Estrogen therapy to treat retinopathy in newborn mice

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Abstract. The aim of the present study was to treat retinopathy of prematurity (ROP) with estrogen (E_2) so as to elucidate the role of E_2 in the pathogenesis of ROP. A total of 120 postnatal 7-day-old (P7) C57BL/6J mice were selected and raised in a high-oxygen environment (75% oxygen) for 5 days, followed by 5 days in normal room air. Different doses of E_2 or normal saline (NS) were injected intraperitoneally during different time-periods, and the mice were divided into 14 groups according dose of E_2 injection (0.5-1.5 μg/0.05 ml) and dosing time. Blood vessel changes and hyperplasia were evaluated in flat-mounted retina and retinal slices. All mice that were exposed to room air, whether they were administered E_2 or NS, showed good vascular development in the flat-mounted retina at P17. No increase in the number of endothelial cell nuclei in the new blood vessels was observed. In ascending order of E_2 dose the numbers of cell nuclei were as follows: 0.18±0.129, 0.28±0.086 and 0.55±0.110. The number in the NS group was 2.12±0.373. When the results of the room-air groups were compared with those of the hyperoxia groups, a highly significant difference was found in each comparison (P<0.0001). All mice showed varying degrees of neovascularization and vascular obstruction in the flat-mounted retina at P17, and it was difficult to compare the blood vessels morphologically among these groups. The number of endothelial cell nuclei decreased following E_2 injection, and the difference from the NS group exposed to hyperoxia was highly significant (P<0.0001). For all dose levels, the number of cell nuclei was the lowest when the drug was administered during P7-16, and the difference from the other two time-periods was statistically significant (P<0.05). When E_2 was administered during P7-16, the number of cell nuclei was 15.5±1.993 in the 0.5-μg group, 14.23±2.49 in the 1.0-μg group and 18.05±1.62 in the 1.5-μg group. No significant difference was found among these three groups (P>0.05). In conclusion, E_2 treatment during the development of retinopathy can improve symptoms in neonatal mice, suggesting that E_2 plays an important role at the two initial stages in the pathogenesis of ROP. This may indicate new pharmacological measures to prevent and treat ROP.

Introduction

Retinopathy of prematurity (ROP) affects premature infants and is a major cause of blindness and visual impairement despite continous improvements in neonatal care (1). There are ~250,000 very low birth weight (VLBW) infants born in China every year. Among these VLBW, ~30,000 will develop ROP, and ~10,000 receive ophthalmological surgery (2). As reported in the International Classification of Retinopathy of Prematurity (3,4) and a multi-center clinical trial on retinopathy of prematurity (ROP) (5-9), current treatments for ROP mainly include cryotherapy and laser photocoagulation for threshold ROP, as well as sclera buckling and vitrectomy at a later stage. Although these procedures do significantly reduce the long-term adverse outcomes of ROP, such as blindness and retinal detachment, they may cause permanent damage to the retinal structure and are associated with life-long eye problems, including visual field defects and refractive error (10-13). Researchers have, therefore, increasingly focused on preventing ROP progression by inhibiting angiogenesis based on the pathogenesis of ROP (14-23). Two distinct phases have been identified: Phase I, involving delayed retinal vascular growth after premature birth; and phase II, concerning uncontrolled proliferative growth of retinal blood vessels. Such approaches have been proven feasible in animal experiments; however, angiogenesis inhibitors may also inhibit normal vascular development and hinder normal ocular anatomical and functional development (24,25). These problems remain unresolved. Furthermore, neither surgical procedures nor angiogenesis inhibitors are capable of preventing the occurrence of ROP, since they are measures taken after the development of ocular lesions.

Estrogen (E_2), or 17β-estradiol, can act on vascular cells and neurons and plays an important role in retinal vascular development. Estrogen receptor (ER)-mediated E_2 is particularly important in normal retinal vascular development and the pathogenesis of ROP (26-32). Preterm infants are prone

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to develop ROP, possibly due to the unique metabolism of E_2 in these infants. Early E_2 replacement administered prior to the occurrence of abnormal vascular lesions may, therefore, promote the development of normal blood vessels and thereby improve the prognosis for ROP. This provides a novel idea for preclinical and clinical trials and may help validate the role of E_2 and ER in normal retinal vascular development and the pathogenesis of ROP from the perspective of treatment.

Materials and methods

Experimental animals. A total of 120 healthy 7-day-old C57BL/6J mice of either gender were selected. The mice were not weaned and were raised with lactating female rats. These were clean-grade animals that were provided by the Department of Anatomy, Histology and Embryology of Fudan University Shanghai Medical College (Shanghai, China). The study protocol was approved by the university Animal Care and Use Committee and conformed to international standards for the humane treatment of experimental animals.

