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Experimental retinopathy of prematurity: angiostatic inhibition by nimodipine, ginkgo-biloba, and dipyridamole, and response to different growth factors

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PURPOSE. *To investigate whether commonly used vasodilating drugs ameliorate angiogenesis in experimental retinopathy of prematurity (ROP), and to study the response of these drugs to different growth factors.*

METHODS. *We used a rat and mouse model of oxygen-induced ischemic retinopathy. Animals were treated with nimodipine, gingko-biloba and dipyridamole intraperitoneally starting the day before exposure to room air (day 1). Controls were injected with vehicle solution only. Eyes were processed histopathologically with serial sections and neovascularization was measured by counting the nuclei within the retinal internal limiting membrane, by a masked observer. Retinal and vitreous tissues were assayed by ELISA for VEGF, PDGF and TGFβ2.*

RESULTS. *Nimodipine significantly inhibited the growth of new vessels in rats. The number of nuclei was 310 ± 69 in the control group (n:14) and 121 ± 53 in the treated ones (n:14), ($p<0.0005$). Similar results were found with ginkgo-biloba extracts: 344 ± 53 (n:15) in controls, and 136 ± 29 (n:11) in treated ones ($p<0.0005$), and with dipyridamole: 303 ± 69 (n:13) in controls, and 131 ± 48.5 in treated rats ($p<0.0005$).*

Results were similar in mice. 186 ± 45 (n:7) nuclei counted in controls against 90 ± 25 (n:6) for dipyridamole treated ($p<0.0005$); and 81 ± 21 for ginkgo-biloba treated animals ($p<0.0005$). A gradual, very significant increase in VEGF values in response to relative hypoxia (room air) contrasted with the significant inhibition noted both with ginkgo-biloba extracts and dipyridamole. TGFβ2 and PDGF both showed a gradual increase in relative hypoxia at days 2 and 4 of room air ($p<0.0005$). Treated animals showed marked inhibition of the three growth factors.

CONCLUSIONS. *All three drugs markedly inhibited angiogenesis in experimental ROP. Growth factors were elevated in hypoxic conditions. Treated animals showed significant decreases of PDGF, VEGF, and TGFβ2 in retinal and vitreous tissues. (Eur J Ophthalmol 2000; 10: 51-9)*

KEY WORDS: *Neovascularization, Angiogenesis, Nimodipine, Ginkgo-biloba, Dipyridamole*

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INTRODUCTION

Our understanding of the pathogenesis of retinopathy of prematurity (ROP) has grown greatly in recent years (1). As a result of increased exposure of the inner retinal layers in the immaturely developed intraretinal vessels to environmental oxygen, vasoobliteration and suppression of normal retinal vascularization occur. The metabolic demands of the growing neural elements, with the decrease in oxygen on returning to room air, render the retina severely hypoxic. In a few cases, this process cannot be reversed, and overproduction of growth factors leads to exuberant neovascularization, severe tractional retinal detachment and blindness (2, 3). Various anti-angiogenic drugs have been tested over the past few years, mainly in experimental settings, but they have been only partially active on inhibiting these newly proliferating blood vessels (4). Recently anti-cancer drugs that inhibit blood supply to tumors (5-7), and a new, orally active inhibitor of protein kinase C and VEGF in experimental laser-induced choroidal neovascularization and ROP, appear to have achieved near-complete inhibition of these newly formed blood vessels (8). In spite of these encouraging results very little is known about the side effects of these experimental drugs, particularly whether they also affect physiological processes such as wound healing, not to mention the normal development of a premature infant.

Since in ROP, oxygen from the choroidal circulation floods the inner retina, which is normally supplied by the retinal vessels (1, 9), and the choroidal circulation has large-caliber vessels, high flow rate, low oxygen clearance and no autoregulation, increasing the choroidal flow with systemic vasodilating drugs may help attenuate this disease non-invasively, as is being attempted with supplemental oxygen (10). Vasodilating drugs which may have this effect are: gingko-biloba extracts, the calcium channel blocker nimodipine, and dipyridamole. In this study, we investigated angiostatic inhibition with these drugs, in rat and murine models of experimental ROP, and the response to different growth factors.

MATERIAL AND METHODS

All experimental procedures adhere to the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research.

