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Effects of lapatinib and trastuzumab on vascular endothelial growth factor in experimental corneal neovascularization

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ABSTRACT

Background: To compare the effects of lapatinib and trastuzumab on vascular endothelial growth factor (VEGF) on experimental corneal neovascularization.

Methods: Thirty-five Wistar albino rats were randomly divided into five groups, each containing seven animals. Corneas of rats in the control group were not cauterized and did not receive any treatment. A silver nitrate pencil was applied on the right corneas of rats in the non-control groups to induce corneal neovascularization. Rats in the sham, lapatinib, trastuzumab and lapatinib+trastuzumab groups were administered systemic saline, 50 mg/kg lapatinib once a day orally by gavage, 4 mg/kg trastuzumab once a day intraperitoneally, or 50 mg/kg lapatinib once a day orally by gavage together with 4 mg/kg trastuzumab once a day intraperitoneally, respectively, for seven days. Rats were sacrificed on the 8th day, and corneas were excised using a 4-mm punch trephine. VEGF immunostaining in the corneal epithelial and stromal layers was evaluated. Staining intensities were determined semi-quantitatively, and corneal VEGF levels were measured by ELISA.

Results: The mean immunostaining intensities of epithelial and stromal VEGF in the control group were significantly lesser than those in the sham group ($p<0.05$). The mean immunostaining intensities of epithelial and stromal VEGF and VEGF ELISA levels of corneas in all treatment groups (lapatinib, trastuzumab and lapatinib+trastuzumab groups) were lesser than those in the sham group ($p<0.05$), however it was similar to those in the control group ($p>0.05$).

Conclusions: This study suggests that systemically administered lapatinib is more effective than systemically administered trastuzumab in preventing corneal neovascularization.

Keywords: Corneal neovascularization; lapatinib; trastuzumab; VEGF; immunostaining.

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INTRODUCTION

Corneal neovascularization (NV), the abnormal growth of blood vessels into the avascular corneal tissue, occurs secondary to chronic corneal hypoxia due to contact lens usage and traumatic, inflammatory, ulcerative or degenerative diseases of the cornea.[1–2] Corneal NV may cause not only a severe reduction of visual acuity (including, in some cases, blindness) due to corneal scarring but also a bad prognosis after corneal transplantation due to immune rejection of corneal grafts. Although the pathogenesis of corneal NV has not yet been clearly defined, it has been hypothesized that multiple angiogenic and anti-angiogenic factors interact to co-regulate NV. Recent studies have shown that vascular endothelial growth factor (VEGF) plays a major role in corneal NV.[3–12]

VEGF is a potent cytokine mainly produced by endothelial cells that has been shown to promote several steps of angiogenesis, including proliferation and migration of vascular endothelial cells. It has recently been shown that VEGF is upregulated in inflamed and vascularized corneas in human and animal models.[5,6,12]

Current treatment modalities for corneal NV include topical corticosteroid and non-steroid anti-inflammatory medications; photodynamic therapy; laser photocoagulation; diathermy; conjunctival, limbal, and amniotic membrane transplantation; and applications of anti-VEGF and various anti-angiogenic drugs. However, these treatments have limited clinical efficacy and also have significant side effects.[13–16]

Trastuzumab is a humanized monoclonal antibody targeted against the extracellular domain of the human epidermal growth factor receptor 2 (HER2). It is currently widely used in the treatment of the women with HER2-overexpressing breast cancer. The mechanism of the action of trastuzumab is not fully understood. However, it has been demonstrated that epidermal growth factor receptor (EGFR) activation is related to angiogenesis, and that this receptor upregulates expression of some potent angiogenic factors such as vascular endothelial growth factor (VEGF).[17–21] In an experimental animal study, Guler et al. demonstrated that systemically administered trastuzumab was effective in preventing corneal NV.[22]

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Lapatinib is a dual tyrosine kinase inhibitor that acts on both the HER2 and EGFR. It is used in combination chemotherapy for HER2-positive early stage breast cancer.[23, 24] HER2 inhibitors (including trastuzumab and lapatinib), inhibit tumour growth and induce apoptosis by targeting HER2 through different molecular mechanisms. Trastuzumab targets the extracellular domain of HER2, while lapatinib inhibits the kinase activity both of HER2 and EGFR. It has been shown that both of them were effective in the treatment of women with HER2-positive breast cancer.[23, 24]

However, there is no data to date concerning whether lapatinib affects VEGF, which plays a dominant role in the development of corneal NV. If lapatinib has a strong effect on VEGF, it may be useful in the prevention of corneal NV.

