Indian J Physiol Pharmacol 2016; 60(3) : 282–290

Original Article

Pharmacological Interventions for Vascular Targeting In Retinopathy of Prematurity : An Experimental Study

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Abstract

Purpose: This study was conducted to evaluate the pharmacological interventions totarget vascular proliferation in the Retinopathy of Prematurity (ROP).

Methods: Protein Kinase C modulator (Bryostatin), tubulin polymerization inhibitor (Dolastatin 10), anti-VEGF (Bevacizumab) and a non-specific VEGF inhibitor (Thalidomide) were screened in Retinopathy of Prematurity (ROP) model. The retinal vasculature was evaluated by calculating the tortuosity indices of vessels and electroretinography responses in terms of 'b' wave amplitude and was recorded from ROP rats on postnatal Day 17 and Day 25.

Results: Retinopathy was seen in the form of tortousity of vessels at the posterior pole with arteries being affected more than veins. Maximum reduction in tortousity of vessels and the highest 'b' wave amplitude noted in bryostatin with a significant correlation between the two.

Conclusion: Bryostatin showed a potential anti-angiogenic effect on the progression of ROP and may hold a promising future in the treatment of ROP.

Introduction

Retinopathy of Prematurity (ROP) is a vasoproliferative disorder that occurs in the incompletely vascularised retina of premature infants and is an important cause of childhood blindness in both the developed and the developing countries (1).

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(Received on April 12, 2015)

ROP is characterised by abnormal blood vessel growth and avascular retina temporal to disc in prematurely born babies. This vasoproliferative disorder is among various retinopathies which revolve around the altered molecular pathway involved in the formation of blood vessel at the time of hypoxia.

Marine isolates such as bryostatin and dolastatin are under various clinical trials for their effect in inhibiting the angiogenesis during the tumour progression and metastatic cancer.Dolastatin, a tubulin binding agent, causes the cell cycle to arrest

at the G2- M phase leading to apoptosis.Bryostatins, another class of Protein kinase- C modulators, are currently in Phase- II trials for solid tumours and haematological malignancies. Bryostatin 1 acts like phorbol ester and binds to C1 domain of PKC for its activation (26–28). Bryostatin 1 exhibits a high affinity for PKC and displaces phorbol esters from PKC at low nano- to picomolar levels but in micromolar concentration it provides longer exposure thus downregulation of PKC as its prolonged exposure results in membrane depletion of PKC and results in inactivation of the PKC activity (29). Thalidomide has been reported to reduce the expressions of angiogenic factors such as VEGF and FGF whereas bevacizumab is an antibody which directly binds and neutralizes the effect of VEGF.

Above mentioned marine isolates and molecule such as thalidomide were never evaluated for their therapeutic effect in the ROP. As these molecules are under investigation for inhibiting angiogenesis in cancer, these could be promising in halting the pathogenesis in the case of retinopathy of prematurity as this disease pathology revolves around angiogenesis. Although, bevacizumab currently occupies the role of an adjuvant to laser photocoagulation in the treatment of ROP, there are no other pharmacological interventions are available. The effect of VEGF on retinal vascular permeability appears to be mediated by the β-isoform of PKC and the inhibition of cell cycle bytubulin binding agents, may lead to regression of the aberrant nascent vessels forming the basis of their use in neovascular ocular diseases. Considering the diverse role of these agents, a search for a safest agent in the developing retina carries paramount importance in the therapy of ROP in infants.

This study was conducted to evaluate the pharmacological interventions totarget vascular proliferation in the Retinopathy of Prematurity (ROP) in experimental animal model using rat neonates. Oxygen induced retinopathy model was used to evaluate protein kinase C modulator (bryostatin), tubulin polymerization inhibitor (Dolastatin 10), anti-VEGF (Bevacizumab) and a non-specific VEGF inhibitor (Thalidomide).