Oxygen-induced ischemic retinopathy in rats

Ischemic retinopathy was induced in rats by a partially modified method of Penn et al (11). New born Sprague-Dawley rats were obtained from Fundación Campomar, Buenos Aires, Argentina. They were raised in our laboratories in similar experimental feeding and lighting conditions, with a regular circadian rhythm. The room temperature was kept constant at 26° ± 2° C. Three litters of newborn rats with their dams, with similar gestational age, were immediately placed in an airtight chamber and exposed to a high-oxygen atmosphere, alternating between 20% and 80%, every 12 hours for 10 days. An oxygen analyser maintained the desired concentration at all times. The rats were then removed from the chamber and exposed to room air for 4 days. For a five-day period (days 10 to 14) the rats were given subcutaneous injections of 0.1 ml of vehicle twice a day, or 0.1 ml of vehicle plus the study drug. Controls were treated with vehicle alone and maintained in room air for the entire 14 days.

After five days, the animals were killed, and their eyes rapidly removed under a dissecting microscope. They were grossly inspected, fixed with 4% paraformaldehyde, and embedded in paraffin.

Murine model of ischemic retinopathy

Ischemic retinopathy was produced in C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine) by the method described by Smith (12). Seven-day-old mice and their mothers (three to four litters at a time) were placed in an airtight incubator and exposed to an atmosphere of 75 ± 5% oxygen for five days, at an incubator temperature of 23 ± 2°C, oxygen was measured every 12 hours with an oxygen analyser. After five days, the mice were removed from the incubator, weighed, and placed in room air for five days. During this five - day period the mice were given an intraperitoneal injection of 0.1 ml vehicle twice a day, or vehicle containing the study drug. After five days, the mice were killed, their eyes were rapidly removed and fixed in 4% paraformaldehyde, then embedded in paraffin.

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Drugs and modality of treatment

Ginkgo-biloba extracts (Phoenix, Argentina) were dissolved in DMSO (70 mg/ml) and diluted in isotonic balanced salt solution (BSS) to a final concentration of 15 mg/ml. Dipyridamole (Boehringer Ingelheim, SA, Argentina) was diluted with BSS to a final concentration of 20 mg/ml. Nimodipine (Lazar - Labianca SA, Argentina) was diluted in BSS to a final concentration of 20 mg/ml. Controls for the three drug groups were given the vehicles alone.

Drugs and control vehicles were adjusted to 0.1 ml volume, and injected subcutaneously to rats every 12 hours for 5 days, from day 9 (still under oxygen) to day 14. The mice were given similar dosages and volumes of drugs and vehicle controls intraperitoneally every 12 hours, starting from day 4 (still under oxygen) and for the next five days of relative hypoxia (room air). Only dipyridamole and ginkgo-biloba extracts were used in mice because nimodipine was toxic even at lower dosages. Autopsy of these animals revealed death from hypovolemic shock.

Quantitation of retinal neovascularization in treated, untreated, and control animals

The eyes were embedded in paraffin, with a pupillary-optic nerve orientation (PO sections) using a dissecting microscope. The blocks were cut in 40-µm serial sections, until the cornea was reached, and sections containing cornea, lens and retina were obtained. Sections were then collected until the opposite edge of the cornea was reached. The diameters of the cornea in mice were consistently about 1500 µm, and in rats about 2500 µm. After staining with hematoxylin and eosin and mounting with Cytoseal (Stephen Scientific), the slides were coded and presented to a masked observer who counted the number of nuclei anterior to the internal limiting membrane. All the serial sections were studied, and for the final counting of new vessels, the values from the chosen ten sections were averaged to give a single experimental value.

For statistical analysis, groups were compared using Student's unpaired/test and ANOVA. Optic nerve sections ± 500 µm in size were excluded from the counting to avoid the hyaloid system.

Growth factors-ELISA

Only rats were used for the study with growth factors and were treated exclusively with Ginkgo-biloba and dipyridamole. TGF-β2, VEGF and PDGF quantikines were obtained from R&D Systems (Minneapolis, MN). Four eyes (two rats) were used for the evaluation of each growth factor. The enucleated eyes were processed on days 2 and 4 of room air, and the control animals on day -1 of room air (day 9 of study, still with oxygen) and days 2 and 4 of hypoxia.