In our study, we aimed to investigate the effect of lapatinib in preventing corneal NV in an experimental rat model and to compare its anti-angiogenic effect with that of trastuzumab.

METHODS

The study was carried out under the permission of the Institutional Animal Care and Use Committee of Firat University. The animals were housed in wire-bottomed cages at room temperature on a 12-hour light-dark cycle at the Institutional Medicine Research Center under appropriate dietary conditions throughout the study. All procedures were performed with strict adherence to the guidelines for animal care and experimentation prepared by the Association for Research in Vision and Ophthalmology and Guidelines for the Housing of Rats in Scientific Institutions.

Groups

Thirty-five Wistar albino rats weighing between 250 and 300 g were randomly assigned to one of five groups of seven animals each: Group I (control), Group II (sham), Group III (lapatinib treatment group), Group IV (trastuzumab treatment group) and Group V (lapatinib+trastuzumab treatment group).

Corneal NV was induced by application of a silver nitrate pencil on the right corneas of all rats except the control subjects; silver nitrate was applied on only one cornea in each rat to allow them to feed easily. A single investigator (M.K.K.) cauterized all

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animals to provide increasing the reproducibility of the burns. The extent of the burn stimulus response for each cornea was scored as follows:

- Grade 0 (no blister and not raised above corneal surface)
- Grade +1 (small blister and raised slightly above the surface)
- Grade +2 (medium blister and raised moderately above the surface)
- Grade +3 (large blister)

In the calculation of the mean burn extents and neovascularization scores in each group, only the corneas with score of Grade +2 or higher were included. Rats in the control group did not receive any treatment. Treatment started immediately after cauterization in the all other groups. Following cauterization, the experimental lapatinib and trastuzumab administrations and dosages were choiced according to studies described by Tracy and Guler with minor modifications. Rats in the sham group were administered systemic saline, and rats in lapatinib group were administered 50 mg/kg lapatinib (Tykerb®; GlaxoSmithKline, Brentford, UK) once a day orally by gavage. Rats in the trastuzumab group were administered 4 mg/kg trastuzumab (Herceptin®, Genentech inc., California, USA) once a day intraperitoneally.[22,25] In the lapatinib+trastuzumab treatment group, rats were administered 50 mg/kg lapatinib once a day orally by gavage together with 4 mg/kg trastuzumab once a day intraperitoneally. All rats excluding those in the control group were treated for seven days. Corneal digital photography was performed on the 8th day. The percentage of NV (area of NV as a percentage of the total corneal surface) was calculated. All animals were sacrificed on the 8th day and corneas were excised using a 4-mm punch trephine. Excised corneas were immunostained for VEGF and staining in corneal epithelial and stromal layers was evaluated. Staining intensities were determined semi-quantitatively, and corneal VEGF levels were measured by ELISA.

Anaesthetization

A combination of 50 mg/kg ketamine hydrochloride (Ketalar; Eczacibasi, Istanbul, Turkey) and 5 mg/kg xylazine hydrochloride (Rompun; Bayer, Istanbul, Turkey) was injected intramuscularly to anaesthetize animals. Before the operation, one drop of

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0.5% proparacaine hydrochloride (Alcaine; Alcon, Istanbul, Turkey) was instilled on the animal's right eyes.

Surgical technique

After induction of anesthesia and analgesia as described above, corneal NV was induced by silver nitrate cauterization as described by Mahoney and Waterbury.[26] A 0.5% solution of proparacaine hydrochloride was used as a topical anaesthetic. After anaesthesia and sedation, an applicator stick with a diameter of 2 mm coated with 75% silver nitrate/25% potassium nitrate (Nitrate D'argent; Botafarma; Ankara, Turkey) was pressed to the central corneas of the right eyes of each animal for 10 seconds under the operating microscope. Residual silver nitrate was removed by rinsing the eyes with 5 ml of saline and then gently blotting the eyes with tissue paper. Treatment was started immediately after a blister was observed during the burning procedure.