Materials and Methods

A prospective, comparative study using 36 neonatal Wistar rat pups was carried out in accordance with the recommendations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.Healthy Wistar rat pups surviving until Day 25 of life were included in the study. We have used custom made hyperoxia chamber in our study. The oxygen chamber, made of acrylic sheet (Widson Scientific, Delhi) was fitted with pro-ox P110 oxygen controller with E702 oxygen sensor (Biospherix, USA). Oxygen cylinder was used for the supply of gas, in order to raise the oxygen levels within the host chamber. A two stage nitrogen pressure regulator was used to deliver oxygen from the cylinder inside the host chamber with a controlled flow of 10 psi/ min, humidity maintained to 37%.

Neonatal rat pups were exposed to 75% hyperbaric oxygen in the above mentioned custom made hyperoxia chamber,from postnatal Day 7 till postnatal Day 12 after which they were transferred to room air with 21% oxygen and given 2 divided doses of subcutaneous injections of drugs 48 hours apart, started from PN 12. The pups were randomised into 5 groups (n=6) of bryostatin (2 µg/15 g), dolastatin (2 µg/15 g), bevacizumab (1.5 mg/15 g), thalidomide (1.5 mg/15 g) and Sham based on the treatment given. On postnatal Day 17 and Day 25, the rat pups were subjected to retinal evaluation using Digital Fundus Imagingand Electroretinographywith the help of Micron III Imaging System (Phoenix Research Laboratories, Pleasanton, CA). The animals were weighed at birth, postnatal Day 17 and Day 25. P 17 was chosen to analyse the effect of drug on developing retina, as the rat pups eyes get fully opened till PN day 17 and as ROP tends to regress after 20-25th day of life irrespective of treatment, we have chosen PN day 25 to analyse this change in all group.

Imaging Protocol

a) Anterior Segment Evaluation:-

Neonatal rat pups were anaesthetized using

Ketamine hydrochloride 50 mg/kg (10 µl/g body weight) and the anterior segments of both eyes were focussed using the MICRON III slit lamp attachment and images were captured using Streampix Software. The presence of cataract and new vessels on iris were looked for.

b) Fundus Photography:-

Eyes of the rat pups were dilated using tropicamide 0.8% and phenylephrine 5% (instilled twice 5 minutes apart). Retina was focussed with the objective lens and posterior pole images of both eyes were captured using Streampix software. The field of view was more than 50°. The retinal images were processed into grey version for appropriate tracing of vessels and the tortuosity index of arteries and veins were calculated using Image J software(available at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) with the standard taken as 100 pixels/mm length as has been described previously by Liu et al (5). The straight length of the vessel, and the actual length were measured by using the above software and their ratio was calculated to get tortuosity index of arteries and veins using the same protocol of Liu et al (5).

c) Fundus Fluorescein Angiography:-

The rat tail vein was cannulated using a 26G needle. The exciter wavelength was opted for blue and barrier was kept for green. Fluorescein sodium (10% w/v) solution at the dose of 0.1 ml was injected through the tail vein. Images were captured soon after dye injection till the diffusion of the dye beyond the retinal vessels.

Electroretinography Protocol

The amplitude and implicit times of ‘a’ and ‘b’ wave were calculated using its inbuilt algorithm. The ERGs were recorded on both 17 and 25 day, both the aspect of ERG wave form i.e. ‘a’ and ‘b’ were evaluated, but the reason being to be focused on ‘b’ wave was that, in ROP retinal blood vessels growth get hampered due to the hyperoxia – normoxia driven pathology, and this ‘b’ wave is originated by the collective response of bipolar, amacrine and ganglion cells, which are the part of the inner layers of retina.

Descriptive statistics to determine mean, standard deviation and standard error of mean was performed with appropriate statistical tests using Sigma Stat software (Version 3.5). ‘p’ value was taken two tailed and a value <0.05 was considered to be statistically significant. Inter-group analysis was done using the unpaired t- test and intra-group analysis was done using Paired t- test.