Immediately after enucleation the eyes were coded and placed at -80°C. Before use, the reagent and the eyes were brought to room temperature. Using a dissecting microscope before processing, all the eye content except sclera and lens was macerated in phosphate buffer saline, centrifuged and, after removing the particles, the remaining fluid was stored at -20°C. Following the manufacturers directions a standard ELISA technique, partially modified by Nieto and Carbonetto, was used (13-15). The study solutions were prepared at 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml and 31.2 pg/ml dilutions. A Microrider, Organon Teknika EL 301 spectrophotometer was used. They were evaluated blind by one of us (HG). The results were expressed in mg/ml for total proteins, in pg/ml for the growth factors, and in pg/ml/mg for the percentage of growth factors with total protein.

RESULTS

On gross inspection and by transillumination, the areas of neovascularization could be seen clearly through the sclera in all the diseased eyes. They had focal deep-blue condensations scattered uniformly throughout the equator. These areas were clinico-pathologically correlated. Control groups in rats (Fig. 2) and mice (Fig. 3) had 100% neovascularization. Treated animals had far fewer new vessels (Fig. 4). Animals allowed to grow in normal conditions had no new vessels, except in the posterior hyaloid system. The new vessels were uniformly distributed, with no regional variability within similar eyes. The new vessels originating from retinal blood vessels could be clearly seen passing

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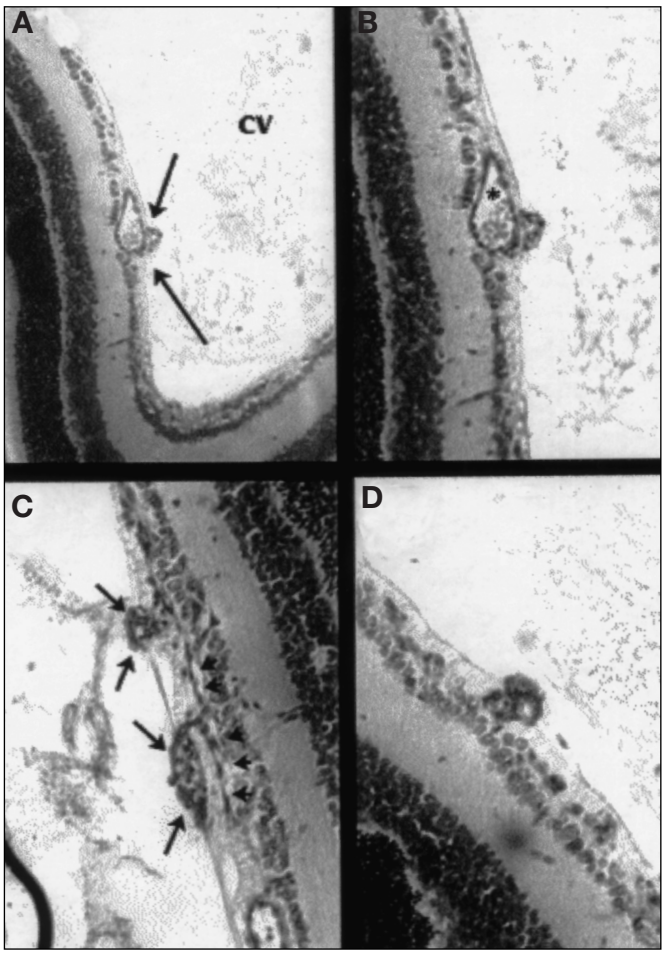

Fig. 1 - *Histopathological findings in rats, day 14.* ***A****: A neovascular crest is observed-black arrows invading the vitreous cavity (CV) - with a congested retinal vessel (HE x 200).* ***B****: The same, magnified, * congested vein (HE x 400).* ***C****: Two neovascular crests are observed: black arrows emerging through the internal limiting membrane (ILM) towards the vitreous cavity, closely connected to a retinal vessel - arrows head (HE x 400).* ***D****: Another small neovascular sprout emerges through the ILM (HE x 400).*

