF-1α seems to be an effective and reliable method to stop neovascularization. More experiments are necessary to fix the best concentration and the best sequence of application of the siRNA to get faster and more robust anti-angiogenic results.

Disclosure section:
No author has a financial or proprietary interest in any material or method mentioned.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2022.109036.

6. REFERENCES

Adams, J.M., Difazio, L.T., Rolandelli, R.H., Lujan, J.J., Hasko, G., Csoka, B., Selmeczy, Z., Nemeth, Z.H., 2009. HIF-1: a key mediator in hypoxia. Acta Physiol Hung 96, 19-28.

Ahn, Y.J., Hwang, H.B., Chung, S.K., 2014. Ranibizumab injection for corneal neovascularization refractory to bevacizumab treatment. Korean J Ophthalmol 28, 177-180.

Al-Debasi, T., Al-Bekairy, A., Al-Katheri, A., Al Harbi, S., Mansour, M., 2017. Topical versus subconjunctival anti-vascular endothelial growth factor therapy (Bevacizumab, Ranibizumab and Aflibercept) for treatment of corneal neovascularization. Saudi J Ophthalmol 31, 99-105.

Auerbach, R., Lewis, R., Shinners, B., Kubai, L., Akhtar, N., 2003. Angiogenesis assays: a critical overview. Clin Chem 49, 32-40.

Auerbach, W., Auerbach, R., 1994. Angiogenesis inhibition: a review. Pharmacol Ther 63, 265-311.

Bachmann, B., Taylor, R.S., Cursiefen, C., 2010. Corneal neovascularization as a risk factor for graft failure and rejection after keratoplasty: an evidence-based meta-analysis. Ophthalmology 117, 1300-1305 e1307.

Balamurugan, K., 2016. HIF-1 at the crossroads of hypoxia, inflammation, and cancer. Int J Cancer 138, 1058-1066.

Basiri, B., Xie, F., Wu, B., Humphreys, S.C., Lade, J.M., Thayer, M.B., Yamaguchi, P., Florio, M., Rock, B.M., 2020. Introducing an In Vitro Liver Stability Assay Capable of Predicting the In Vivo Pharmacodynamic Efficacy of siRNAs for IVIVC. Mol Ther Nucleic Acids 21, 725-736.

Bhattarai, D., Xu, X., Lee, K., 2018. Hypoxia-inducible factor-1 (HIF-1) inhibitors from the last decade (2007 to 2016): A "structure-activity relationship" perspective. Med Res Rev 38, 1404-1442.

Campochiaro, P.A., 2006. Potential applications for RNAi to probe pathogenesis and develop new treatments for ocular disorders. Gene Ther 13, 559-562.

Chan, A.T., Ulate, R., Goldich, Y., Rootman, D.S., Chan, C.C., 2015. Terrien Marginal Degeneration: Clinical Characteristics and Outcomes. Am J Ophthalmol 160, 867-872 e861.

Chang, J.H., Gabison, E.E., Kato, T., Azar, D.T., 2001. Corneal neovascularization. Curr Opin Ophthalmol 12, 242-249.

Chappelow, A.V., Kaiser, P.K., 2008. Neovascular age-related macular degeneration: potential therapies. Drugs 68, 1029-1036.

Chen, P., Yin, H., Wang, Y., Wang, Y., Xie, L., 2012. Inhibition of VEGF expression and corneal neovascularization by shRNA targeting HIF-1alpha in a mouse model of closed eye contact lens wear. Mol Vis 18, 864-873.

Edelhauser, H.F., Ubels, J.L., 2004. Cornea and Sclerotica, in Kaufman PL, Alm A (eds): Adler's Physiology of the Eye. Mosby Inc. and Elsevier, pp 47-114.

Epstein, R.J., Stulting, R.D., Hendricks, R.L., Harris, D.M., 1987. Corneal neovascularization. Pathogenesis and inhibition. Cornea 6, 250-257.

Ferrari, G., Dastjerdi, M.H., Okanobo, A., Cheng, S.F., Amparo, F., Nallasamy, N., Dana, R., 2013. Topical ranibizumab as a treatment of corneal neovascularization. Cornea 32, 992-997.

Folkman, J., 1995. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1, 27-31.