Results

A total of 36 neonatal rat pups were studied by dividing them into various groups based on the treatment administered and compared to room air. The pups were weighed at birth, postnatal Day 12, 17 and postnatal Day 25 to look for any growth retardation in terms of weight loss. The weights were compared to the room- air raised rats. The average body weight of rat pups at day 1 were 4.40 gm (±1.5 gm), at day 12 the avg. body weight of normal rat pups were 18.9 gm (±1.7 gm), whereas the avg. weight of hyperoxia treated group was 16 gm (±1.2 gm) which was lesser than the normal but not significant. Upon treatment with various drugs the avg. weight of bryostatin, dolastatin, bevacizumab and thalidomide at postnatal day 17 were 17.5±0.92, 15.8±0.8, 15.5±0.5 and 19.3±0.3 respectively, where the avg. weight of disease control and normoxia pups at day 17 were 18.9±1.5 and 24.6±0.21 respectively. Although the decrease in weight was seen in all the hyperoxic groups but it wasn’t significantly less when compared to normal signifying minimal or no apparent side effect of the drugs administered.

Anterior Segment Imaging:-

An increased dilatation and tortuosity of iris vessels was seen in all the groups when compared to room air. No evidence of iris neovascularization was seen. Transient lens opacities and corneal haze was noted after administration of ketamine which resolved with the wearing- off of the effect of anaesthesia (Fig. 1).

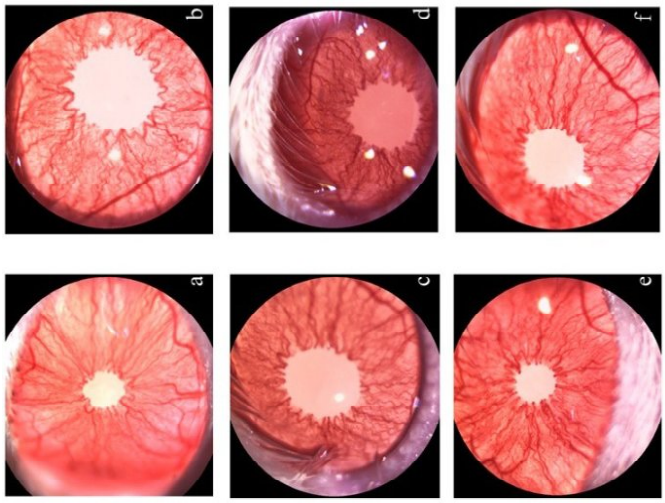

Fig. 1 : Anterior Segment images of rat pups of various groups on Day 17 using MICRON-III retinal imaging system showing an increased dilatation and tortuosity of iris vessels in all the groups as compared to room-air. No evidence of iris neovascularization was present. a) Normoxia b) Sham c) Bevacizumab d)Thalidomide e) Bryostatin f) Dolastatin.

Fundus photography:-

Fundus photographs were taken using MICRON-III retinal imaging system on both Days 17 and Day 25 and the groups were compared (Fig. 2).

(i) Day 17:-

On comparing the tortuosity index of arteries (TI_A) and veins (TI_V), it was noted that the tortuosity index of arteries was much higher than veins showing a greater effect of the disease process on arteries than veins in the rat model of ROP. When compared to room air raised rats, the tortuosity index of arteries in Sham was 31% higher than room air (TI_A = 1.029) which was statistically significant (p=0.002), hence the disease model of ROP was reliably produced in our laboratory. The tortuosity of vessels at the posterior pole was higher in all the groups kept on hyperbaric oxygen as compared to room air. Maximum tortuosity of arteries was noted in Sham (TI_A = 1.349) and minimum was seen with bryostatin (TI_A = 1.126) and bevacizumab (TI_A = 1.167). When compared to Sham, the tortuosity index of arteries was found to be lower in all the other drug treated groups with results achieving statistical significance with bevacizumab with a 13.5% decrease (p=0.040)