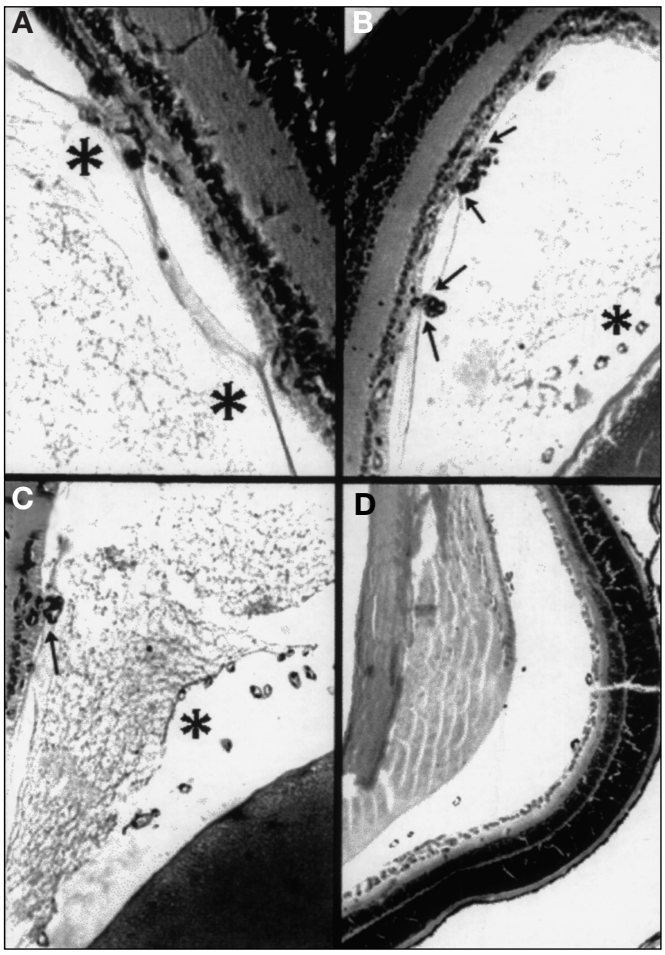

Fig. 2 - *Histopathological findings of eyes of control mice, day 10.* ***A****: New blood vessels - marked with *- appear to infiltrate the vitreous, with traction and detachment of the posterior hyaloid membrane resembling what occurs in human diabetic retinopathy (HE x 400).* ***B*** *and* ***C****: The same: black arrows show new vessels invading the vitreous, and * indicate new vessels behind the lens (HE x 400).* ***D****: A view of the new vessels at lower magnification (HE x 150).*

through the internal limiting membrane (ILM), and becoming adherent to the extracellular matrix in the vitreous (Figs. 1, 2). The newly formed vessels appeared to proliferate from the inner retina as well as the hyaloid system. The new vessels from the retina in both species extended beyond the equator going through the lens and even behind the iris (Figs. 2, 3). At no point were adhesions of these sprouts of new vessels seen to the iris, to the lens or rubeosis iridis. There were occasional focal areas of intraretinal hemorrhage along the new formed vessels, but no evidence of inflammatory cells.

Nimodipine significantly inhibited the growth of new vessels in rats, as shown in Table I. Similar results were found with Ginkgo-biloba extracts and in the dipyridamole group (Tab. I).

In the mice the results were similar. Dipyridamole controls had significantly more nuclei than the treated group, and so did Ginkgo-biloba controls (Tab. II). Even though the counting of nuclei excluded the optic nerve cuts, and hence the majority of the hyaloid system, the drugs also clearly inhibited this system.

The growth factor responses are shown in Table III. A gradual, very significant increase in VEGF in

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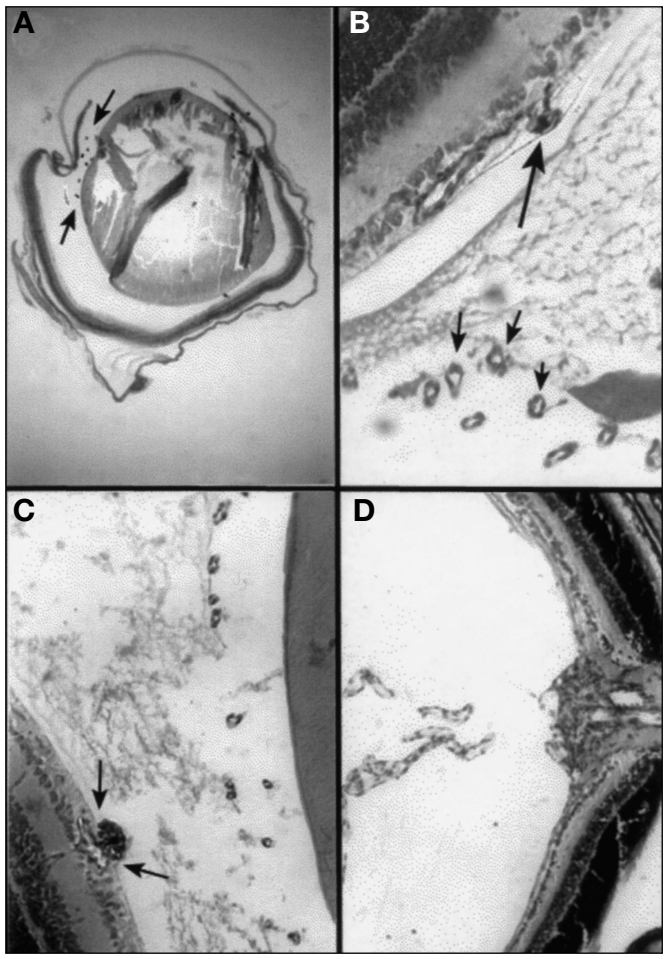

