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VEGF-induced corneal neovascularisation in a rabbit experimental model

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Abstract

Introduction and Purpose: Various conditions may cause vascularization of the normally avascular cornea. The aim of the present study was to create a reproducible experimental model that could enable the investigation of the phenomena leading to corneal vascularization. This involved creating a software to record the experimental data, enabling a subsequent digital analysis based on the growth models. The VEGF-induced pattern of neovascularization was also investigated. *Material and Methods*: Twenty-seven rabbits divided in groups were used for the purposes of the present study. Some of them underwent intracorneal implants with or without vascular endothelial growth factor (VEGF) pellets, using an original microsurgical technique. Central and peripheral corneal burns were induced to other groups of animals in order to mimic the neovascularization process induced by inflammation. Finally, Dexamethasone (Maxidex) was given intraocularly, on days 1 and 3 after the onset of neovascularisation, in rabbit groups with both corneal burns and VEGF-implants. Video recording and data analysis of the corneal vascularization were made with an advanced biomicroscope, a computerized imaging system and a special software. A histochemical study of the animals' eyes was also carried out. *Results and Discussion*: The recorded data showed the simplicity and reproducibility of the present experimental model. The results showed the importance of VEGF as an initiator and promoter of corneal vascularization through a non-inflammatory mechanism, quite different from the inflammation illustrated by the corneal burn. At the same time, Dexamethasone therapy proved its effectiveness in corneal angiogenesis induced by thermal burn, but not by VEGF-implant.

***Keywords*:** corneal vascularization, VEGF, corneal burn, corneal inflammatory process.

Introduction

The issue of angiogenesis is relevant not only in physiology, but also in the physiopathology of many illnesses. The genesis of neovessels is crucial in normal growth and development of any tissue. This process is particularly significant in the setting of tumor growth and metastasis, of chronic inflammatory and/or metabolic diseases. During adulthood, this vascularization is important for wound healing and reproduction. The development of the angiogenetic phenotype is a complex process, involving the ability of normal or tumoral cells to secrete angiogenetic factors [1]. Angiogenesis is practically a synonym to vascular sprouting. The sprouting of new vessel segments follows a well-defined program: degradation of basement membrane, endothelial cell proliferation, formation of solid sprouts of endothelial cells connecting a neighboring vessel, and restructuring of the sprout into a lumen lined by endothelial cells and integrated in the vascular network. [2]. This process results in the apparition of intact neovessels, with a permeable channel. It is controlled by a large number of molecules, classified as angiogenic and angiostatic [3]. The angiogenic and angiostatic factors are ennumerated elsewhere (Chang JH *et al.*, 2001) [4]. Angiogenic agents include various growth factors and cytokines, such as b-FGF, VEGF, IL-8, selectine E. Angiostatic molecules, such as thrombospodine, angiostatine, and platelet factor 4, hold the same significance [4–7].

The term neovascularization might be used to describe the installation of new vascular structures in previously avascular areas.

Within the eye, neovessels are directly responsible for most of the destructive events that are characteristic for certain diseases, such as proliferative retinopathies and age-induced macular degeneration [8–12].

Several laboratories have tried to explain the main role of VEGF as regulator of normal and abnormal angiogenesis [13–16]. Complete and detailed information on the biologic and clinical implications of VEGF are given in the reviews published by Ferrara N *et al.* [14, 17, 18].

VEGF (also named VEGF-A) belongs to a gene family that includes placental growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D.

VEGF-A binds to VEGFR1 and VEGFR2, while placental growth factor (PlGF) and VEGF-B binds only to VEGFR1. VEGF-C and VEGF-D bind to VEGFR2 and VEGFR3 [18]. VEGFR1, VEGFR2 and VEGFR3 are receptor tyrosine kinases.

VEGFs are released as growth factor peptides generated by alternative splicing in five isoforms in humans (VEGF115, VEGF121, VEGF165, VEGF189, and VEGF206) [4].

During embryogenesis, loss of a single allele may result in embryonic death. This proves the essential role of VEGF in the differentiation and proliferation of the normal vascular system. On the other hand, it was experimentally proven that VEGF-induced angiogenesis might show therapeutic benefits in animal models with coronary or leg ischemia, and even in one patient with severe leg ischemia [19–21].

VEGF is released by macrophages, T-cells, retinal pigmentary epithelial cells, astrocytes, and smooth muscle cells. Induction of VEGF has been shown in hypoxic and inflammatory settings. Additionally, VEGF promotes several steps of angiogenesis, including proteolysis (dissolution of the membrane of the original vessel), endothelial cell proliferation, migration, and capillary tube formation [4].

Corneal vascularization

The normal absence of vessels within the cornea is explained by several facts: the compact structure of the stroma, which the vessels cannot penetrate (proven by the presence of vessels within the edematous cornea), and/or the abundance of mucopolysaccharides (MPZ) that, together with proteins and water, form a barrier against the penetration of vessels.

Abnormal corneal vascularization is the major expression of corneal pathology [22]. Even if vascularization plays a role in early stages of corneal repair, its persistence becomes pathological. Local injury is the most common trigger. Small local mechanical and chemical injuries result in apparition of neovessels. Extensive injury results in generalized stromal edema with circumferential vascular invasion. Other triggers of corneal vascularization include infections such as trachoma or herpes simplex with keratitis. In some cases, it may complicate corneal transplant. In diseases of superficial cornea, blood vessels come from the limbic areas that cross the sclerocornean limbus from the conjunctiva, whereas in diseases of the stroma, neovessels come from depth, i.e. from the anastomosis of deep arteries.

Several factors influence neocapillary growth. Diffusible mediators, able to initiate directional capillary growth, are found in many normal and disease tissues. Several authors have emphasized the existence of such factors in the vascularized cornea vs. the normal one. Furthermore, competition for oxygen and nutriments between certain cell groups, together with the accumulation of large amounts of metabolites, may stimulate the release of growth factors capable of inducing directional vascular growth, with the purpose of correcting these imbalances. Given that the corneal stroma is relatively acellular, the question is whether the cornea could acquire a high cellularity, which might result in neovessels. Recent research has emphasized the fact that leukocytes could play a crucial role in this process. Thus, several experiments have showed that corneal vascularization is invariably accompanied by an inflammatory infiltrate in the corneal stroma.