Calculation of corneal NV area

All corneas were evaluated using a slit-lamp biomicroscope on the eighth day of the experiment. Corneas were photographed with a Sony digital camera with a ×40 magnification (CCD-IRIS model DXC 107 AP, Sony Company, Tokyo, Japan) attached to the slit-lamp biomicroscope. NV area in each cornea was calculated as a percentage of the total cornea area (neovascularization area X 100/entire corneal area) using digital computer image analysis software (Topcon Image Net 2000, Tokyo, Japan).[27]

Tissue preparation and VEGF immunostaining

Following anaesthesia and sedation on the 8th day, the animals were sacrificed and corneal excision was performed.

Five μm-thickened paraffin sections that included the burned cornea were cut. Sections were placed on slides with poly-L-lysine. Then, they were deparaffinized using the EZ Prep kit (Ventana Medical Systems) at 75°C for four minutes and subjected to antigen retrieval for eight minutes at 95°C in Tris buffer cell conditioning solution and treated at 37°C for four minutes with the UltraView

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Inhibitor to block endogenous peroxidase. Slides were incubated for one hour at 37°C by the use of a monoclonal mouse antibody to VEGF applied to the slide at a 1:750 dilution in antibody diluents (Santa Cruz Biotechnology, CA, USA). The complex was visualized by the use of hydrogen peroxide and DAB (3,3 diaminobenzidine tetrahydrochloride) as substrate and chromogen, respectively. The slides were rinsed by the use of a Tris-based buffer solution and then they were counter-stained with haematoxylin, post-counterstained with the aqueous solution of buffered lithium carbonate (Bluing Reagent, Ventana Medical Systems). Finally, they were mounted for histopathological examination. Negative controls were prepared by replacing the primary antibody with normal mouse serum.

Slides were evaluated under a light microscope (Olympus BX-50 Photomicroscope). Digital photographs of tissues were taken using a digital camera with ×40 magnification attached to the same microscope.

Semi-quantitative assessment of VEGF immunostaining intensities

VEGF immunostaining intensities in the epithelium and the stroma of each cornea were determined by a standardized semi-quantitative method previously described by Philip et al[28] and VEGF immunostaining intensities were scored as follows:

- Grade 0: No staining
- Grade 1: Weak staining
- Grade 2: Moderate staining
- Grade 3: Intense staining

Because healthy corneal endothelium also shows VEGF immunostaining and because of the difficulty of performing a semi-quantitative evaluation in a single endothelium layer, endothelial VEGF immunostaining was not evaluated.

ELISA levels of corneal VEGF

To evaluate corneal VEGF levels, corneal samples were taken using a punch trephine having a diameter of 4 mm to obtain samples of equal areas. For protein extractions from corneal buttons, corneal samples were first homogenized using the Magna Lyser Instrument (Roche Diagnostics, Istanbul, Turkey) in 2.0 ml tubes containing ceramic beads and 100 µl of cell lysis buffer (Invent Biotechnologies, Inc.,

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Minnesota, USA.). Then, protein extraction was performed using the Minute™ Total Protein Extraction Kit (Invent Biotechnologies, Inc., Minnesota, USA.). Corneal VEGF levels were determined by a commercially available rat VEGF ELISA kit (RayBio, RayBiotech Inc., USA). The assay was performed according to the manufacturer's instructions. Briefly, standards and samples were pipetted into wells that contained immobilized anti-VEGF antibodies, which bound to VEGF (if present) in the standard or sample. The wells were washed, and biotinylated anti-Rat VEGF antibody was added. After washing away unbound biotinylated antibody, horseradish peroxidase-conjugated streptavidin was added to the wells. The wells were again washed; a tetramethylbenzidine substrate solution was added to the wells, and colour developed in the wells in proportion to the amount of VEGF bound. The Stop Solution changed the colour from blue to yellow, and the intensity of the colour in each well was measured at 450 nm by an ELISA reader device. VEGF levels were determined and recorded in units of picograms/cornea.