Fig. 3 - *Histopathological findings in eyes of control mice, day 10.* ***A****: PO section of the whole eye. The eye is smaller than the rat eye. Black arrows point to anterior neovascularization behind the iris and the ciliary body (HE x 100).* ***B****: Neovascular crest pressing against the ILM - big arrow. Small arrows indicate vitreal neovascularization (HE x 400).* ***C****: New vessels clearly originating from a retinal vessel, erupting through the ILM (HE x 400).* ***D****: New vessels originating in the posterior hyaloid, from the optic nerve head towards the vitreous cavity (HE x 400).*

response to relative hypoxia (room air) contrasts with the significant inhibition by Ginkgo-biloba extracts and dipyridamole ($p < 0.0005$) (Fig. 5). TGFβ2 showed a gradual increase in relative hypoxia at days 2 and 4 of room air. Ginkgo-biloba and dipyridamole both markedly inhibited this growth factor to the point that at day 4 the inhibition was comparable to controls ($p < 0.0005$) (Fig. 6). With PDGF, there was also a gradual rise when the animals were in relative hypoxia, i.e. room air, on days 2 and 4 ($p < 0.0005$). (Fig. 7).

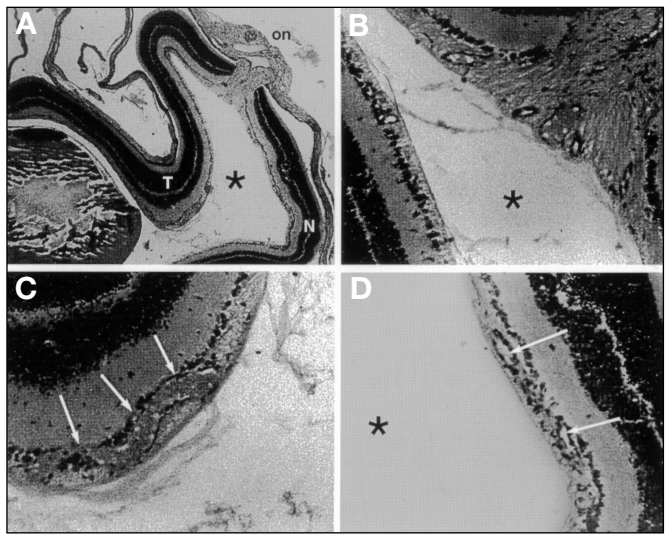

Fig. 4 - *Histopathological findings in rats, treated with nimodipine day 14.* ***A****: PO section, low magnification showing an almost empty vitreous (*). ON, optic nerve, T, temporal; N, nasal (HE x 100).* ***B****: High-power view of an optic nerve section, largely devoid of hyaloid system (*).* ***C****: Temporal, and* ***D****: Nasal retina of same animal with congested vessels (white arrows), vitreous remnants without gross neovascularization (HE x 400).*

TABLE I - MEAN ENDOTHELIAL CELL NUCLEUS COUNTS ANTERIOR TO THE INTERNAL LIMITING MEMBRANE (ILM) PER RETINAL SECTION IN RAT CONTROLS AND TREATED GROUPS

Drug	Control	With treatment
Ginkgo-biloba	344 ± 53 (n:15)	136 ± 29 (n:11)*
Dipyridamole	303 ± 69 (n:13)	131 ± 48.5 (n:11)*
Nimodipine	310 ± 69 (n:14)	121 ± 53 (n:16)*