Several factors are involved in the onset of neovascularization. One of them is the release of embryonary substances. One hypothesis claims that corneal lesions release an embryonary agent that diffuses into the limbus, where it gives birth to a vascular tree. Another factor is hypoxia, resulting in the accumulation of vasoforming metabolites, balanced however by the stromal density, which inhibits corneal vascularization, in normal circumstances. Edema causes a decrease in the stromal density, favoring the apparition of neovessels. With the development of neovascularization, the oxygen supply increases, suppresses the neoforming stimuli, and the new vessels do not persist. The accumulation of lactic and pyruvic acid also represents a noxious stimulus for the apparition of neo-vessels.

The consequences of corneal vascularization are equally important [23]. In early stages, it may be seen as a defense mechanism, because it increases the supply of oxygen and metabolites, activating the metabolic exchanges required for tissue repair. The metabolism of the vascular cornea is similar to that of the normally avascular cornea. Vascularization brings with itself immune elements such as phagocytes, immunocompetent cells, and antibodies. The intense blood flow in this tissue is followed by growth of the specific intercellular signal density in the cornea. This triggers a sequence of cascade events, which support and increase corneal angiogenesis. Several studies have analyzed the evolution of corneal hemangiogenesis or lymphangiogenesis [24, 25].

The corneal angiogenesis assay is still considered one of the best in vivo assays for the formation of new blood vessels [26]. Any vessels seen within the cornea after stimulation by angiogenesis-inducing tissues or factors are new, because the cornea is normally avascular. The original method was developed on rabbit eyes [27], but has since been adapted to mice [28]. It involves creation of a corneal pocket, where test tumors or tissues are introduced, eliciting the growth of new vessels from the peripheral limbic vasculature.

VEGF can induce corneal angiogenesis *in vivo* (e.g., in the rabbit cornea [29, 30], or in mice cornea [31]).

It was shown that vascular endothelial growth factor (VEGF) was up-regulated in inflamed and vascularized cornea, both in humans and in animal models [29, 32, 33].

In one model of inflammatory neovascularization in mice (suture-induced inflammatory corneal neovascularization), VEGF-A recruitment of monocytes/macrophages might play a crucial role in inducing inflammatory neovascularization [31].

Interestingly, the essential role of VEGF in corneal angiogenesis was shown by the inhibition of neovascularization after stromal implantation of anti-VEGF blocking antibody or peptides in rat and rabbit corneal models [4].

New medical and surgical treatments, including angiostatic steroids, non-steroidal inflammatory agents, anti-VEGF agents, argon laser photocoagulation, and photodynamic therapy, have proven effective in inhibiting corneal neovascularization in animal models [4, 34, 35].

Material and Methods

The vascular endothelial growth factor (VEGF) was prepared in Toulouse (France), in the Laboratory of Molecular Biology of Eukaryotes, the angiogenesis focus group. We used isoform $VEGF_{165}$ (with 165 amino acids), originally prepared by genetic recombination on a baculovirus and then purified. Afterwards, it was dialyzed and lyophilized in water. It was transported in plastic micro-recipients, each containing 5 μg VEGF. It could be used with any buffer solution. In order to be implanted in the cornea, VEGF had to be brought in a semisolid state, enabling surgical implantation and gradual release of the factor. Pellets (implants) with VEGF and other conditioning elements were prepared. They were manufactured by the aid of a slow-release polymer (Hydron or poly-Hema), and contain either VEGF, Sucralfate, or both. Approx. 100 pellets were prepared out of two dosages of 5 μg VEGF. Only similarly sized pellets were selected under microscope in order to be implanted. Therefore, each pellet contained either about 100 ng of VEGF, or double. The technical details of pellet preparation are available upon request from the corresponding authors.

Implantation of the pellets

The rabbits were anaesthetized with 5% ketamine hydrochloride (Calypsol) 15 mg/kbw, and injected intramuscularly in the hind leg. After 20 minutes, 20% chloralhydrate 0.25 mg/kbw was intraperitoneally injected. A local anesthetic, 2% lydocaine or 4% cocaine, was instilled intraocularly. A binocular surgical microscope (Carl Zeiss) and a surgical table with a rotative stand were used. The animals were placed in a contention device designed by us. The pellet implantation procedure started with a linear intrastromal incision, parallel to the corneoscleral limbus (linear keratotomy), using a surgical blade (Bard–Parker). The preparation of the corneal pocket for the pellet implant was made with a Von Graefe knife with small edge and 45^0-angle blade. The implant was introduced through the keratotomy line, parallel to the corneal epithelium and under it, in the external third of the stroma, up to 0.5 mm from the limbus. One single pellet was extracted from the Petri plaque and then introduced in the corneal pocket, close to the corneoscleral limbus, at the base of the pocket, with the knife blade. Finally, an antibiotic solution was instilled, in order to prevent suprainfection and reduce irritation.

Animals

Twenty-seven healthy rabbits, 16 males and 11 females, weighing between 2.2 and 2.4 kg, were used. They were divided into several study groups, four with six rabbits, and one with three rabbits. Pellets with or without VEGF were implanted in the right or left eyes of the rabbits. In certain cases, central corneal burns were made, and/or Maxidex (Dexamethasone 0.1%) was given intraocularly (two drops in each conjunctival sac, four times daily).

Pellets were implanted as follows: Hydron and Sucralfate, Hydron and VEGF, Hydron with Sucralfate and VEGF (100 ng or 200 ng). Peripheral corneal burns were carried out in six animals, and central corneal burns were performed in other six, respectively. The detailed description of each rabbit eye treatment is shown in Table 1. All treatments were performed with the approval of the University Animal Experimentation Ethics Board, as per the requirements of the Romanian National Law on the Use of Experimental Animals.