Fu, Y.C., Xin, Z.M., 2019. Inhibited corneal neovascularization in rabbits following corneal alkali burn by double-target interference for VEGF and HIF-1alpha. Biosci Rep 39.

Guillon, M., Bilton, S., Bleshoy, H., 1985. Limbal changes associated with hydrogelcontact lens wear. J Br Contact Lens Assoc 8, 15-19.

Gupta, D., Illingworth, C., 2011. Treatments for corneal neovascularization: a review. Cornea 30, 927-938.

Han, H., Son, S., Son, S., Kim, N., Yhee, J.Y., Lee, J.H., Choi, J.S., Joo, C.K., Lee, H., Lee, D., Kim, W.J., Kim, S.H., Kwon, I.C., Kim, H., Kim, K., 2016. Reducible Polyethylenimine Nanoparticles for Efficient siRNA Delivery in Corneal Neovascularization Therapy. Macromol Biosci 16, 1583-1597.

Hirota, E., Yan, L., Tsunoda, T., Ashida, S., Fujime, M., Shuin, T., Miki, T., Nakamura, Y., Katagiri, T., 2006. Genome-wide gene expression profiles of clear cell renal cell carcinoma: identification of molecular targets for treatment of renal cell carcinoma. Int J Oncol 29, 799-827.

Holden, B.A., Sweeney, D.F., Swarbrick, H.A., 1986. The vascular response to long-term extended contact lens wear. Clin Exp Optom 69, 112-196.

Huang, Y.F., Yang, C.H., Huang, C.C., Tai, M.H., Hsu, K.S., 2010. Pharmacological and genetic accumulation of hypoxia-inducible factor-1alpha enhances excitatory synaptic transmission in hippocampal neurons through the production of vascular endothelial growth factor. J Neurosci 30, 6080-6093.

Iwase, T., Fu, J., Yoshida, T., Muramatsu, D., Miki, A., Hashida, N., Lu, L., Oveson, B., Lima e Silva, R., Seidel, C., Yang, M., Connelly, S., Shen, J., Han, B., Wu, M., Semenza, G.L., Hanes, J., Campochiaro, P.A., 2013. Sustained delivery of a HIF-1 antagonist for ocular neovascularization. J Control Release 172, 625-633.

Jansson-Löfmark, R., Hjorth, S., Gabrielsson, J., 2020. Does In Vitro Potency Predict Clinically Efficacious Concentrations? Clin Pharmacol Ther 108, 298-305.

Kaiser, P.K., Symons, R.C., Shah, S.M., Quinlan, E.J., Tabandeh, H., Do, D.V., Reisen, G., Lockridge, J.A., Short, B., Guerciolini, R., Nguyen, Q.D., Sirna-027 Study, I., 2010. RNAi-based treatment for neovascular age-related macular degeneration by Sirna-027. Am J Ophthalmol 150, 33-39 e32.

Kather, J.N., Kroll, J., 2014. Transgenic mouse models of corneal neovascularization: new perspectives for angiogenesis research. Invest Ophthalmol Vis Sci 55, 7637-7651.

Kim, B., Tang, Q., Biswas, P.S., Xu, J., Schiffelers, R.M., Xie, F.Y., Ansari, A.M., Scaria, P.V., Woodle, M.C., Lu, P., Rouse, B.T., 2004. Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial growth factor pathway genes: therapeutic strategy for herpetic stromal keratitis. Am J Pathol 165, 2177-2185.

Krock, B.L., Skuli, N., Simon, M.C., 2011. Hypoxia-induced angiogenesis: good and evil. Genes Cancer 2, 1117-1133.

Lee, P., Wang, C.C., Adamis, A.P., 1998. Ocular neovascularization: an epidemiologic review. Surv Ophthalmol 43, 245-269.

Liesegang, T.J., 2001. Herpes simplex virus epidemiology and ocular importance. Cornea 20, 1-13.

Lin, A., Patel, N., Yoo, D., DeMartelaere, S., Bouchard, C., 2011. Management of ocular conditions in the burn unit: thermal and chemical burns and Stevens-Johnson syndrome/toxic epidermal necrolysis. J Burn Care Res 32, 547-560.

Maguire, M.G., Fine, S.L., Ying, G.S., 2005. Intravitreal injection of VEGF siRNA. Retina 25, 101-102.