*P<0.0005 for control and treated group

TABLE II - MEAN ENDOTHELIAL CELL NUCLEUS COUNTS ANTERIOR TO THE ILM PER RETINAL SECTION IN MOUSE CONTROLS AND TREATED GROUPS

Ginkgo-biloba	81 ± 21 (n:6)*
Dipyridamole	90 ± 25 (n:6)**
Control	186 ± 46 (n:7)***

*, ** vs *** (p<0.0005)

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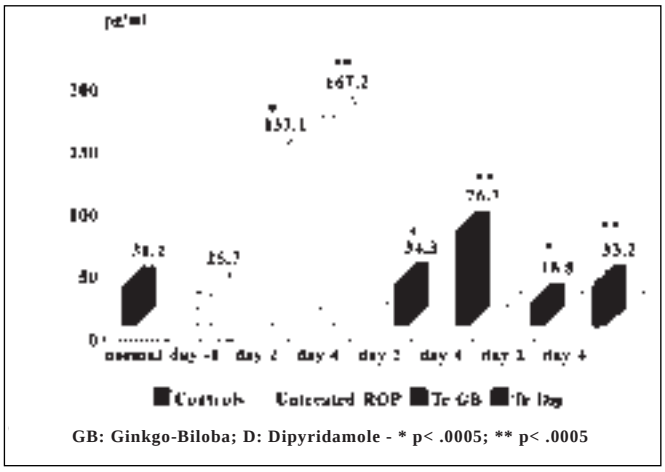
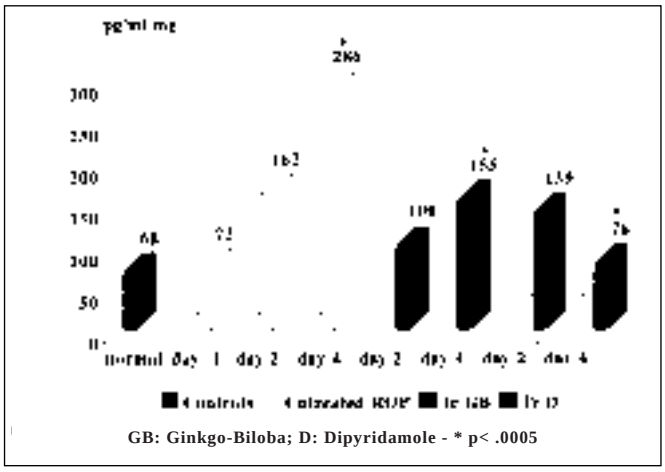


GB: Ginkgo-Biloba; D: Dipyridamole - * p< .0005; ** p< .0005

Fig. 5 - *VEGF levels by ELISA, on experimental ROP in rats treated and controls.*

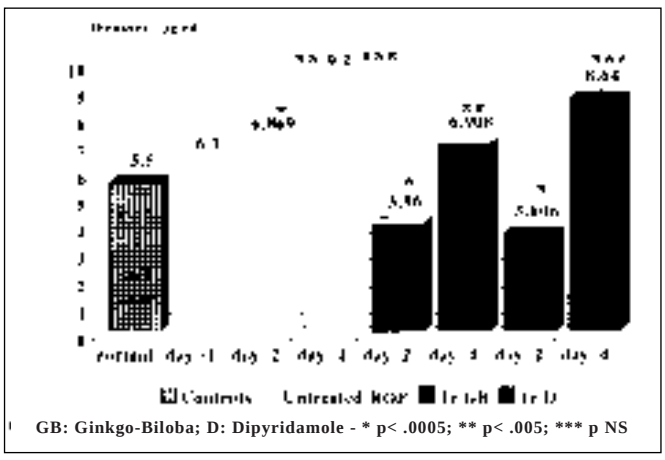


GB: Ginkgo-Biloba; D: Dipyridamole - * p< .0005

Fig. 6 - *TGF-β2 levels by ELISA, on experimental ROP in rats treated and controls.*

DISCUSSION

Nimodipine, gingko-biloba and dipyridamole clearly inhibited the neovascularization induced in the cyclic oxygen model in rats and mice. Except with gingko-biloba (16), to the best of our knowledge, no previous studies of these drugs in experimental ROP have been described. The fact that no complete inibition of angiogenesis was observed agrees with other studies showing similar percentages (17, 18). These results probably reflect the extremely complex process of neovascularization involving endothelial activation, migration and invasion of the extracellular matrix, a process that can be arrested at multiple and intricate points (8, 17-19).