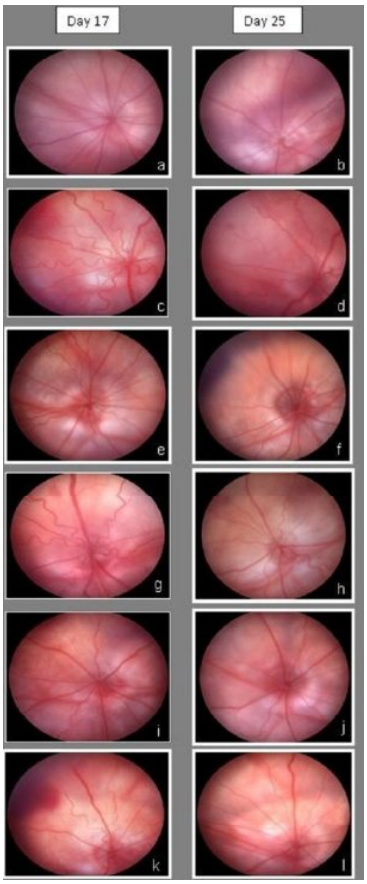

Fig. 2 : Fundus photographs of rat pups of various groups on Day 17 and Day 25 using MICRON-III Retinal imaging system.Showing a decreased tortuosity of posterior pole vessels in drug treated groups in comparison to Sham on day 17. A further reduction in tortuosity of vessels is noted on Day 25 in all the groups.a-b: Normoxia, c-d: Sham, e-f: Bevacizumab, g-h: Thalidomide, i-j: Bryostatin, k-l: Dolastatin.

and bryostatin with a 16.7% decrease (p=0.018) with no significant difference between the two (p=0.394, Fig. 4). There was a mild reduction of tortuosity of arteries in dolastatin by 3.5% which was not statistically significant (Fig. 3).

The tortuosity index of veins was higher in Sham (TI_V = 1.064) as compared to room air raised rats (TI_V = 1.032) though the difference was not statistically significant (p=0.067). The tortuosity index of veins in the drug treated groups was lower as compared to Sham but not to a statistically significant degree (p>0.1). Hence, the tortuosity of veins of the posterior pole was comparable between the groups and did not exhibit a significant difference on Postnatal Day 17 (Fig. 4).

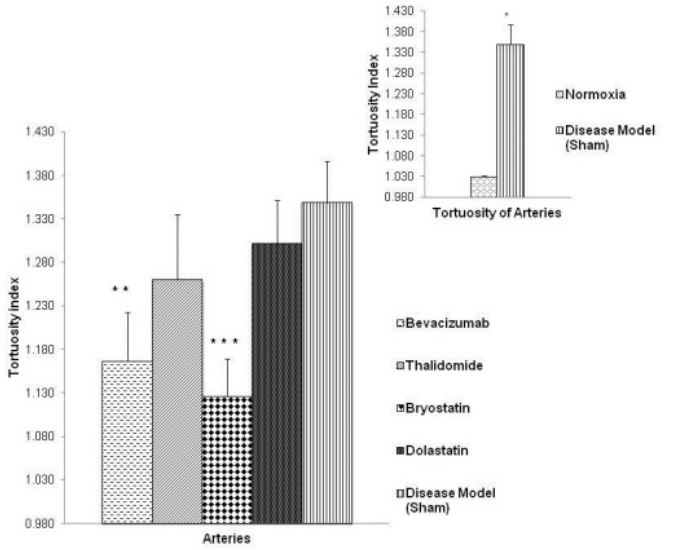

Fig. 3 : Tortuosity of arteries in rat pups on Day 17 (n=6) using Unpaired t- test .Tortuosity of arteries in disease control is higher than room-air *(p=0.002), while in drug treated groups maximum reduction seen in bevacizumab **(p=0.040) and bryostatin ***(p=0.018) in comparison to disease control.

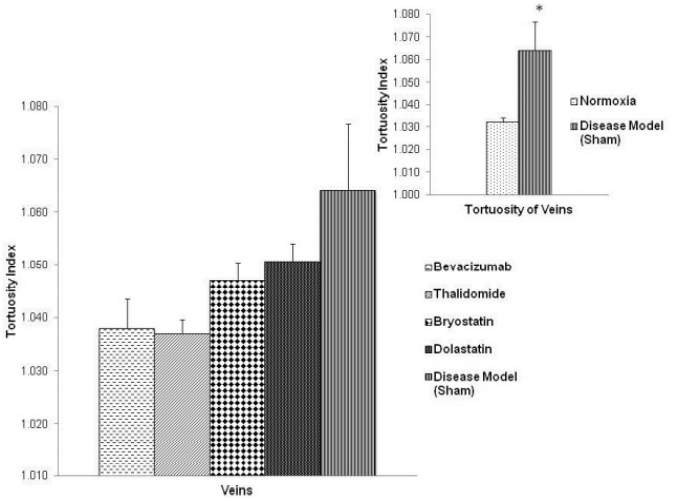

Fig. 4 : Tortuosity of veins in rat pups on Day 17 (n=6). Tortuosity of veins in Sham is higher than room-air *(p=0.067) Tortuosity of veins in drug treated groups is lower as compared to Sham (not statically significant).