Manire, G.P., 1997. Biological characteristics of Chlamydiae, in Hobson D, Homes KK: Nongonococcal Urethritis and Related Infection. . American Society for Microbiology, 67-175.

Martinez, T., Gonzalez, M.V., Roehl, I., Wright, N., Paneda, C., Jimenez, A.I., 2014. In vitro and in vivo efficacy of SYL040012, a novel siRNA compound for treatment of glaucoma. Mol Ther 22, 81-91.

Mendicute, J., Aldazabal, P., Saornil Mª, A., Emparanza, J., Ostolaza, J., Durán, J., 1997. Diseño de un modelo experimental de causticación corneal con compromiso límbico (grado III, Thof). Arch. Sociedad española de Oftalmología 72, 49-58.

Nguyen, Q.D., Schachar, R.A., Nduaka, C.I., Sperling, M., Basile, A.S., Klamerus, K.J., Chi-Burris, K., Yan, E., Paggiarino, D.A., Rosenblatt, I., Khan, A., Aitchison, R., Erlich, S.S., Group, P.F.S., 2012a. Phase 1 dose-escalation study of a siRNA targeting the RTP801 gene in age-related macular degeneration patients. Eye (Lond) 26, 1099-1105.

Nguyen, Q.D., Schachar, R.A., Nduaka, C.I., Sperling, M., Klamerus, K.J., Chi-Burris, K., Yan, E., Paggiarino, D.A., Rosenblatt, I., Aitchison, R., Erlich, S.S., Group, M.C.S., 2012b. Evaluation of the siRNA PF-04523655 versus ranibizumab for the treatment of neovascular age-related macular degeneration (MONET Study). Ophthalmology 119, 1867-1873.

Otsu, N., 1979. A Threshold Selection Method from Gray-Level Histograms. IEEE Transactions on Systems, Man, and Cybernetics 9, 62-66.

Papas, E., 1998. On the Relationship Between Soft Contact Lens Oxygen Transmissibility and Induced Limbal Hyperaemia. Experimental Eye Research 67, 125-131.

Papas, E.B., 2003. The role of hypoxia in the limbal vascular response to soft contact lens wear. Eye Contact Lens 29, S72-74; discussion S83-74, S192-194.

Pugh, C.W., Ratcliffe, P.J., 2003. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 9, 677-684.

Seta, F., Patil, K., Bellner, L., Mezentsev, A., Kemp, R., Dunn, M.W., Schwartzman, M.L., 2007. Inhibition of VEGF expression and corneal neovascularization by siRNA targeting cytochrome P450 4B1. Prostaglandins Other Lipid Mediat 84, 116-127.

Singh, N., Higgins, E., Amin, S., Jani, P., Richter, E., Patel, A., Kaur, R., Wang, J., Ambati, J., Dong, Z., Ambati, B.K., 2007. Unique homologous siRNA blocks hypoxia-induced VEGF upregulation in human corneal cells and inhibits and regresses murine corneal neovascularization. Cornea 26, 65-72.

Supe, S., Upadhya, A., Singh, K., 2021. Role of small interfering RNA (siRNA) in targeting ocular neovascularization: A review. Exp Eye Res 202, 108329.

Tabbara, K.F., Ross-Degnan, D., 1986. Blindness in Saudi Arabia. JAMA 255, 3378-3384.

Taylor, H.R., M., D.E., 1996. Ocular onchocerciasis, in Tabbara KF, Hyndiuk RA: Infections of the eye. Little, Brown and Co, 673-683.

Tolentino, M.J., Brucker, A.J., Fosnot, J., Ying, G.S., Wu, I.H., Malik, G., Wan, S., Reich, S.J., 2004. Intravitreal injection of vascular endothelial growth factor small interfering RNA inhibits growth and leakage in a nonhuman primate, laser-induced model of choroidal neovascularization. Retina 24, 660.

Zuo, L., Fan, Y., Wang, F., Gu, Q., Xu, X., 2010. A siRNA targeting vascular endothelial growth factor-A inhibiting experimental corneal neovascularization. Curr Eye Res 35, 375-384.