GB: Ginkgo-Biloba; D: Dipyridamole - * p< .0005; ** p< .005; *** p NS

Fig. 7 - *PDGF levels by ELISA, on experimental ROP in rats treated and controls.*

TABLE III - GROWTH FACTORS AND TOTAL PROTEINS IN RAT EYES

Sample	Total protein	VEGF		TGF-β2		PDGF-AB	
	µg/ml	pg/ml	pg/ml/mg	pg/ml	pg/ml/mg	pg/ml	pg/ml/mg
No O_2 exposure	62.5	31.2	0.49	68	1.09	5500	88.0
O_2 exposure day 1	70.4	25.7	0.40	71	1.02	6100	86.6
O_2 exposure day 2	64	133.1	1.89	162	2.53	6860	107.2
O_2 exposure day 4	72.6	167.2	2.31	286	3.94	9200	126.7
GB day 2	65.3	34.3	0.53	100	1.53	3960	60.6
GB day 4	94	76.7	0.81	155	1.65	6908	73.5
D day 2	79.9	18.6	0.23	139	1.74	3606	45.1
D day 4	69.3	33.2	0.48	78.0	2.35	8640	124.6

GB: Gingko-biloba - D: Dipyridamole

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We cannot conlcude that the inhibition of newly formed blood vessels is solely due to choroidal vasodilation bringing more oxygen to the hypoxic inner retinal layers. Besides their vasodilating properties, these drugs have multiple actions at the cellular level behaving as antiinflammatory and in some cases as antiproliferative agents, like in the case of gingko-biloba (20). The three study drugs are anti-platelet aggregating factors (PAF), inhibiting common intracellular mediators and proto-oncogenes at the molecular level (21-24), and acting through PAF receptors in their membranes (25). They have been isolated on the cesik retina (26), and in the retina and choroid in the rat (27). In the rat retina they have been found mainly in the retinal ganglion and microglial cells (27). Microglia are related to immune responses producing different cytokines and are scavengers for apoptotic cells (28). Intravitreal injection of PAF in rabbits induced immediate rupture of the blood-retinal barrier, generalized vascular thrombosis with total vascular occlusion 3 or 4 hours after the injection (29).

The idea of increasing the retinal and choroidal blood flow with a view to improving a possible ischemic component has recently been exploited in glaucoma (30-35), and in age-related macular degeneration (36), but these studies paid little attention to the possible neuroprotective effects of these medications. Both nimodipine (31, 32) and ginkgo-biloba extracts (35) seemed to improve visual functions and increase retinal and choroidal blood flow. *In vitro* studies show that dipyridamole induced concentration-dependent relaxation and suggest the use of this drug in patients with ocular and ophthalmic vascular dysfunctions (37). Campochiaro et al reported that a single intraocular injection of dipyridamole in animal models caused a reversible, marked dilation of retinal vessels. However, to our knowledge, there are no reports of dipyridamole's effects on the choroidal vasculature (38). A partial inhibition of the normal developing hyaloid system with these drugs is also worth noting. In our study nimodipine was toxic to mice, secondary to what appeared to be peripheral vasodilation and hypovolemic shock in the autopsied animals. Presumably due to a difference in species susceptibility this complication was not observed in rats.

The growth factors assessment in this study reflects their gross accumulation in isolated tissues. They were measured in a blind fashion by a single observer and even though they have no topographical meaning, they may indicate a total accumulation in reponse to the ischemic injury and to the drugs. As regards VEGF the progressive increase of hypoxia in the control animals agrees with previous reports of the release of this growth factor under ischemic conditions (16, 17, 39, 40). The significant arrest produced by ginkgo-biloba and dipyridamole may reflect an improvement in the hypoxia itself as well as inhibition of the transduction mechanism and proto-oncogenes like C-Fos, C-Jun, NF-AT, and NF-KB (21-24, 41).

In conclusion ginkgo-biloba extracts, dipyridamole and nimodipine had a neovascular inhibitory effect in rat and mouse ROP models. This effect on retinal neovascularization might be explained either by direct regulation of stimulatory or inhibitory growth factors, or indirectly, by increasing retinal and choroidal blood flow, or a combination of both.

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