(ii) Day 25:

A decrease in tortuosity of vessels was found in all the groups on Day 25 as compared to Day 17 in both veins and arteries signifying the natural course of regression of the disease process with time. The amount of decrease seen however was not statistically significant in any of the groups (p>0.1). On Day 25, though a 16% reduction in the tortuosity of arteries in Sham ($TI_A = 1.13$) was noted as compared to Day 17 ($TI_A = 1.349$), it still remained significantly higher than room air raised rats ($TI_A = 1.028$, p=0.017) with a 10% higher tortuosity index as compared to room air. The tortuosity index of arteries in all the groups was lower than Sham with a maximum decrease seen again with bryostatin ($TI_A = 1.098$) though not to a statistically significant degree (p=0.366).The tortuosity index of veins was comparable among all groups on Day 25.

Fundus Fluorescein Angiography:-

Fundus fluorescein angiography was performed on Day 17 but the procedure was abandoned in view of an early leak observed from the vessels due to immaturity of the vessel wall, an increased choroidal flush obscuring the details of the retinal vessels and difficulty in cannulating the tail vein of rat pups with age due to excessive fibrosis.

Electroretinography:-

The ‘a’ and ‘b’ waves were analysed on Day 17 and Day 25 for any neural changes in response to hyperbaric oxygen (representative ERG wave form Fig. 5A & 5B).

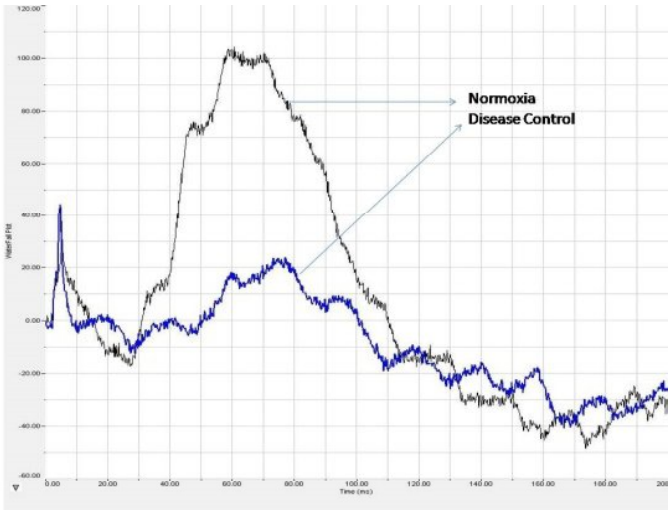

Fig. 5A : Representative ERG waveform of Disease control vs Normoxia. X-axis represents Time (msec) and Y-axis represents amplitude in microvolt.

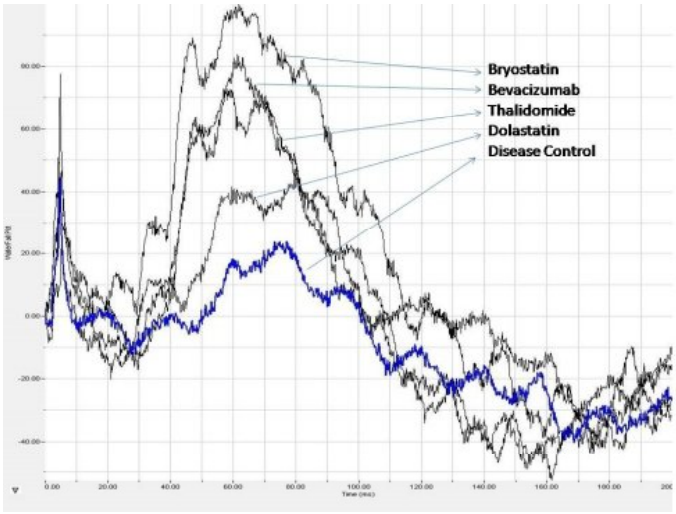

Fig. 5B : Representative ERG wave form of Disease Control Vs Various treatment group. X-axis represents Time (msec) and Y-axis represents amplitude in microvolt.