Figures

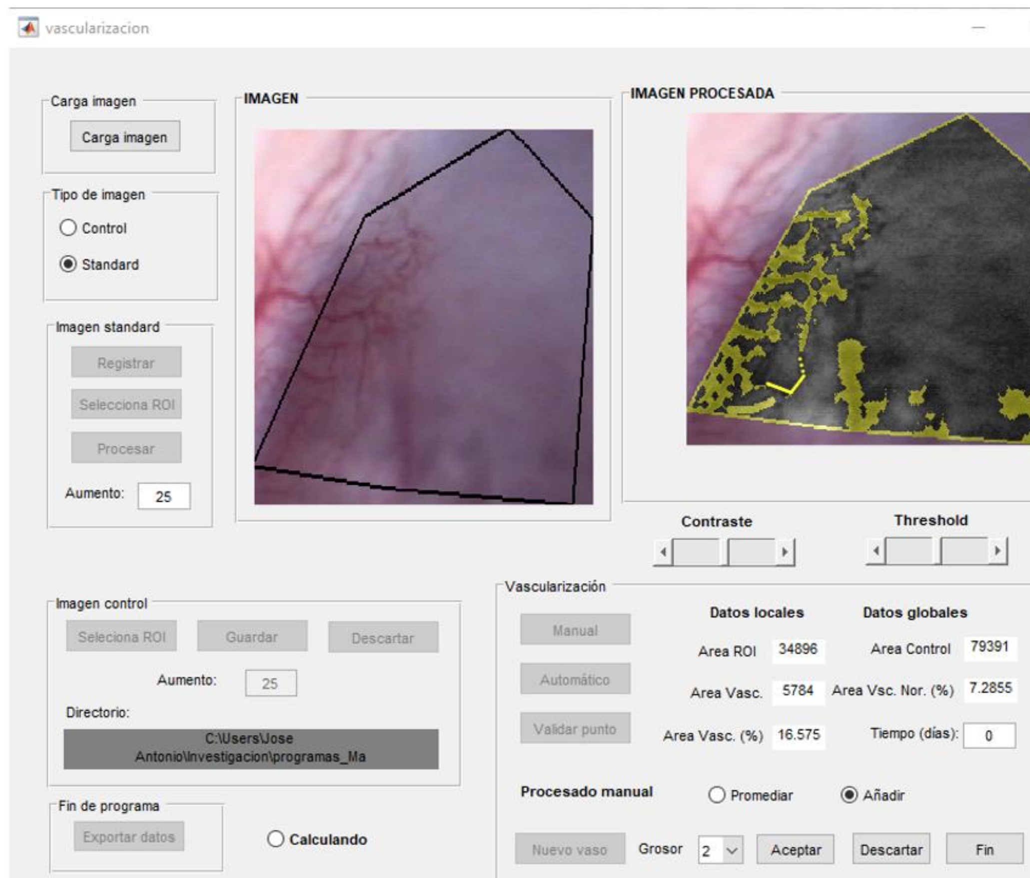

Fig. 1. Capture of the vascularization program GUI (Guided User Interface). Up at the right it can be seen the high contrast image (processed image) with the detected vascularization area (gold) superposed. A yellow line indicating the trajectory of an undetected vessel which is being manually added by the user, can be also seen. Down at the right, it can be seen two groups of data. Local data where a ROI, to account for the total area where the blood vessels grow, the vascularization area and the percentage of neovascularization are shown. Global date where it can be seen the control area (area of the injury defined for the control image) and the normalized vascularization area. (For interpretation of

the references to colour in this figure legend, the reader is referred to the Web version of this article.)