Statistical analysis

Results were given as means and standard deviations. Statistical analysis was performed using SPSS version 13.0 software (SPSS Inc., Chicago, Illinois, USA). A Mann–Whitney U test was used to evaluate the differences between the control and treated eyes and a Kruskal–Wallis test was used to evaluate the differences within the treatment groups. A p value less than 0.05 was considered to be significant.

RESULTS

Mean intensities of epithelial and stromal VEGF staining, mean corneal VEGF ELISA levels and mean percentages of NV (areas of NV as a percentage of total corneal surface areas) in the study groups are given in Table 1. We also determined normal corneal VEGF levels by measuring levels in the control group. The mean percentages of NV in Groups III, IV and V were lower than that of the sham group ($p<0.01$, $p<0.01$, and $p<0.01$, respectively). The mean NV areas in Groups III and IV were similar to each other ($p=0.31$,) but were lower than that of Group IV ($p=0.038$, $p=0.011$).

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The mean epithelial VEGF immunostaining intensity in the control group was significantly lesser than that in the sham group (p=0.01). The mean epithelial VEGF immunostaining intensities of corneas in Groups III, IV and V were lesser than that in the sham group (p<0.01, p=0.01, and p=0.02, respectively) but were similar to that in the control group (p=0.53, p=0.12, and p=0.53, respectively). The mean epithelial VEGF immunostaining intensity of corneas in Group IV was higher than those of Groups III and V (p=0.03 and p=0.02, respectively). There was no significant difference between the mean epithelial VEGF immunostaining between the Groups III, IV, V and the control group (p=0.53, p=0.63 and p=0.98).

The mean stromal VEGF immunostaining intensity in the control group was significantly lesser than that in the sham group (p<0.01). The mean stromal VEGF immunostaining intensities of corneas in Groups 3, 4 and 5 were lower than that of the sham group (p<0.01, p=0.01 and p=0.02, respectively) but similar to that of the control group (p=0.71, p=0.73, and p=0.71, respectively). The mean stromal VEGF immunostaining intensity of corneas in Group IV was higher than those of Groups V and III (p=0.03 and p=0.03, respectively). There was no significant difference among the mean stromal VEGF immunostaining intensities between the Groups III, IV, V and the control group (p=0.71, p=0.71, p=0.92, respectively).

The mean corneal VEGF ELISA levels in the control group was significantly lesser than that in the sham group (p<0.01). The mean corneal VEGF ELISA levels in Groups III, IV and V were lesser than that in the sham group (p<0.01, p<0.01, and p<0.01, respectively) but similar to that of the control group (p=0.07, p=0.71, and p=0.07, respectively). The mean corneal VEGF ELISA levels in Group IV was higher than those of Groups V and III (p=0.03 and p<0.01, respectively). There was no significant difference between the mean stromal VEGF ELISA levels between the Groups III, IV, V and the control group (p=0.07, p=0.07 and p=0.92).

The corneal photographs (Figures 1 and 2) and the microphotographs of immunostained corneal tissue (Figures 3 and 4) of the sham and treatment groups showed significant changes from those of the control group.

DISCUSSION

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Recent studies have supported a central role of VEGF, a potent angiogenic factor, in corneal NV, as evidenced by the strong inhibition of NV by VEGF inhibitors.[3,5-9,28-30] Normal corneal VEGF levels was determined by the control group. EGFR activation is also linked to angiogenesis, and EGFR upregulates expression of VEGF**.** Human EGFR-1 has tyrosine kinase activity. HER-1 is one of 19 subgroups in this EGFR family. The activation of these receptors is very important for cell activation and proliferation. HER-1 is mainly activated by EGF and TGF-α. Binding of EGFR to its ligand causes dimerization. The stimulation of HER-1 activates protein kinase and phosphotidil-inositol-3-OH kinase pathways via TK activation. Recent studies have demonstrated that the activation of HER1 is effective in preventing cell migration, proliferation, adhesion and apoptosis and that it causes NV. It is known that EGFR activation induces angiogenesis by increasing the abundance of angiogenic molecules such as VEGF. In recent studies, it was also demonstrated that EGFR regulates VEGF expression and also that angiogenesis might be prevented with EGFR inhibitors.[23, 31-37]