Table 1 – ***Classification of study animals by groups and specific treatment***

No.	Right eye	Left eye
1.	VEGF (100 ng)	Central burn
2.	VEGF (100 ng)	Central burn
3.	VEGF (100 ng)	Central burn
4.	VEGF (100 ng)	Central burn
5.	VEGF (100 ng)	Central burn
6.	VEGF (100 ng)	Central burn
7.	Peripheral burn	Peripheral burn + Maxidex
8.	Peripheral burn	Peripheral burn + Maxidex
9.	Peripheral burn	Peripheral burn + Maxidex
10.	Peripheral burn	Peripheral burn + Maxidex
11.	Peripheral burn	Peripheral burn + Maxidex
12.	Peripheral burn	Peripheral burn + Maxidex
13.	VEGF (200 ng) + Sucralfate	VEGF (200 ng) + Maxidex from first day
14.	VEGF (200 ng) + Sucralfate	VEGF (200 ng) + Maxidex from first day
15.	VEGF (200 ng) + Sucralfate	VEGF (200 ng) + Maxidex from first day
16.	VEGF (200 ng) + Sucralfate	VEGF (200 ng) + Maxidex from first day
17.	VEGF (200 ng) + Sucralfate	VEGF (200 ng) + Maxidex from first day
18.	VEGF (200 ng) + Sucralfate	VEGF (200 ng) + Maxidex from first day
19.	VEGF (100 ng) + Sucralfate	VEGF (200 ng) + Maxidex from three days
20.	VEGF (100 ng) + Sucralfate	VEGF (200 ng) + Maxidex from three days
21.	VEGF (100 ng) + Sucralfate	VEGF (200 ng) + Maxidex from three days
22.	VEGF (100 ng) + Sucralfate	VEGF (200 ng) + Maxidex from three days
23.	VEGF (100 ng) + Sucralfate	VEGF (200 ng) + Maxidex from three days
24.	VEGF (100 ng) + Sucralfate	VEGF (200 ng) + Maxidex from three days
25.	Hydron + Sucralfate	Hydron + VEGF (100 ng) alone
26.	Hydron + Sucralfate	Hydron + VEGF (100 ng) alone
27.	Hydron + Sucralfate	Hydron + VEGF (100 ng) alone

Follow-up

After the pellets were implanted, all study animals were followed-up. They were first anaesthetized with chloral-hydrate intraperitoneally, and after 20 minutes each animal eye was examined with the slit-light biomicroscope after exposure in proptose, with the aid of a blepharostate. Before the examination, one drop of Atropine sulphate 1% was instilled in each eye, and Chloramphenicol 5% ophthalmic ointment was applied afterwards. The clinical evolution of the implants and of the ocular lesions was recorded by the presence of corneal reactions, such as corneal edema, the intensity of the corneal cellular infiltrate, the total area of neovascularization.

The computerized system of image recording, processing, and analysis

Each day the animals underwent anesthesia, and the anterior ocular pole images were computer analyzed. An advanced video camera (Sony 450X) connected to a color video monitor and a computer with video-bluster and special capture software were used to record the anterior ocular pole images. In order to extract the vascular tree from every image, some graphic processing was required:

- adjustment of contrast and brightness, in order to highlight the vascular tree;
- image conversion in a gray scale format (8 bytes for a pixel);
- image extraction of the vascular tree (skeletonisation).

Corel Photo Paint and Corel Draw software were used for these purposes. Manual skeletonisation was used, because Corel Trace caused too many errors. The trajectory of the vascular tree was traced over the processed BMP (Bit Map) image, finally only retaining the image corresponding to the corneal vessels. A special software was also created, processing the skeletonisated image to characterize it (length, width, number of pixels), and generating a data folder that contained these coordinates and the number of pairs.

The histopathological examination

At certain time, intervals the animals were sacrificed by the intraperitoneal injection of a toxic dose of chloral-hydrate associated with ether inhalation. Shortly after death, the ocular globes were enucleated, in order to prevent as much as possible the onset of postmortem structural damage. The second step was histomorphological fixation, in order to stop the vital tissular phenomena and capture certain changes present within the tissue at prelevation. The final step was the inclusion in paraffin blocks, solidified in ice. These blocks were cut, with a microtome, into serial sections of 5 μm each. These sections were then placed on albumin–glycerin lubrified slides. Hematoxylin–Eosin and Van Gieson stains were performed. The sections were placed, using Canada balm, between slides, resulting in a large number of samples. The permanent microscope samples were examined with a DOCUVAL (Carl Zeiss Jena) microscope, and color microphotographs were taken.

Results

At the biomicroscopic examination, healing of the epithelium was seen 24 hours after the intervention. Moderate corneal edema was also seen. The pellets containing either Sucralfate alone or VEGF (100 ng) alone did not result in neovascularization (n=6 eyes). On day 3, in the eyes implanted with the combination VEGF (200 ng) and Sucralfate, the limbic vessels advanced to the cornea. In the previous days, we noticed that the normal limbic vessels (branches of the long ciliary arteries) dilated, with minor congestion of the corneo-scleral area corresponding to the corneal implant. In the following two days, the neovessels front advanced to the pellet, reaching on the fifth postoperative day the length (VL) of 1.19 mm and the surface (VS) of 16.89 mm^2. On day 6, these neovessels invaded the pellet (VL=1.50 mm, VS=18.29 mm^2). Starting with day 7, the neovascular reaction stabilized (Figure 1). This neovascular reaction was intense, well localized and reproducible. The eyes implanted with pellets containing a smaller dosage of VEGF (100 ng) showed significantly lower neovascular reactions (Figure 2). Moreover, the VEGF (200 ng) pellet induced, starting with day 7, a slight stromal oedema and tissular hemorrhage, phenomena that were absent at the lower dose of VEGF (100 ng).

The microscopic examination was carried out on a large number of tissue sections. These sections were carefully directed in order to include the most representative aspects of the phenomena. The Hematoxylin–Eosin and Van Gieson stains showed different elements supporting positive and differential diagnosis.

Daily observation, at the same hours, of the effects induced by thermal injury, showed that any small focal corneal injury resulted in a tendency of the blood vessels to grow towards the lesion. Vessels came from the periphery of the cornea and occupied an area similar to an isosceles triangle, with the apex at the lesion and the base at the corneoscleral limbus.