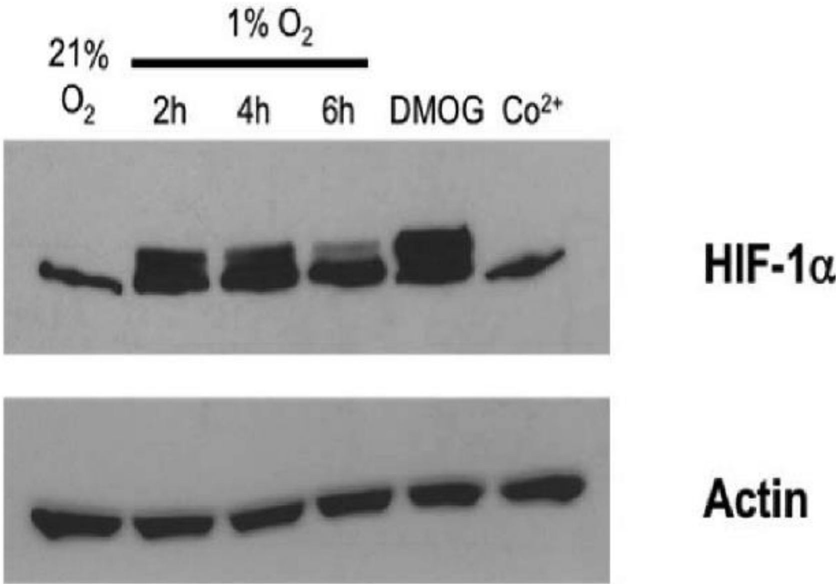

Fig. 2A. Expression level of HIF-1ɑ _in normoxia conditions (21% O2) and in hypoxia conditions (1% O2) after 2, 4 and 6 h of hypoxia are shown in Fig. 2A. Cells were incubated for 6 h with two hypoxia mimetic compounds, dimethyloxalylglycine (DMOG, 1 mM) and cobalt chloride (Co2+, 100 μM).

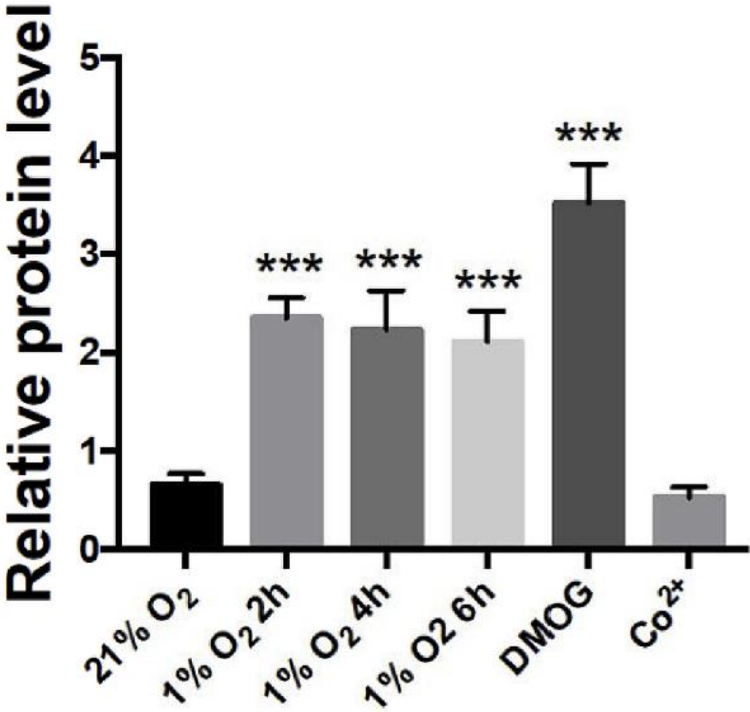

Fig. 2B. Data of the relative protein level of Fig. 2A, represented in a bar graph.

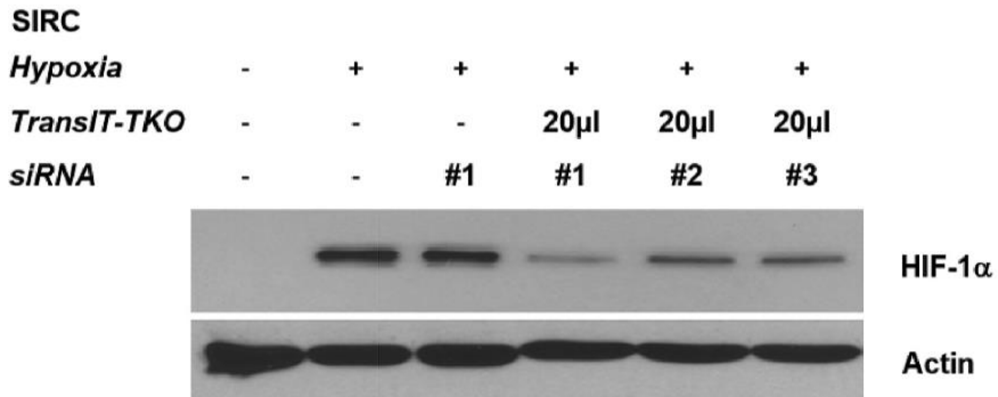

Fig. 3A. Effect of three siRNAs #1, #2 and #3 against HIF-1α _after 4 h of hypoxia in SIRC cell line.