Human EGFR-2 is another receptor from EGFR family that is also affected by lapatinib. Trastuzumab only affects this receptor. The activation of HER-2 induces the binding of EGF to its receptor. Consequently, multiple cellular signal pathways including mitogenic activated protein kinase and phosphotidil-inositol-3-OH kinase. HER-2 plays a critical role in the cellular proliferation and differentiation. The activation of HER-2 is correlated with the increasing of cellular proliferation. The HER-2 and VEGF signalling pathways are linked with each other at a molecular level. HER-2 and VEGF indirectly or directly affect various processes such as cellular proliferation and invasion, increased tumour motility, inhibition of apoptosis and angiogenesis. In a recent study, Han et al. demonstrated that treatment with a drug that selectively inhibits EGFR tyrosine phosphorylation led to a reduction of VEGF mRNA to 50% of the control level, which confirms that EGFR kinase activity influences VEGF mRNA regulation[38]. *In vitro* studies have demonstrated that lapatinib inhibits both HER-1 and HER-2 and that lapatinib alone is more effective than HER-1 blockers.[32- 36]

Trastuzumab, a humanized monoclonal antibody specifically targeted against the extracellular domain of the HER2–is currently widely used in the treatment of HER2-

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overexpressing early or metastatic breast cancer. The HER2 is a member of the EGFR family of receptor tyrosine kinases. Pure HER2 inhibitor (including trastuzumab) and the dual EGFR/HER2 inhibitor (lapatinib) inhibit tumour growth and induce apoptosis through different molecular mechanisms. It has been demonstrated that both trastuzumab and lapatinib are effective in the combination chemotherapy in women with HER2-positive breast cancer.[23, 24]

It is known that trastuzumab reduces expression of pro-angiogenic factors and increases expression of anti-angiogenic factors.[17-21] In a recent experimental study, Guler et al. demonstrated that trastuzumab was effective in preventing corneal NV and that systemically administered trastuzumab led to decreased VEGF staining intensities of corneal epithelial and endothelial layers compared to a sham control.[22] Our findings concerning trastuzumab are consistent with theirs. In the present study, our main findings were the suppression of corneal NV and the reduction in VEGF immunostaining by both lapatinib and trastuzumab. However, to our knowledge, ours is the first to report the inhibition of corneal NV by lapatinib.

Lapatinib is an orally bioavailable small molecule dual tyrosine kinase inhibitor of HER2, EGFR, and HER1. Inhibition of HER2 and EGFR results in blockade of downstream signalling pathways and has been shown to lead to cessation of cell proliferation and cell death in preclinical studies.[39,40] However, there is no previously published data concerning whether lapatinib prevents corneal NV. Because lapatinib inhibits both HER-1 and HER-2, we initially speculated that the simultaneous inhibition of both receptors by lapatinib might be more effective in preventing corneal NV. In our study, we found that corneal NV area, VEGF immunostaining intensities and corneal VEGF ELISA levels were indeed significantly decreased compared to those of trastuzumab group. In the other words, lapatinib prevented corneal NV more effectively than did trastuzumab. However, there is no data to date concerning whether lapatinib affects VEGF, which plays a dominant role in corneal NV. It is possible that lapatinib, an indirect anti-VEGF drug, binds and blocks proteins that respond to VEGF, thereby preventing VEGF function via a secondary mechanism.

In recent clinical studies on breast cancer, it was demonstrated that subjecting patients who were refractory to trastuzumab to further treatment with lapatinib

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improved the success of therapy. Upregulation of insulin-like growth factor due to HER-2 inhibition is one of the mechanisms of resistance to trastuzumab. The pathways activated by this upregulation were inhibited by lapatinib.[33-40] In our study, we demonstrated that corneal NV was significantly regressed with lapatinib compared to trastuzumab. However, we found that combination treatment including lapatinib and trastuzumab did not differ from single lapatinib treatment as regards inhibition of both NV and VEGF. It seems that lapatinib might be enough to treat corneal NV.

