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ORIGINAL RESEARCH

Intraocular and systemic levels of vascular endothelial growth factor in advanced cases of retinopathy of prematurity

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Purpose: To measure vitreous, aqueous, subretinal fluid and plasma levels of vascular endothelial growth factor in late stages of retinopathy of prematurity.

Methods: Interventional study. We enrolled patients with clinical diagnoses of bilateral stage V retinopathy of prematurity, confirmed by b-scan ultrasound and programmed for vitrectomy. During surgery we took samples from blood, aqueous, vitreous, and subretinal fluids. The vascular endothelial growth factor concentration in each sample was measured by ELISA reaction. A control sample of aqueous, vitreous and blood was taken from patients with congenital cataract programmed for phacoemulsification. For statistical analysis, a Mann–Whitney and a Wilcoxon W test was done with a significant P value of 0.05.

Results: We took samples of 16 consecutive patients who met the inclusion criteria. The vascular endothelial growth factor levels in the study group were: aqueous, 76.81 ± 61.89 pg/mL; vitreous, 118.53 ± 65.87 pg/mL; subretinal fluid, 1636.58 ± 356.47 pg/mL; and plasma, 74.64 ± 43.94 pg/mL. There was a statistical difference between the study and the control group ($P < 0.001$) in the aqueous and vitreous samples.

Conclusion: Stage 5 retinopathy of prematurity has elevated intraocular levels of vascular endothelial growth factor, which remains high despite severe retinal lesion. There was no statistical difference in plasma levels of the molecule between the control and study group.

Keywords: VEGF, late stages, ROP

Introduction

Retinopathy of prematurity (ROP) is an ischemia-induced vasoproliferative disease of multifactorial etiology.[1–3] The hallmark of this pathology is the lack of development of normal peripheral retinal vasculature, secondary to exposure to high oxygen levels during the neonatal period. This leads to vascular changes, hypoxia, and overexpression of vascular endothelial growth factor (VEGF), which favors the formation of peripheral neovascularization, and finally retinal detachment.[4,5] Despite the traditional role that has been given to the high levels of oxygen in the genesis of ROP, it is now known that there are other risk factors like low birth weight (less than 1500 g), short gestational age (GA) (32 weeks or less), neonatal hypoxia, hypotension, acidosis, neonatal sepsis, the need of mechanical ventilation, ventricular hemorrhages, the use of steroids after birth, blood transfusion, the presence of patent ductus arteriosus, apnea, and production of other molecules such as erythropoietin and other cytokines that could also increase the risk of presenting the disease.[2,3,6–9]

VEGF is a dimeric glycoprotein, normally expressed in epithelial and neoplastic cells.[10] Under hypoxic conditions, it is produced by pericytes, retinal pigment

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epithelium, and glial cells.[4,10–12] Its biological activity is mediated by high affinity receptors (Flt-1 [VEGFR1], Flk-1/KDR [VEGFR2]), and it is an essential factor for retina angiogenesis, which under physiologic conditions results in the formation of the superficial and deep capillary plexus of the retina.[5,10,13–15]

The relationship between VEGF and ROP has been studied before.[5,10,16–21] Vitreous concentrations were found elevated in all stages of the disease.[11,22,23] However the opportunity to measure VEGF concentration in various ocular compartments at the same time is rare, since this represents, in most cases, an additional risk for the patient that is not necessarily justified. Therefore, the aim of this study is to measure the VEGF concentration in aqueous, vitreous, subretinal fluid (SRF), and plasma of patients with stage 5 ROP, programmed for open-sky vitrectomy and compare them with a control group of healthy patients diagnosed with congenital cataract and programmed for phacoemulsification.

Patients and methods

The study was reviewed and approved by the hospital internal review board, and all procedures were performed according to the tenets of the Declaration of Helsinki. An informed consent form was obtained from all parents or legal guardians of the patients after a complete explanation about nature, severity, possible consequences and poor visual and anatomical prognosis of each particular case, and after they had received psychiatric counseling. The study was conducted entirely in our hospital facilities (level-3 ophthalmology hospital) which have no neonatal care. The patients included in this study were selected from the cases referred to our institution from other hospitals and private offices.

We screened patients with medical history of prematurity (32 weeks of gestation or less), low birth weight (2500 mg or less), use of supplemental oxygen after delivery and clinical diagnosis of bilateral ROP stage 5, confirmed by b-scan ultrasound, according to the international classification of ROP from 1984 and 1987[24,25] who were programmed for open-sky vitrectomy. In addition, patients were classified into 2 groups: vascular-active ROP and vascular-inactive ROP in a similar manner to that made by Sonmez et al,[5] but in ROP stage 5 during surgery. The presence of retinal vascular changes (dilatation, tortuosity, or vitreous hemorrhages), as well as the persistence of neovascularization in the zone of traction, were the criteria for considering the case as active. We excluded patients with a history of previous treatment for ROP (laser, cryotherapy, or anti-VEGF drugs), surgery, and other significant systemic or ocular pathologies. The decision about which eye to operate on was made by the surgeon (HQM, GGA) depending on the appearance of the retinal detachment in the ultrasound at the operating room. All surgeries were done under general anesthesia.