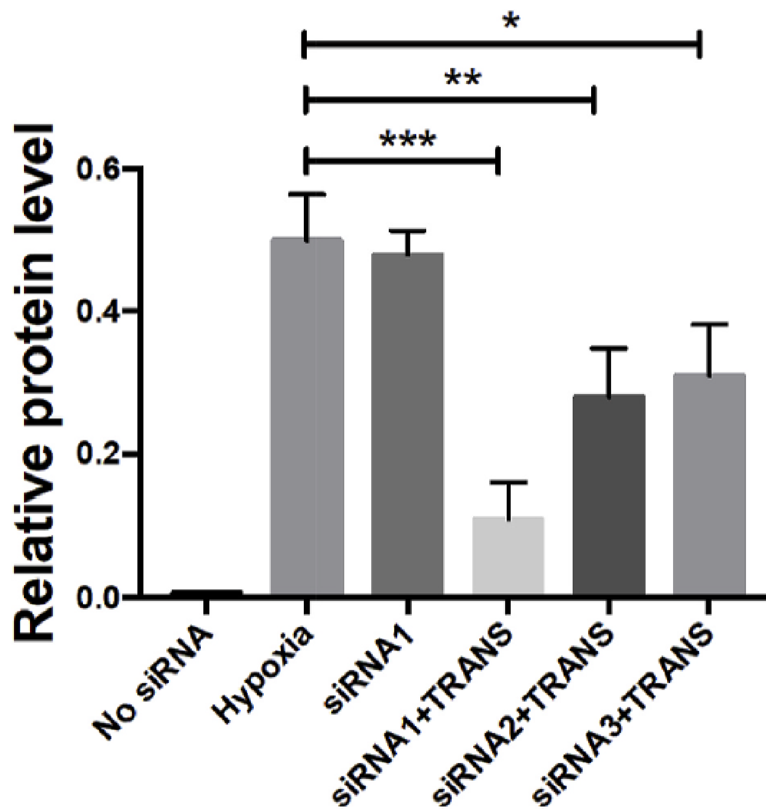

Fig. 3B. Data of the relative protein level of Fig. 3A, represented in a bar graph.

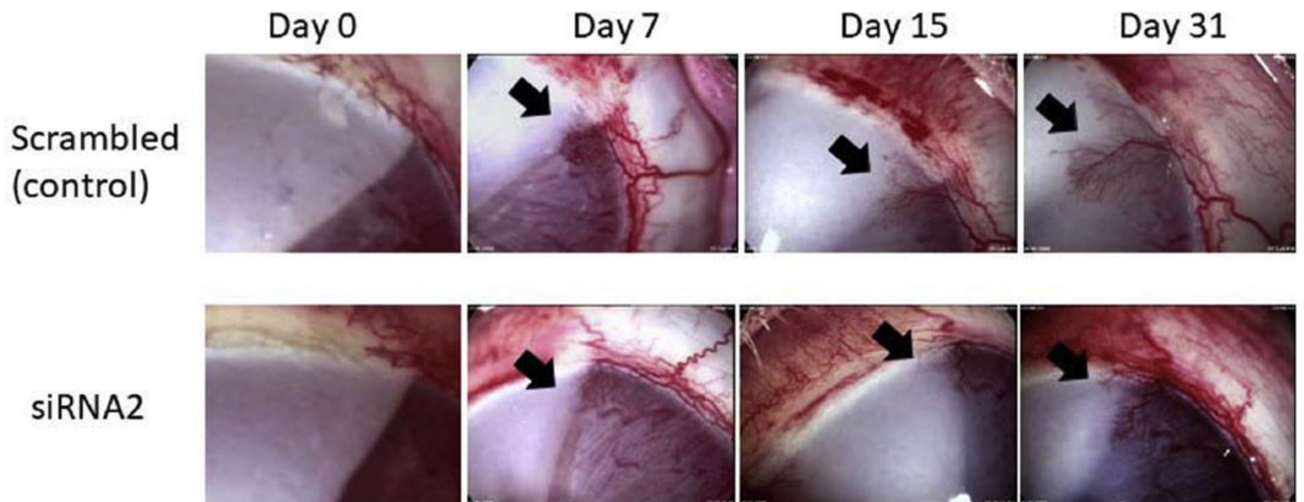

Fig. 4A. Experiments in vivo with a scramble siRNA (control) and siRNA#2 at day 0, 7, 15 and 31.