(i) Day 17:

A 66% decrease in 'b' wave amplitude was noted in Sham (11.62 µV) as compared to room air raised rats (33.96 µV)which was statistically significant (p=0.002). The 'b' wave amplitude of drug treated rat pups on Day 17 were significantly higher than Sham signifying the preserved retinal function in the drug treated groups (p<0.05, Fig. 6). The maximum amplitude was seen with bryostatin and bevacizumab

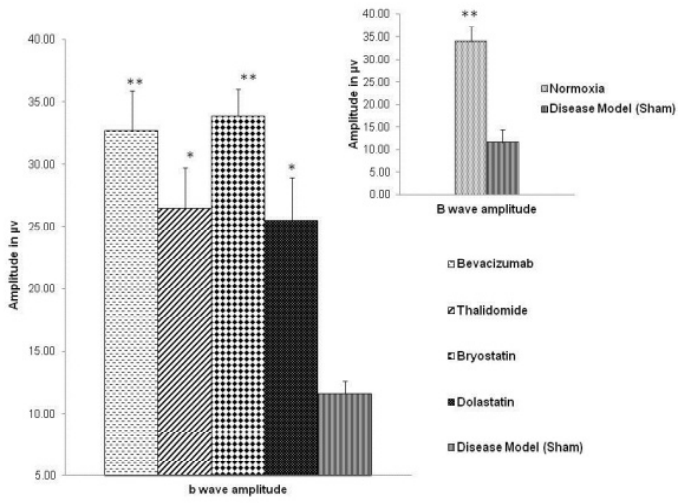

Fig. 6 : ERG response ('b' wave amplitude) in rat pups on Postnatal Day 17(n=6). a) 'b' wave amplitude of Sham is lower than room air **(p=0.002). b) 'b' wave amplitude of drug treated groups is higher when compared to Sham *(p<0.02), **(p=0.002) using unpaired t-test.

(33.86 µV) with a 67% higher amplitude than Sham (p=0.002). Dolastatin also showed a significantly higher 'b' wave amplitude (25.51 µV, p=0.02).No significant difference in the 'a' wave amplitudes and implicit times of 'a' and 'b' wave were noted between the groups signifying a probable intact photoreceptor cells and lack of conduction defect.

(ii) Day 25:

A recovery of 'b' wave amplitude among all the groups was noted and the 'b' wave amplitudes were higher than on Day 17 though the rise was not statistically significant (p>0.1).A 58% recovery of 'b' wave amplitude was noted in Sham on Day 25 (18.43 µV) in comparison to Day 17 (11.62 µV).Though there was a recovery of 'b' wave amplitude in Sham, it remained lower than the room air raised rat pups (34.02 µV) but not to a statistically significant degree (p = 0.124) and the 'b' wave amplitude of the drug treated groups remained higher than Sham though not significant (Fig. 7).

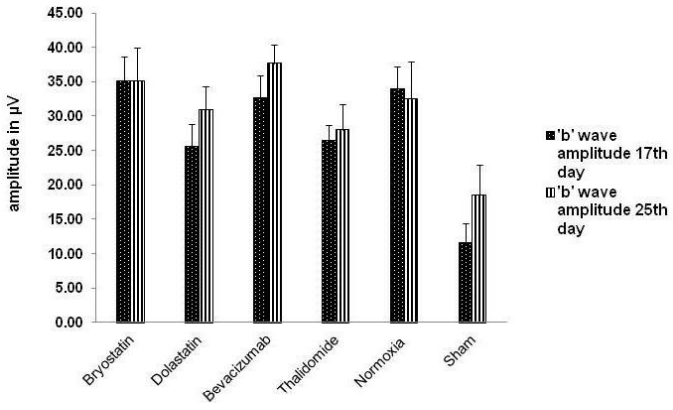

Fig. 7 : ERG response ('b' wave amplitude) in rat pups (n=6) on Postnatal Day 17 vs .Postnatal Day 25 (n=6). 'b' wave amplitude improved at PN 25 (no statistical difference found using paired t-test).