For the control group, we enrolled patients with clinical diagnosis of congenital cataract, with no history of prematurity, normal birth weight (2500–3500 mg) and no significant systemic or ocular pathologies, who were programmed for phacoemulsification with posterior capsulorhexis and intraocular lens implantation. An additional informed consent form was used for the control group.

Sample collection

In the study group, undiluted aqueous, vitreous, and SRF samples were collected from each eye during surgery. Special care was taken to not contaminate the samples with tissue or blood during the extraction process. The aqueous sample was obtained with a 30GA needle by clear corneal paracenthesis, prior to the placement of viscoelastic and corneal trephination. The vitreous sample was obtained after lens extraction and retrolental membrane dissection by 23GA open-sky vitrectomy and manual suction. Finally the SRF sample was obtained by external needle drainage through a radial sclerotomy to avoid sample contamination.

The samples were then placed in eppendorf sterile tubes (Eppendorf AG, Hamburg, Germany) and stored at −70°C until analysis. The blood samples were collected by the anesthesiologist when gaining IV access for the anesthetic procedure. The sample was placed in a sterile tube with EDTA (Vacutainer™, Becton Dickinson and Co, Franklin Lakes, NJ, USA) for immediate centrifugation at 5000 rpm for 5–10 minutes, until a clear separation between serum and the cell component was seen. The serum was then transferred into an eppendorf sterile tube and stored at −70°C until analysis.

In the control group, for each eye, a 2-port limbal phacoemulsification was used. An undiluted aqueous sample was collected by corneal parasenthesis with a 30GA needle before port construction. After lens aspiration, the anterior chamber was completely filled with viscoelástico material (Amvisc® Bausch and Lomb, Rochester, NY, USA). A posterior continuous curvilinear capsulorhexis was performed and undiluted vitreous sample was collected during the anterior vitrectomy. The samples were then placed in eppendorf sterile tubes and stored at −70°C until assay. The

blood sample was taken by the anesthesiologist and processed in the same way as in the study group.

All samples were processed in the laboratory of the Department of Cellular and Molecular Neurobiology, Universidad Nacional Autónoma de México, Queretaro, Mexico. After centrifugation at 5000 rpm for 10 minutes, the supernatant was discarded and an enzyme-linked immunosorbent assay (ELISA) reaction was made with a commercial kit (Quantikine™ R&D Systems Minneapolis, USA) according to the manufacturer's instructions, with a sensitivity of up to 9.0 pg/mL of VEGF. We used 50 µL of each sample for VEGF ELISA reaction. The optical density was determined at 450 nm, using an absorption spectrophotometer. The mean values of 2 readings were used for quantitative analysis.

The statistical analysis was performed using SPSS software, version 16.0 (Sigma Stat; Systat Software, Inc., San Jose, CA, USA). Data are presented as median and standard deviation. A Friedman/Wilcoxon *W* test and Mann–Whitney *U* test was used to compare VEGF concentrations between groups. A *P* value of less than 0.05 was considered to be statistically significant. The study was registered as a clinical trial (http://www.clinicaltrials.gov).

Results

We included a total of 16 consecutive patients (10 males) in the study group and 8 patients (5 males) in the control group. 7 patients (43.75%) of the study group were classified as vascular-active and 9 patients (56.25%) as vascular-inactive. The demographic data of the study and control group are summarized in Table 1. The median ± standard deviation of the GA at delivery, in the study group was 29.19 ± 2.23 weeks and in the control group 40.63 ± 1.06 weeks ($P < 0.001$). The median birth weight of patients in the study group was 1274.81 ± 140.83 g and in the control group 3311.88 ± 235.95 g ($P < 0.001$). The median age in the study group at the moment of surgery was 8.56 ± 2.61 months of age and in the control group 70.63 ± 12.40 months ($P < 0.001$). The ELISA results for VEGF of the control and study group are summarized in Table 2. There was a statistical difference between the concentrations of VEGF in aqueous and vitreous samples