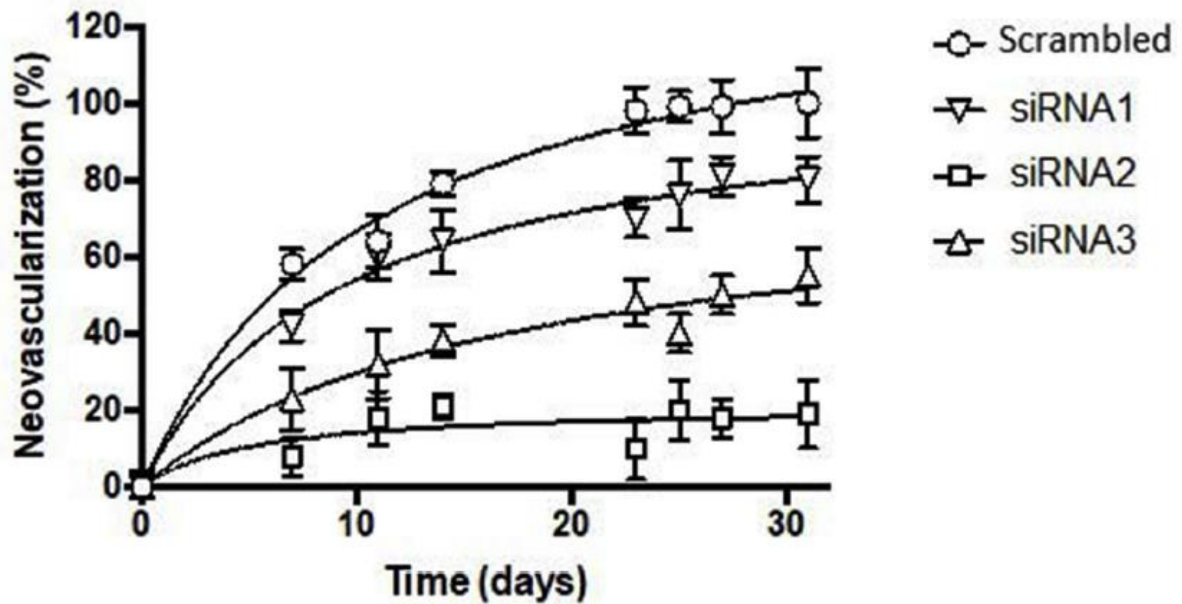

Fig. 4B. Percentage of neovascularization (NV) along time. N = 6 for each siRNAs and the experiment was replicated 3 times.

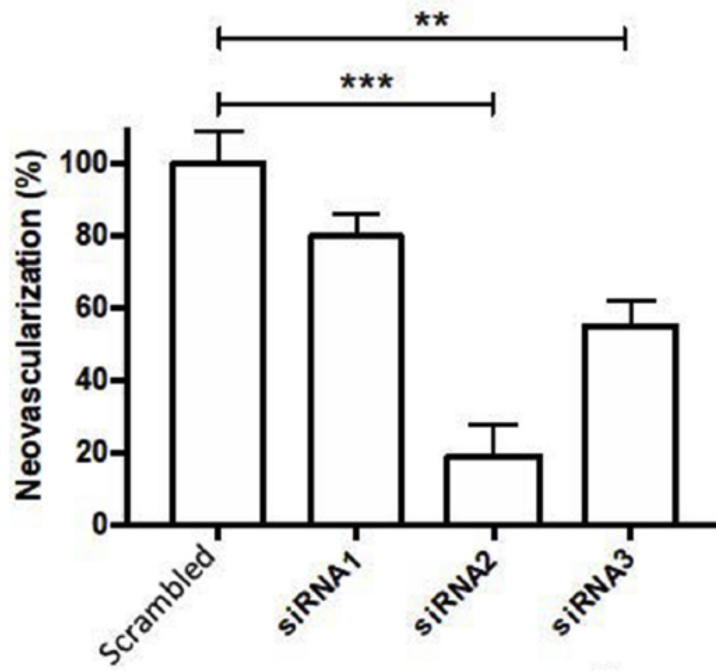

Fig. 4C. Bar graph over the effect of siRNAs in neovascularization.