The decrease in tortousity of vessels correlated well with higher 'b' wave amplitude in the drug treated groups as compared to Sham and was especially seen with bryostatin. Hence, there was a positive correlation between the anatomical changes seen in fundus with the neural responses seen on electroretinography in this ROP model (Fig. 8).

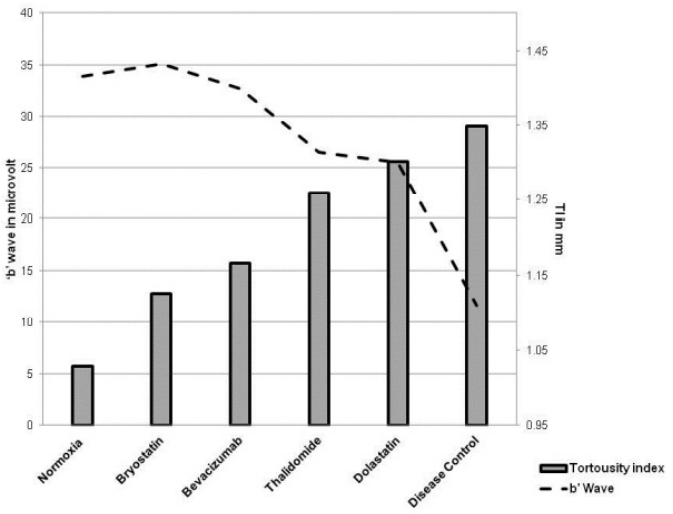

Fig. 8 : Comparison of 'b' wave response (in microvolt) with the tortousity index of arteries (in mm) of various test groups at day 17.

Discussion

Our study was conducted on Wistar rats in which Retinopathy of Prematurity was successfully produced in the rat pups under hyperbaric oxygen conditions made in custom designed chamber in our laboratory. This study was designed based on the observations from previous animal studies reported in the literature (5, 6). Animal model of ROP with 75-80% oxygen from day 7 to day 12 has been well documented in the literature (30, 5, 6). While working on this model preliminary studies were done like FITC-Dextran labeled whole flatmount (Data not shown) to ensure the reproducibility of the model, this model also creates peripheral neovascularization in rat retina, although in the present study the rats were not sacrificed, the retina was assessed through fundus camera and retinopathy was assessed in form of tortousity of retinal vessels.This model produced a form of avascular retina in the periphery with neovascularization at the junction of perfused and unperfused retina and posterior pole tortuosity of vessels suggestive of Plus disease of ROP. Plus disease is characterized by abnormal dilatation and tortuosity of the retinal arteries and veins at the posterior part of the retina of the eye and is an important indicator of disease activity, severity and risk of progression. Decrease in plus disease signifies disease regression.

Our study has included newer therapeutic targets like protein kinase C modulator (bryostatin-1), tubulin binding (dolastatin) against the previously proven ones like direct VEGF binding agent (bevacizumab), VEGF/ FGF signal interfering agent (thalidomide) and compared them to disease control.

Previous studies carried out with Protein kinase- C inhibitors like riluzole (4) and ruboxistaurin (3) and with tubulin binding agents like combretastatin (9) showed a decrease in ROP but so far no studies had been conducted with bryostatin and dolastatin which are currently under phase- II trials for solid tumours and haematological malignancies. Also, these agents tested previously were not compared with proven anti-VEGF agents like bevacizumab which currently occupies the status of an effective adjuvant to laser photocoagulation in the treatment of ROP.

Although, in earlier studies (7, 8) an intravitreal route was preferred for drug delivery in thalidomide over intraperitoneal route, we went ahead with systemic route as all rat pups did not open their eyes on Day 12. Initially, when Intraperitoneal route was attempted, increased mortality of pups was noted which could have been due to rapid absorption of the drug from the peritoneum. Hence, we resorted to subcutaneous injection of drugs in two divided doses 48 hours apart rather than daily dosage was done in previous studies.

MICRON- III retinal imaging system was used in our study which helped to carry out the anterior segment imaging, fundus photography, fluorescein angiography as well as focal electroretinography all by one instrument with different attachments which could be carried out with a single dose of anaesthesia.