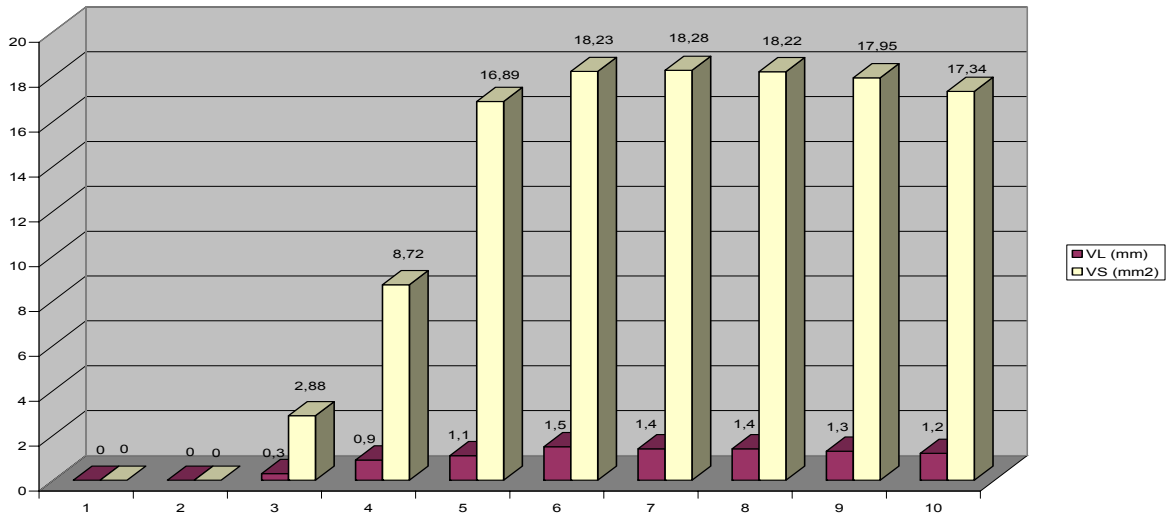

Figure 1 – ***Evolution of vascular length (VL) and surface (VS) in eyes with VEGF (200 ng) and Sucralfate implants.***

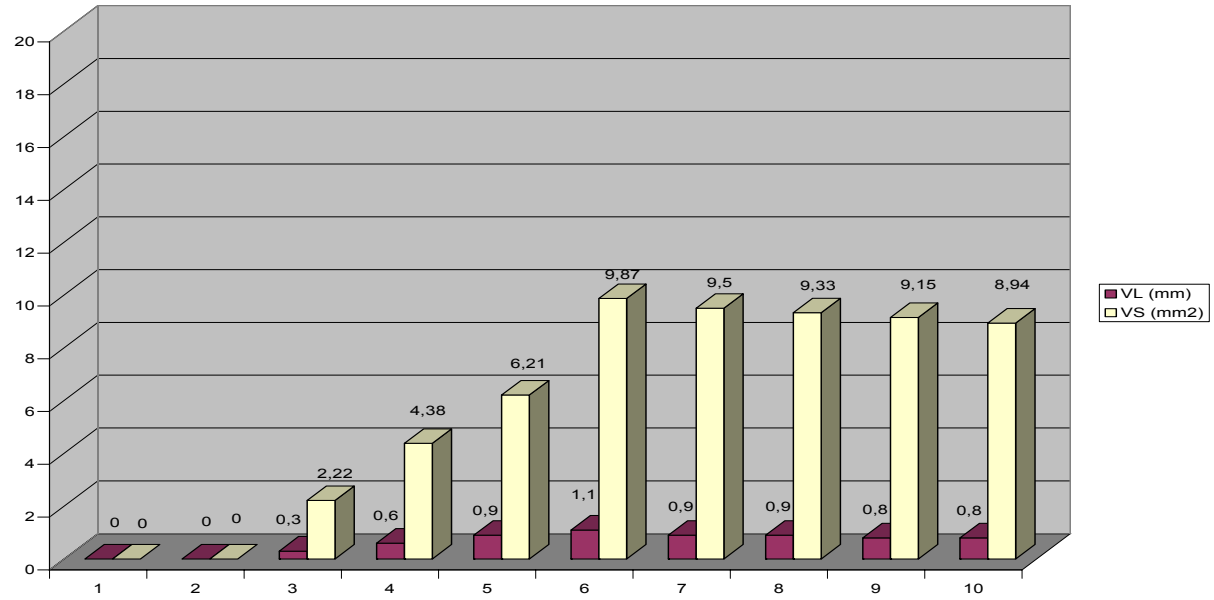

Figure 2 – ***Evolution of vascular length (VL) and surface (VS) in eyes with VEGF (100 ng) and Sucralfate implants.***

We also noticed that the onset of corneal neovascularization depended on the proximity of the lesion to the corneoscleral limbus. In the eyes where the thermal burn was induced in the center of the cornea, corneal neovascularization did not occur. In those eyes where burns were induced in the same circumstances, but near the corneoscleral limbus, a rich network of neovessels appeared rapidly, from the first day after injury (Figure 3).

Furthermore, we tried to investigate the relationship between the onset of inflammation and corneal angiogenesis.

In eyes where we performed peripheral corneal cauterization, a rich inflammatory infiltrate with numerous leukocytes and interstitial clinical and histological edema was seen, starting with the first hours after injury, in the neighborhood of the corneal lesion. In eyes where we performed central corneal cauterization, far from the corneoscleral limbus, these phenomena did not occur.

We have also recorded a significant correlation between the onset and intensity of angiogenesis and the onset of an inflammatory infiltrate. Moreover, treatment with Maxidex (a well-known anti-inflammatory steroid) showed a significant effect only in eyes with peripheral corneal burn (Figure 4). It became certain that, if the cells have not penetrated the corneal stroma, this remains avascular.

After the VEGF implants, neovessels appeared and grew progressively from day 3 to day 6, and stabilized thereafter. The intensity of the neovascular reaction depended on the VEGF dosage of the pellet.

The administration of Maxidex after the VEGF implant showed no effect on neovascularization, which developed with approximately the same intensity, whether the eyes had been treated with Maxidex or not (Figures 5 and 6).