In conclusion, we have demonstrated that systemically administered lapatinib may be beneficial in preventing new blood vessel formation in an *in vivo* corneal NV model by means of an anti-VEGF effect. We consider lapatinib as having potential use in the treatment of corneal NV. With systemic administration, the dose delivered to the cornea may be low, but similar studies with antibodies have shown that systemic administration is effective.[22] We also choiced systemic administration route to be sure that all animals received therapeutic dosage. When the study was conducted, orally administration by gavage was only way to use lapatinib.

Further studies are needed to measure the levels of lapatinib and trastuzumab in cornea achieved by systemic administration and to explain the exact mechanism of the anti-angiogenic effect of lapatinib and whether its topical administration is effective in preventing corneal NV.

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FIGURE LEGENDS

Figure 1: Normal rat clear cornea. Arrows with intermittent lines indicate the pupillary border, while the thick arrow indicates the border of normal iris vasculature.

Figure 2: Corneal neovascularization (NV) and its regression in the groups. Thin arrows indicate NV; thick arrows indicate the iris vessels. Arrows with intermittent lines indicate the pupillary border of the iris. Total corneal NV and central corneal scarring in the sham group (A). Minimal corneal NV and central corneal scarring in a rat from the lapatinib treatment group (B). Minimal corneal NV and central corneal scarring in a rat from the trastuzumab treatment group (C). Partial corneal NV in a rat from lapatinib+trastuzumab treatment group (D).

Figure 3: Minimal VEGF immunostaining is seen in normal rat cornea (Mayers hematoxyline ×100).

Figure 4: Histopathological microphotographs of corneas from the sham and treatment groups show immunohistochemical staining of VEGF (Mayers hematoxyline ×100): Sham (A), lapatinib treatment group (B), trastuzumab treatment group (C) and lapatinib+trastuzumab treatment group (D).

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TABLE

Table 1: Mean intensities of epithelial and stromal VEGF staining, mean corneal VEGF ELISA levels, and mean percentages of NV areas in the study groups.

Study Groups	Epithelial VEGF Staining Score Mean±SD	Stromal VEGF Staining Score Mean±SD	Corneal VEGF ELISA Levels Mean±SD	NV % Score Mean±SD
Control	1.285±0.75	0.710±0.48	15.428±2.50	0.00±0.00
Sham	2.857±0.37	2.571±0.53	70.571±4.89	63.71±6.07
Lapatinib	1.000±0.81	0.571±0.53	12.714±2.28	16.28±3.59
Trastuzumab	2.000±0.57	1.428±0.53	16.142±2.19	22.85±7.15
Lapatinib+Trastuzumab	1.000±0.81	0.571±0.53	12.857±1.34	14.71±2.21