The previous studies on animal models of ROP had not studied the anterior segment for new vessels on iris which is an important component of plus disease. In our study, although we did not find any new vessels on iris, an increased dilatation and tortuosity of iris vessels was noted which showed ischemia affecting the anterior segment.

Very few studies have been conducted demonstrating the effect of ROP on the function of the neural retina and the relationship between anatomical changes in

retinal vasculature and neural responses on ERG (5, 10). Also, the effect of drug administration on the ERG responses has not been evaluated for the test compounds of our study.

In our study, ERG showed a significant decrease of 'b' wave amplitude in Sham group as compared to room air showing evidenced due to ischemia. A recovery of 'b' wave amplitude on Day 25 was seen signifying that the transient damage to the inner retinal layers due to ischemia also occurred which might have recovered within a week. The 'a' wave amplitude was within the normal range and comparable to room air signifying that the outer retinal layer were probably less affected due to ischemic damage as it is getting supply from the choroidal plexus. On comparing the drug treated groups with Sham, we found significantly higher 'b' wave amplitudes on the electroretinogram on Day 17 which showed that these drugs might have helped in inhibiting or reversing the ischemic damage to inner retinal layers. The decrease in tortousity of vessels in the drug treated groups correlated well with the higher 'b' wave amplitude especially with bryostatin. Thus, ROP affected both the vascular component and the neural retina and drugs which act on ROP led to an improvement in both the components.

The retina was also evaluated structurally to assess the amount of retinopathy, through calculating the tortousity index of blood vessels by method adopted by liu et al., 2006. The retinal arteriolevessels were more affected as compare to venules in terms of tortousity index, which were co relating with the work of liu et al. Tortousity of blood vessels were evident in disease control as compare to normal air raised pups suggesting the establishment of the ROP model. When disease control retinal vessels TI was compared with other treatment group, a decrease in TI was seen in all treatment groups. Maximum reduction of TI was seen in bryostatin followed by bevacizumab, dolastatin and thalidomide when compared to disease control at PN day 17. At PN day 25, the tortousity of retinal vessels regressed in all the groups, although the TI improvement was not statistically significant when compared to day 17 in all the groups.

Significant positive results were obtained with bevacizumab and bryostatin with no statistically significant difference between the two drugs suggesting that the drugs acting through the newer pathway by protein kinase C modulation might be as efficacious as bevacizumab.

Dolastatin failed to show a significant reduction in tortousity of vessels at the dose tested but showed a significant improvement in 'b' wave amplitude, hence elaborating some effect on angiogenesis in the ROP model. The ineffective reduction of retinopathy by dolastatin may be attributed to inadequate dose of the drug, failure to cross the blood-retinal barrier and immature liver metabolism similar to that seen with thalidomide (8). Hence, dolastatin also might prove to have a significant anti-angiogenic effect with a higher dosage and route of delivery.

Thereduction in tortousity index was observed in bryostatin followed by bevacizumab, thalidomide and dolastatin when compared to disease control, the data was supported by the improved 'b' wave response from the retina of various treatment groups, where maximum improved response was seen in the same fashion i.e.bryostatin followed by bevacizumab, thalidomide and dolastatin. The reason could be preserved neuronal cells due to less ischemia driven trauma of the inner retina (Fig. 8).

For the first time, the present study showed the protective effect of PKC modulation by bryostatin in the hyperbaric oxygen model in rat pups. This reveals the possibility of developing protein kinase C modulator as a candidate for ROP with low toxicity for its safer use in infants.

The present study documented the effect of diverse pharmacological targets in the experimental model of hyperbaric oxygen induced ROP in rat pups. It has evaluated vascular changes after drug administration and correlated it with neural responses on electroretinography.Among new pathways attempted, protein kinase C modulation by bryostatin proved to have more positive effect on the reduction of ROP. In our study tubulin binding agents like dolastatin has also demonstrated a reduction of Retinopathy of Prematurity in the rat model, though not to a significant degree, but its potential as an anti-angiogenic agent cannot be overlooked.

To conclude, this study showed protein kinase-C modulatorwould be having a potential role in the treatment of ROP. However, further preclinical studies are required to strengthen the hypothesis for their evaluation in clinical studies.

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