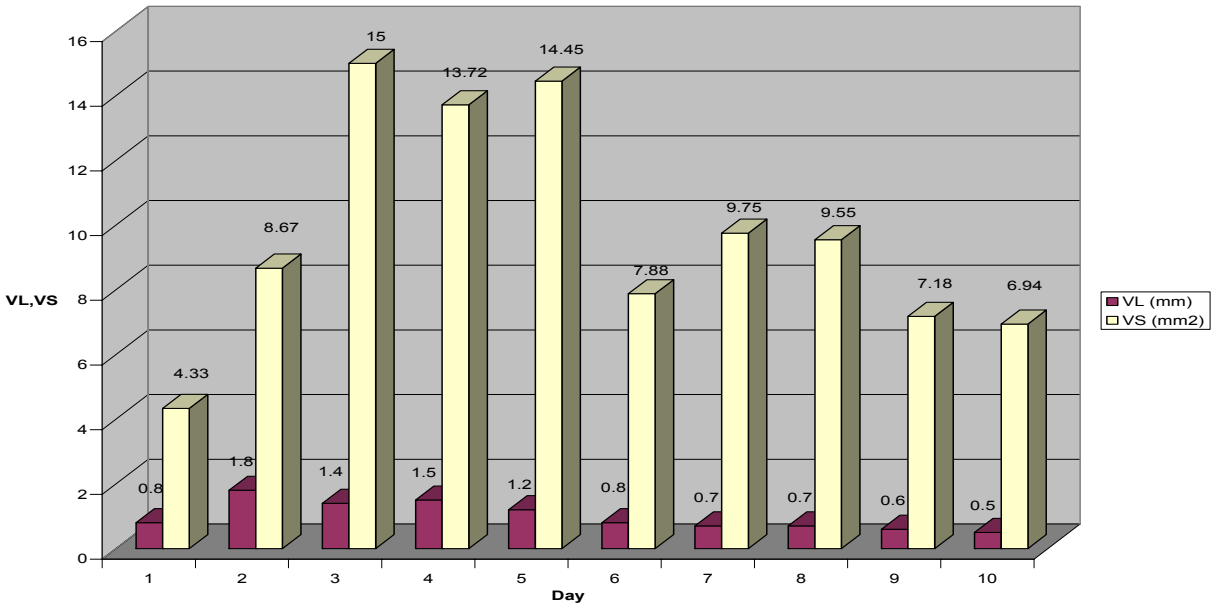

Figure 3 – ***Evolution of vascular length (VL) and surface (VS) in eyes with peripheral corneal burns.***

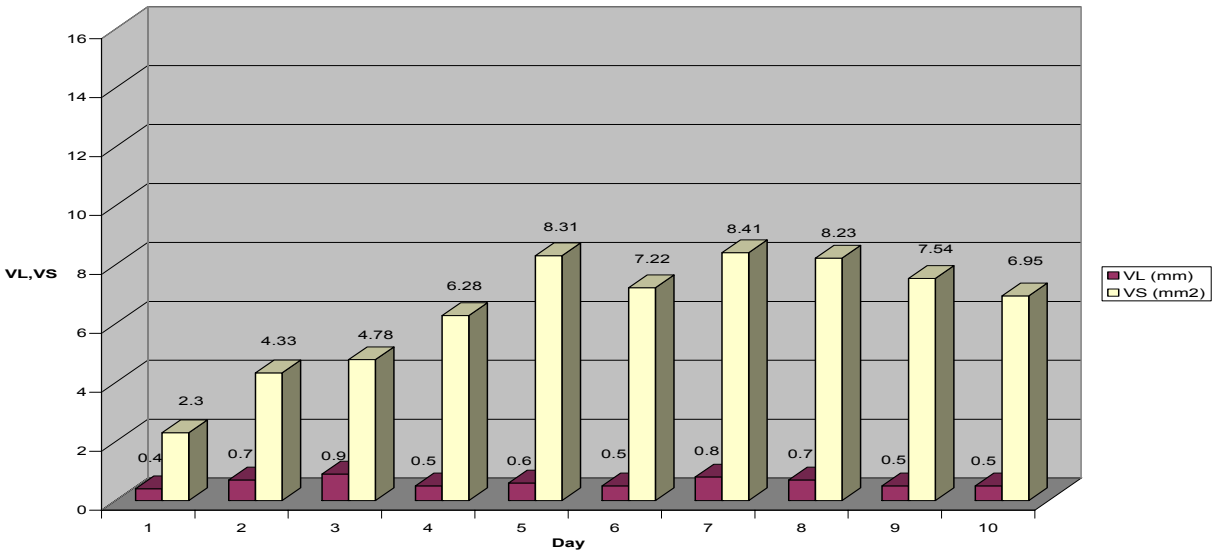

Figure 4 – ***Evolution of vascular length (VL) and surface (VS) in eyes with corneal burns treated with Maxidex.***

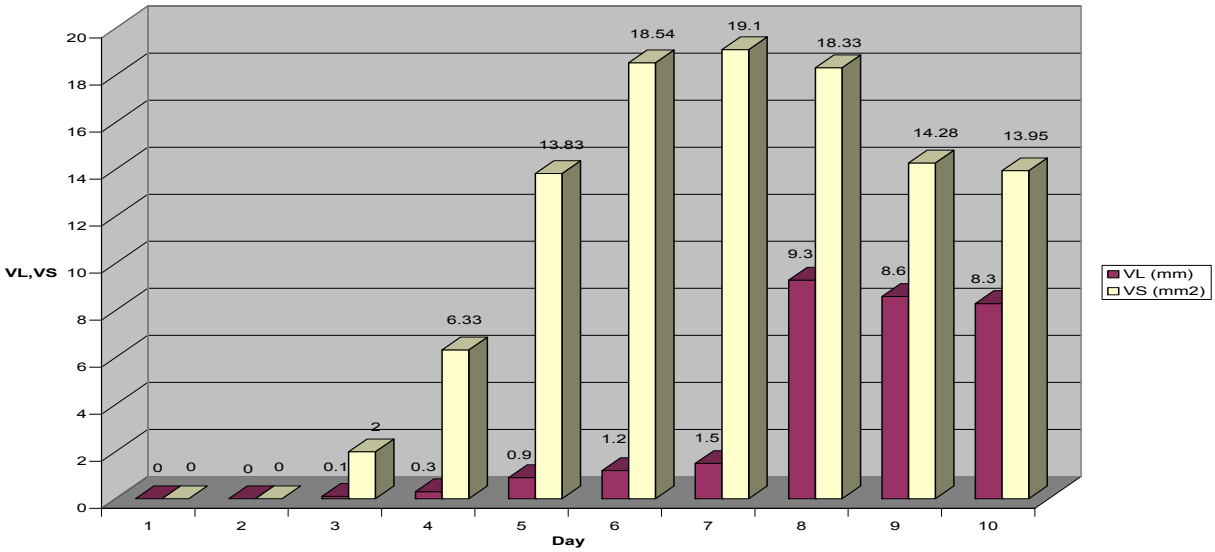

Figure 5 – ***Evolution of vascular length (VL) and surface (VS) in eyes implanted with VEGF (200 ng) pellets and treated with Maxidex immediately after implant.***