Table 1 General demographic data

	No.	Gender	Gestational age (weeks at delivery)	Birth weight (gr)	Age at surgery (month)	Disease status
Study group	1	M	29	1135	9	VI
	2	F	26	1040	7	VA
	3	M	26	1352	10	VA
	4	F	27	1441	9	VI
	5	M	27	1200	10	VI
	6	M	31	1501	12	VI
	7	M	32	1280	3	VI
	8	M	32	1370	11	VA
	9	F	32	1180	11	VA
	10	M	31	1205	10	VA
	11	M	30	1386	8	VI
	12	M	28	1328	12	VI
	13	F	28	1090	8	VA
	14	F	28	1164	6	VI
	15	M	28	1490	5	VI
	16	F	32	1235	6	VA
Control group	1	M	39	3450	63	ND
	2	M	40	3200	72	ND
	3	M	41	3500	84	ND
	4	F	42	3650	61	ND
	5	M	40	3015	76	ND
	6	F	41	3260	86	ND
	7	F	40	2990	49	ND
	8	M	42	3430	74	ND

Notes: The gestational age and the birth weight were data extracted from the clinical history told by the parents and not from a medical record. Therefore the data may have some final variation.

Abbreviations: M, male; F, female; VA, vascular-active; VI, vascular-inactive; ND, no disease.

Table 2 Study and control group VEGF results

	No	Aqueous (pg/mL)	Vitreous (pg/mL)	SRF (pg/mL)	Plasma (pg/mL)
Study group	1	86.98	37.00	1326.48	98.78
	2	117.36	203.25	1850.63	68.23
	3	103.20	164.52	2035.54	119.42
	4	138.01	175.12	1957.3	184.94
	5	95.54			69.88
	6	37.73	113.57	1092.08	87.55
	7	49.08	215.37	1133.08	52.19
	8	259.51	158.76	2057.32	42.15
	9	16.50	61.4	1523.56	67.26
	10	26.15	70.02	1881.00	12.46
	11	65.45	205.12	1431.83	98.76
	12	79.61	68.95	1658.34	130.59
	13	83.54		2124.32	42.95
	14	16.13	88.50	1259.30	37.59
	15	16.20	42.3	1333.92	38.89
	16	38.03	55.6	1883.95	41.05
Control group	1	12.97	12.2		44.1
	2	14.52	7.8		62.8
	3	16.07	5.1		28.4
	4	13.36	0		42.16
	5	16.46	14.7		32.61
	6	18.14	8.2		45.22
	7	13.25	15.1		58.38
	8	16.42	11.4		51.14

Notes: The blanks in the study group correspond to samples that were discarded due to contamination during sampling. The control group does not have SRF sample.
Abbreviations: VEGF, vascular endothelial growth factor; SRF, subretinal fluid.

of the study versus the control group ($P < 0.005$) but not between groups for plasma. During the collection of samples, vitreous samples of patients 5 and 13 and SRF sample of patient 5 had to be discarded due to contamination with the patient's blood. Finally, we regrouped the study group according to vascular-active or inactive status. There was no statistical difference in the VEGF levels of the studied samples (Table 3).

Discussion

The vascularization of the retina begins during the 14th week of gestation. This consists of 2 well recognized stages. In the vasculogenesis stage, precursor cells of mesenchymal origins enter the retina through the optic nerve. These cells are responsible for the formation of the main retinal vessels.[26] In the angiogenesis stage, the number of capillary vessels increases and the peripheral retina becomes vascularized.[27,28] In order to carry out the angiogenesis stage, it is necessary for the following events to occur: the stimulation, proliferation, and migration of endothelial cells; the proteolytic breakdown of the endothelial basement membrane; the degradation of the adjacent extracellular matrix; the recruitment of support cells (pericytes); and finally the close of the vascular circuit.[29]

Growth factors are molecules with different biological activities. After secretion by cells they can have autocrine, paracrine and endocrine activity.[28,29] There are several growth factors identified so far. Some of them have a regulatory role in the vascularization of the retina by stimulating or inhibiting the formation of vessels in either normal or pathological conditions.[1,26,30] VEGF is a dimeric glycoprotein overexpressed under hypoxic conditions.[10–12,26] It directly stimulates the development of internal and external eye vasculature and also acts as a vascular permeability factor.[26,29] In ROP, there is an arrest of the normal development of peripheral retinal vascularization due to a series of factors that induce vascular changes. After delivery and after the primary insult ceases, the retinal oxygen demand suddenly increases. The lack of peripheral vessels prevents the oxygen supply needed to meet the new demand. Therefore the retina enters into a state of acute hypoxia which favors overexpression of VEGF. In an attempt to counteract the deleterious effects of an excessive level of VEGF, the retina starts producing vasoinhibins – a group of molecules derived from prolactin (ie, 16 Kd-PRL) that inhibit angiogenesis and vascular functions.[30–33] However, VEGF production is high enough that it easily surpasses the production of inhibitors. This series of events leads to the formation of a demarcation line between the vascular and avascular retina, the growth of new vessels toward the