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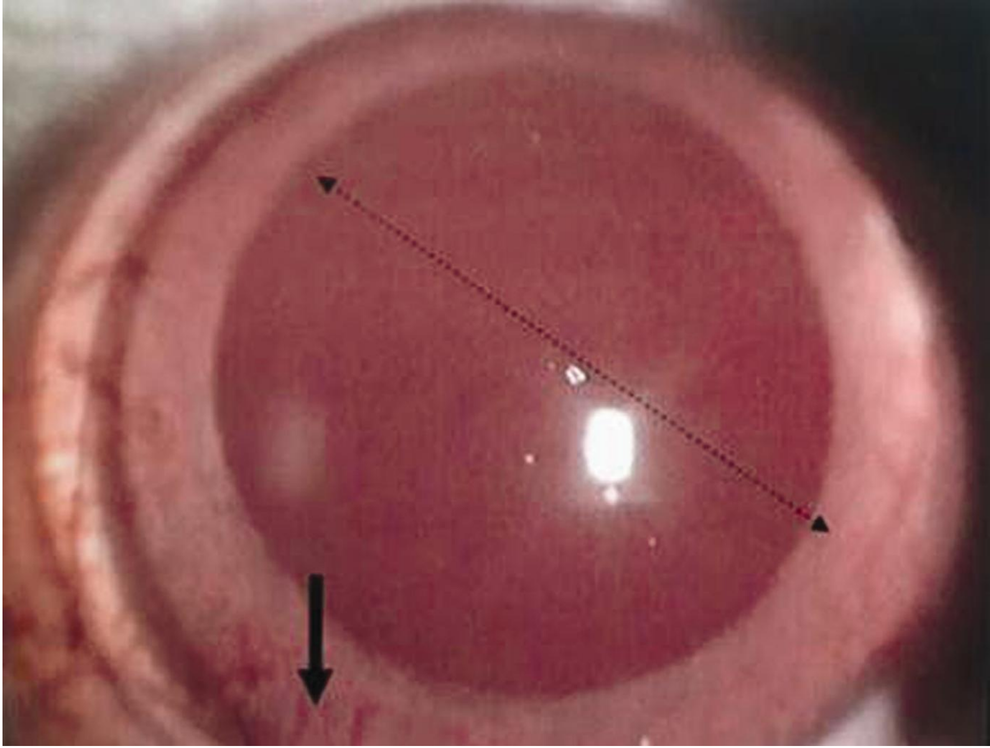

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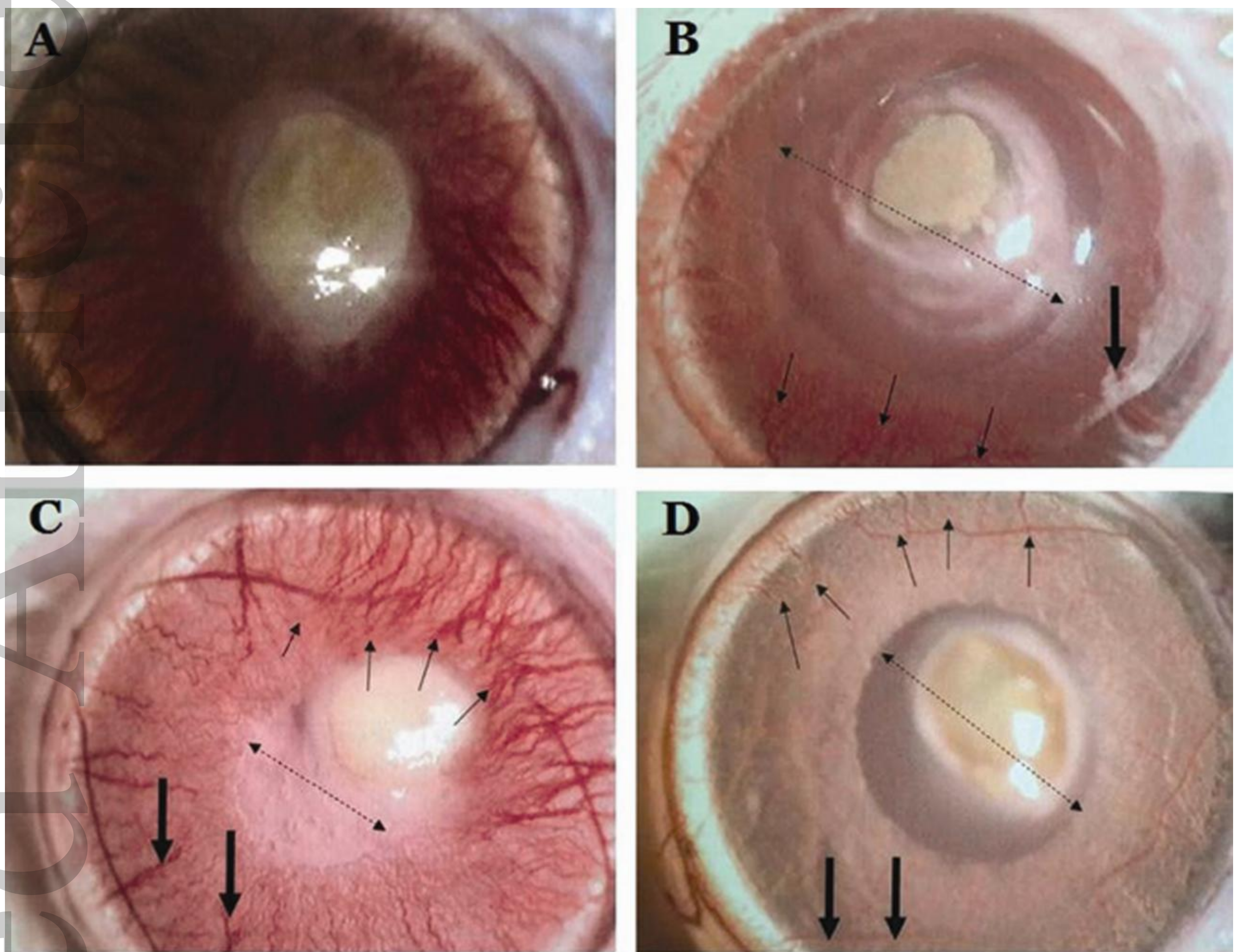