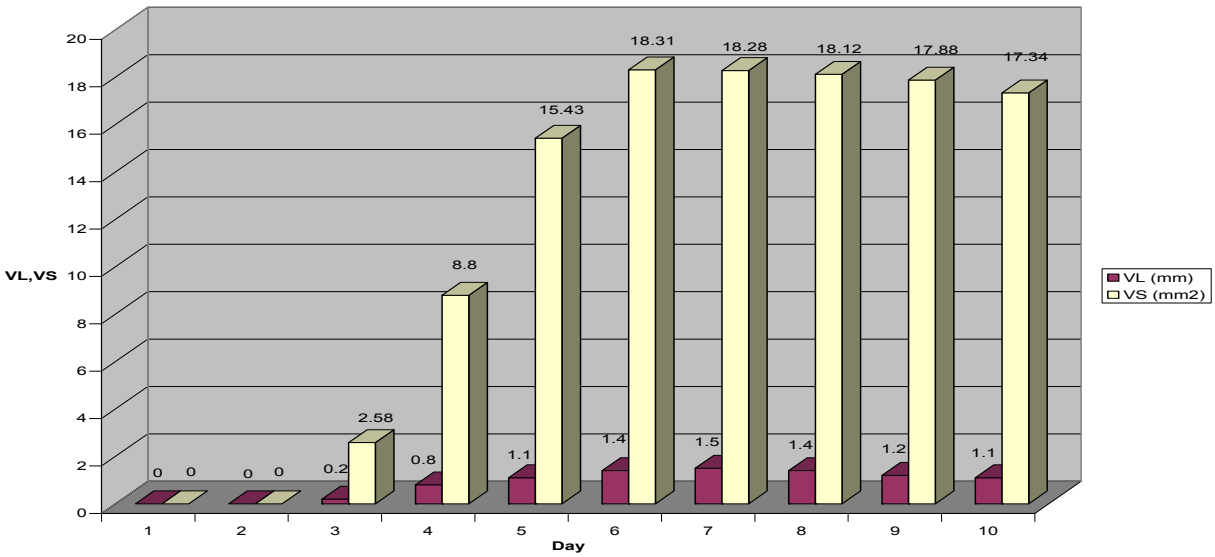

Figure 6 – ***Evolution of vascular length (VL) and surface (VS) in eyes implanted with VEGF (200 ng) pellets and treated with Maxidex from day 3 after implant (onset of neovessels).***

Corneal invasion with neovessels could be initiated by the reduction in the thickness of the surrounding tissue. If the corneal lamellae were separated by edema, neovessels would be able to penetrate the cornea (Figure 7A). Even more, it is obvious that a cornea with neovessels not only does it have the collagen lamellae separated from one another, but also contains a chemotactic factor for capillary growth (Figure 7B). In our experiment, we used VEGF as a major angiogenetic factor, but the possibility of other active vasoforming agents was not ruled out. It is possible that corneal vascularization may depend on the presence of one or several diffusible factors, able to direct capillary growth, and absent from the normal cornea (Figure 7C).

In our experiment, we noticed that, when neovascularization was induced by thermal injury or VEGF implant, it was strictly limited to the corneal area corresponding to the lesion, and was dependent on the VEGF-dosage. On the other hand, it can be supposed that any corneal injury causes a certain imbalance. An agent that normally inhibits vascularization might be destroyed in certain situations, allowing the invasion of cornea with neovessels (Figure 7D).

The possibility of combined action of the above-mentioned factors should also be considered. For example, normal cornea may contain both types of compounds: some promoting vascularization and others inhibiting neovascular invasion (Figure 7E).

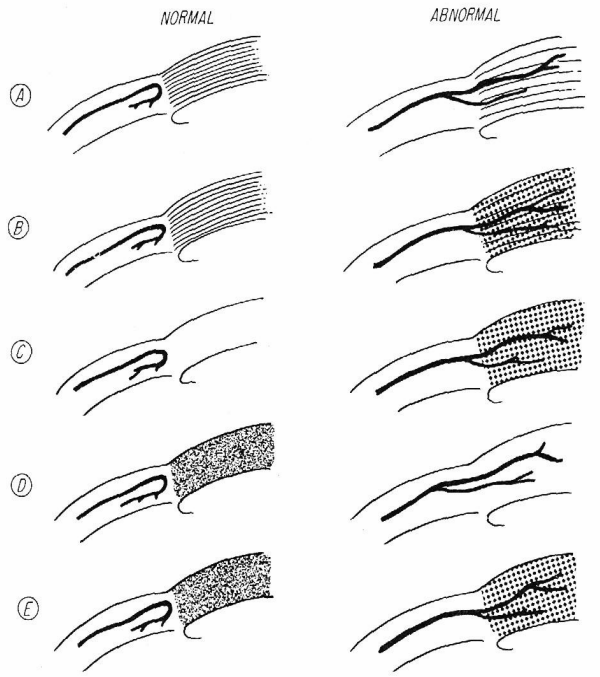

Figure 7 – ***Various models of corneal progression of neovessels.***

This biomicroscopic evidence suggested that the anti-inflammatory effect of Maxidex could not be correlated with the angiogenesis induced by VEGF. After the histopathological examination of eyes with VEGF-implants and treated with Maxidex (Figures 8–10), no typical inflammatory infiltrate was seen, as in eyes with corneal thermal burns (Figure 11).

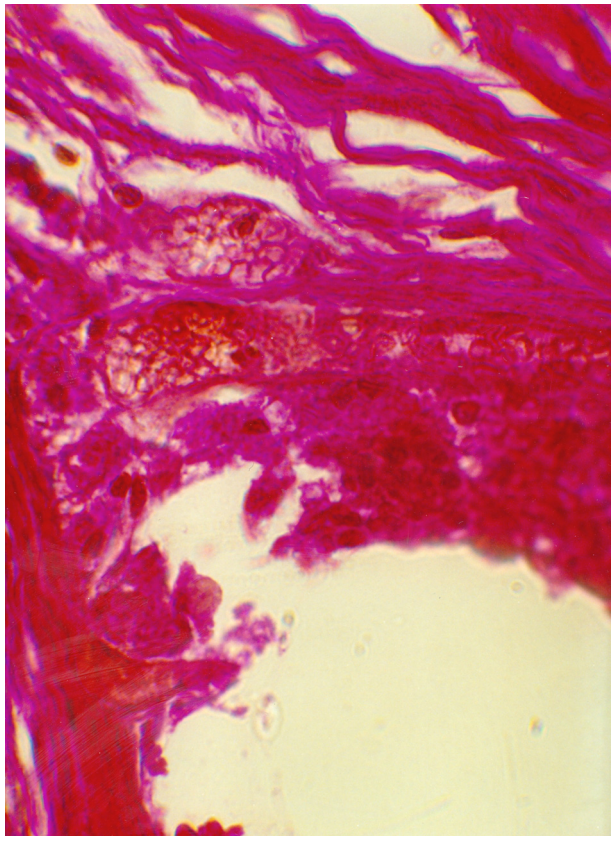

Figure 8 – ***Section through neovessels filled with hypochromic conglomerated red blood cells. Edema and lymphoplasmocytic infiltrate are absent.***