Table 3 Vascular-active and vascular-inactive results

Group	Gestational age	Aqueous VEGF (pg/mL)	Vitreous VEGF (pg/mL)	SFR VEGF (pg/mL)	Plasma VEGF (pg/mL)
Vascular-active	29.57 ± 2.82	92.04 ± 83.52	118.93 ± 64.01	1908.05 ± 199.06	56.22 ± 33.62
Vascular-inactive	28.89 ± 1.76	64.97 ± 39.88	118.24 ± 71.63	1399.04 ± 286.35	88.80 ± 47.44
Alpha value	0.66	0.53	0.94	0.09	0.2

Notes: The comparison between the results of vascular-active and vascular-inactive patients with a Friedman/Wilcoxon *W* test and Mann–Whitney *U* test did not show a statistical difference between these 2 groups.
Abbreviations: VEGF, vascular endothelial growth factor; SRF, subretinal fluid.

vitreous, and finally in a tractional retinal detachment with severe visual impairment.[5,34]

The results of this study confirm the existence of elevated VEGF levels in vitreous, aqueous and SRF in eyes with stage 5 ROP, which remain high despite the significant damage to the intraocular structures. Although concentration in vitreous and SRF have been studied previously, there is little information about the quantities of the molecule in aqueous humor in this disease. VEGF is considered a key molecule in the regulation of angiogenesis.[5] However, it is also found in increased amounts in other non-neovascular pathologies like retinal detachment and some types of uveitis.[35,36] The high levels of VEGF in aqueous have even been associated with the etiology of primary open-angle glaucoma, pseudoexfoliation glaucoma, and neovascular glaucoma.[37,38] Some studies have suggested that these kinds of glaucoma are causes of visual impairment in patients with advanced cases of ROP, even despite a successful surgical treatment.[39]

In the paper published by Sonmez et al, the authors measured the concentrations of VEGF in vitreous in patients with stage 4 ROP.[5] It is remarkable that they found a statistical difference between the patients with a vascular-active disease and the patients considered to have a vascular-inactive disease.[5] In our study, we tried to apply a similar approach to classify the patients in the study group as active or inactive. However, when comparing the results between these 2 papers, the VEGF levels in the vitreous of our active group were significantly lower than those reported previously by Sonmez (3454 versus 118.93 pg/mL). In fact, if we compare the combined results of our 2 groups, both vascular-active and vascular-inactive, the vitreous VEGF levels are more similar to those found in the vascular-inactive patients in the study by Sonmez et al (316 versus 118.53 pg/mL). This makes us question whether in fact what we classified as vascular-active disease was simply remnants of the retinal vasculature rather than active neovessels.

In another similar paper by Lashkari et al,[10] the authors measured the VEGF concentration in SRF in patients with stage 4 and 5 ROP. Once again, our results were significantly lower (14770 versus 1638.58 pg/mL) despite only taking into account the vascular-inactive group.[10] A significant difference between the Lashkari et al paper and ours is that Lashkari et al did not mention the ages of the stage 5 patients, which might have been the cause of the difference between the results. In our study group, the average age at the time of surgery was 8.56 ± 2.61 months. If the patients in the Lashkari study were younger at the time of sample collection that could explain the higher levels of VEGF in SRF.

There are a few limitations in this study to address. First, the limited number of patients in the study group makes the study lack sufficient statistical power to make more definitive statements, and the statistical analysis available is very limited. A more comprehensive statistical analysis, such as a correlation between ROP risk factors and VEGF levels would have been desirable, but the fact that the control group was not well matched with the study group would have introduced a confounding factor. Despite observing significant differences in the VEGF levels of all intraocular spaces between the study and control groups, the information provided so far by our study does not allow a clear differentiation between in-situ VEGF production or accumulation of the molecule in the intraocular spaces due to an increased permeability secondary to chronic injury. A more appropiate approach would have been to simultaneously measure basal levels of a protein that has a constant presence in plasma. If the concentration of this protein had also been high in the intraocular spaces, it would be due to an increased permeability rather than in-situ production. Finally, the control group was neither age nor weight matched.

In summary, ROP is a public health problem in developing countries and is a major cause of visual disability among children. The ophthalmologic disorders occurring in patients after stage 5 ROP are not well described. The results of this study detect consistently high levels of VEGF in all studied fluids, which were statistically higher than those detected in the control group. However, the results were also consistently lower than those reported in similar series. The question of whether the increased VEGF level detected were due to a residual in-situ production or an increase in permeability of the damaged tissue was not answered with this study. Further studies are needed to complete the information obtained in this study.

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Disclosure

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