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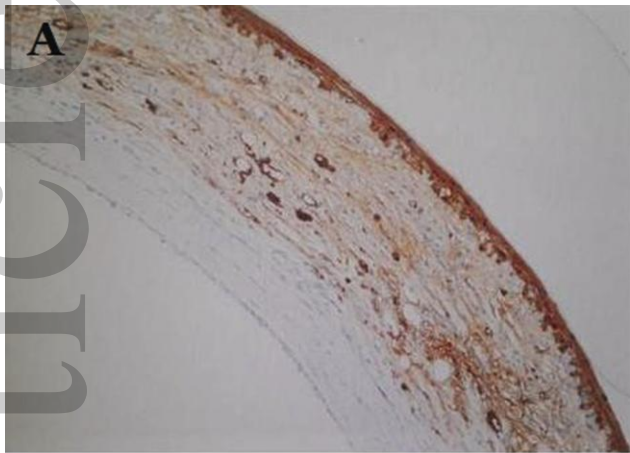

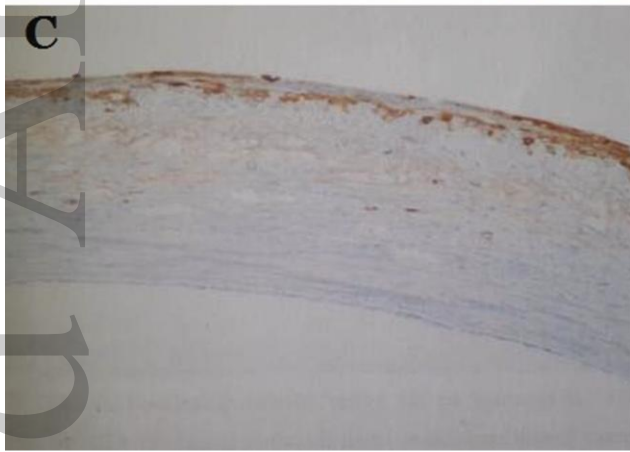

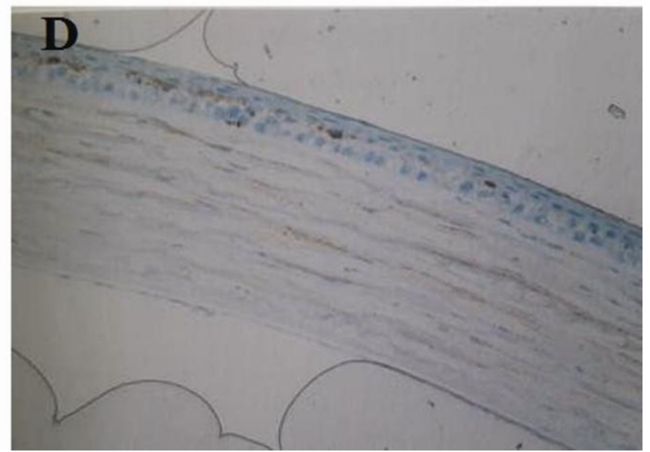

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