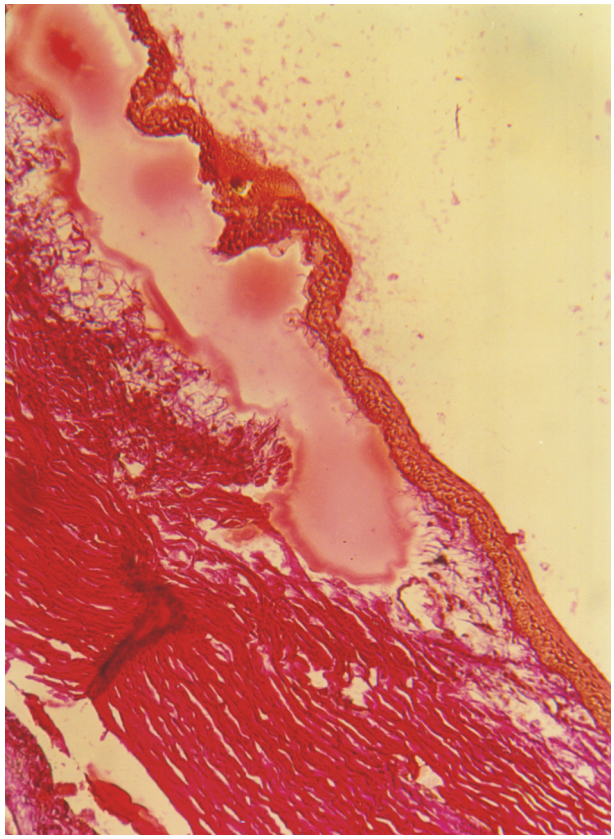

Figure 9 – ***Longitudinal section through a neovessel, at the periphery of the pellet. Other neovessels appear in its neighborhood, some of them in transversal section.***

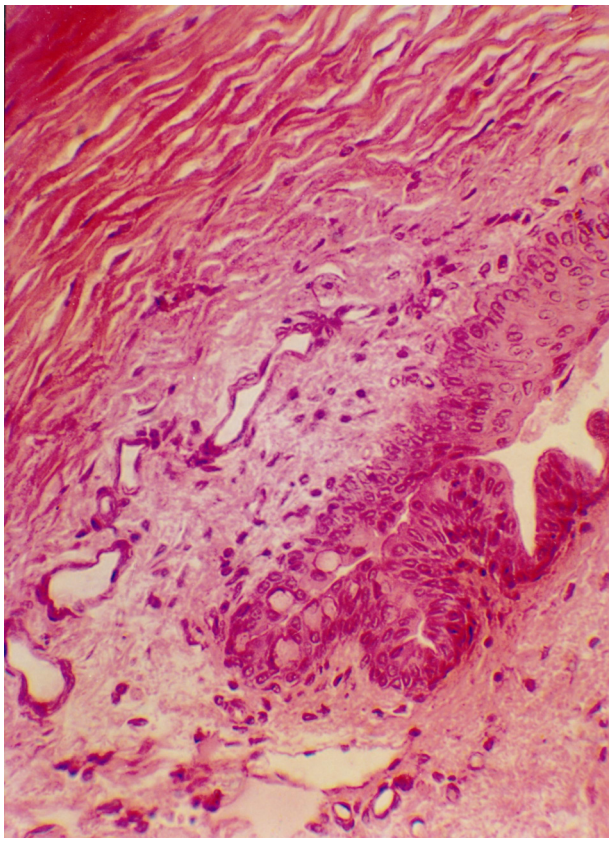

Figure 10 – ***Image of a VEGF pellet; around this pellet, tubular structures filled with macrophages with a hyperchromic, elongated nucleus can be seen.***

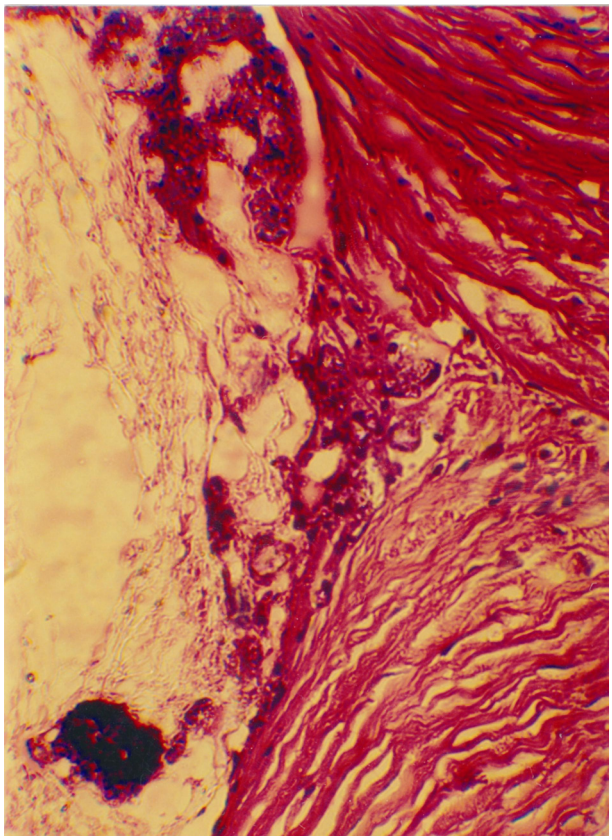

Figure 11 – ***Corneal section, including a fragment of the periphery of the thermal injury corneal area, with moderate edema and lymphoplasmocytic infiltrate.***

Discussion

Several experimental models have reproduced corneal vascularization; they include various means of damaging the cornea (chemical agents, physical means, exogenous infections, immunological reactions, intracorneal inoculation of different agents, toxic states, or nutritional deficits). Such models vary in their reproducibility.

Two different ways of inducing neoangiogenesis were used in the present study: thermal burns of the central or peripheral cornea, and implantation of VEGF pellets. Both induced a neovascular reaction, but its etiopathogeny was different in each case. This affirmation is supported by the above-described findings.

The angiogenesis model is considered reliable, because the angiogenetic stimulus is directly induced by VEGF and so the activity of angiogenesis inhibitors may be easily monitored. A large number of serial observations and measurements in the corneal sector of the neovascular reaction were performed on the slit special biomicroscope. The direct nature and action of the angiogenetic stimulus (VEGF) enabled us to rule out inflammation as the trigger of angiogenesis.

Our experimental model used VEGF as pellets, alone or in combination with Sucralfate, for the purposes of stabilizing the active biological form of VEGF and slowing down its release from the pellets. We used two types of control pellets: either with Sucralfate alone or with VEGF alone and they did not induce neoangiogenesis. The combination of the two agents (VEGF and Sucralfate) did however induce a significant angiogenetic answer, probably due to the potentiating effect of Sucralfate on VEGF.

Corneal injury by thermal burn was shown to be an angiogenic model of inflammation, because it resulted in a leukocyte infiltrate of the corneal stroma near the limbus, followed by the onset of corneal neoangiogenesis.

In our rabbit cornea experimental model, non-inflammatory neongiogenesis was different from that induced by thermal burn, fact that was proven directly by the findings of the histopathological examination, and indirectly by pharmacologic evidence.

Histopathologically, VEGF-induced the invasion of the three-dimensional collagen matrix by capillary endothelial cells, and its organization in tubules similar to that of the blood capillaries. This network subsequently became permeable to the red blood cells. This effect depended on the dosage and the proximity of the implant to the corneoscleral limbus.

Pharmacologically, Dexamethasone 0.1% solution, instilled in the conjunctival sac of the rabbit eye, inhibited angiogenesis in rabbits with peripheral corneal burns. Optimal results, with an almost complete inhibition of neovascularization, were seen when administration was started on day 1 after the thermal injury. Dexamethasone administered in the same way showed no effects on angiogenesis developed in eyes implanted with VEGF.

The computer analysis of the images captured by the video camera had two major benefits: the accuracy of

the analysis of the neovascularization process, by the measurement of the vascular length and surface, and the possibility of using fractal analysis. This analysis, in this particular case, might open new perspectives in the understanding of angiogenetic processes and their follow-up.

Three important issues may rise from these findings. One is the role of the *inflammatory infiltrate*, another is the role of *edema,* and the third would be the role of *tissular hypoxia,* in the corneal neovascularization.

As shown by the histological examination, leukocytes penetrated the cornea before the capillaries. The presence of an *inflammatory infiltrate* in a certain area should be followed by a corneal vascularization reaction, with certain localization, depth and direction of the vascular invasion of the stroma. Therefore, we can suppose that the presence of leukocytes in the lesion area is important, but not essential, for the development of the neovascular network, because it still appeared. The administration of Maxidex from day 3 after cauterization showed no significant effects on angiogenesis, even if the evolution of the inflammatory process was improved. It may be stated that leukocytes play an important role in the onset of angiogenesis, but less in its progression. We may presume the existence of a "cascade of events" in corneal angiogenesis, because once installed, it supports itself, and leukocytes certainly play a role in its initiation, significantly reduced thereafter.

The *edema* caused by an increase in vascular permeability of the vessels ofthe corneoscleral limbus almost invariably precedes the corneal vascular invasion. The question is whether the angiogenetic growth factors are present within inflammatory exudates together with the serum, or they come from the lesion area, released by the corneal cells altered by the new environment. A full answer is not yet available.

At the histopathological examination, we noticed that, if in some cases stromal edema preceded neovascularization, in most cases such edema did not occur. We may presume that the stage of inflammatory edema was overcome in such cases, leaving only a disorganization of the stromal fibers that lost their parallel alignment and separated.

As neovessels do grow preferentially in tissular spaces with low resistance, it may be said that stromal edema precedes and rarely accompanies corneal vascularization, and that the loss of stromal network that follows edema offers tissular space for the capillaries to extend. Proteolysis of the extracellular matrix, intermediated by fibrinolytic system, also contributes to this phenomenon.

Taking into account the role of *tissular hypoxia* in corneal vascularization, a correlation could be made with the retina, where neovessels proliferate as an answer to the accumulation of certain chemical mediators, produced by anaerobic metabolism [36]. Normal cornea has a small number of cells with minimal metabolic requirements that can be met by diffusion only. However, in the presence of a corneal cellular infiltrate, obviously the metabolic requirements will rise. This situation is similar the embryonary or malign tissues [7, 37, 38], where the high cellularity causes neovascularization. Even if the cornea is able of taking a sufficient amount of oxygen from the atmosphere in order to satisfy its increased needs, the corneal tissue is not able to use it, due to the lack of other essential metabolites. For example, in the case of the peripheral corneal burn induced in our experiment, the massive inflammatory infiltrate, with a high cellularity, may play the role of initiator in the equation hypoxia–neovascularization. In eyes implanted with VEGF, we saw, on the histological sections, a low cellularity. Nevertheless, the neovascular network was considerable, with vascular length and surface greater than after the thermal injury. This fact might prove that hypoxia alone was not the major element in the onset and maintenance of angiogenesis, at least in the corneal tissue [7, 22].

Conclusions

The experimental model the authors have described and carried out is original, due to the induction of corneal neoangiogenesis in rabbits after intracorneal implant with vascular endothelial growth factor (VEGF).

The proposed model is special, due to the facility of the surgical approach, the originality of the implant preparation, the accuracy in dosing the vascular factor, and its reproducibility.

VEGF-induced angiogenesis occurred through a different mechanism than that induced by corneal cauterization.

Maxidex (Dexamethasone 0.1%) solution showed no effect on VEGF-induced angiogenesis, due to the absence of any inflammatory infiltrate.

Leukocytes did not play a significant role in VEGF-induced angiogenesis even if the vascularization was lower, while in other angiogenesis models their presence proved to be more important.

Through this experiment, we established a method that proved indirectly, by means of Maxidex treatment that VEGF-induced angiogenesis was of a non-inflammatory nature.

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