Materials and instruments. The E_2 used in the study was estr a-1,3,5(10)-triene-3,17β-diol (Sigma-Aldrich, St. Louis, MO, USA). The molecular formula was $C_{18}H_{24}O_2$ and the molecular weight was 272.4 Da. We customized a 50x40x25-cm glass container with three round holes in the lid. Of the three holes, one was for inserting an air inlet pipe connecting the oxygen cylinder and the nitrogen cylinder, one was an air outlet hole and the remaining hole was connected to an oxygen analyzer. The bottom of the container was covered in soda lime to keep the container dry. A common mouse cage was placed in the container. The room temperature was maintained at 23±2°C and room luminosity did not exceed 300 Lux with illumination for 12 h a day.

Experimental methods
Grouping. In the study group, ROP was induced in C57BL/6 mice by exposing postnatal 7-day-old (P7) mice to 75% oxygen (hyperoxia) for 5 days, followed by 5 days in normal room air. The mice in the control group were raised in room air for 10 days. The mice in the control and hyperoxia groups received an intraperitoneal injection of either E_2 or normal saline (NS) once per day and were further grouped according to the administered agent, dose and dosing time, as shown in Table I.
Flat mounting. The flat-mounted retinas were prepared as follows. For the three groups with an injection time of P7-11, retinas were separated and flat-mounted on the slides on days 0, 2 and 5 (i.e., P12, 14 and 17), respectively, after the mice were removed from the oxygen container. Six eyes were enucleated at each time-point from 6 mice. For the remaining groups, 6 eyes were enucleated from 6 mice, and the flat-mounted retinas were prepared on P17 to observe the profile of retinal vascular development and proliferation. The specific method of retina flat-mounting and ADPase enzyme histochemistry is as detailed below. For anesthesia, the mice received a perito-neal injection of 2,2,2-tribromoethanol (Avertin®; 0.5 ml/15 g; Sigma-Aldrich). For perfusion, the chest cavity of each mouse was opened and the heart was exposed. Through an infusion needle inserted in the left ventricle, NS was infused for 2-3 min

followed by infusion of 4% paraformaldehyde solution for ~5 min. The eyeballs of the mice were enucleated and fixed in 4% paraformaldehyde solution. For sampling, the limbus was cut open circularly at 1 mm posterior to the limbus. The cornea was removed and the lens was extracted. Four to five radial incisions were made using the optic papilla as the center. The sclera and choroid were removed, and the vitreous body and the retinal pigment epithelial layer were removed by a brush. The samples were then rinsed with 0.05 mol Tris-maleate buffer (pH 7.2; Sigma-Aldrich) five times (15 min each time). Next, the samples were incubated in the reaction solution at 37°C for 15 min. For color development, the samples were reacted with 10% (1:10) ammonium sulfide for 5 min. The results were observed with an optical microscope.

Preparation and observation of paraffin sections. Six eyes of 6 mice were collected from each group. The mice were sacrificed by cervical dislocation 5 days after the removal of the mice from the oxygen container. The eyeballs were enucle-ated and directions were marked. For fixation, the enucleated eyeball was placed in 4% paraformaldehyde solution and fixed for 24 h. Samples were dehydrated with gradient alcohol and made transparent with xylene. The samples were then embedded in soft paraffin, hard paraffin I and hard paraffin II for 30 min, 1 h and 1 h, respectively. Serial sections of 6 μm were sliced parallel to the sagittal plane from the cornea to the optic papilla. Slices were then flattened in warm water and mounted on their slide. The samples were dewaxed with xylene and dehydrated with gradient alcohol. Conventional hematoxylin and eosin staining was then performed. Finally, an optical microscope was used to observe the results.

Endothelial cell nucleus count. The endothelial cell nucleus count in the new retinal blood vessels was performed as follows. Ten pathological slices were taken intermittently from each eyeball. The interval between two adjacent sections was 60 μm (10 slices). The endothelial cell nuclei of the blood vessels that broke through the internal limiting membrane of the retina were counted by section and by eyeball. Only the cell nuclei of blood vessels near the internal limiting membrane, rather than those of blood vessels in the vitreous cavity not associated with the internal limiting membrane, were counted.

Statistical analysis. All data were analyzed with the statis-tical analysis software SPSS 11.0 for Windows (SPSS, Inc., Chicago, IL, USA). Analysis of variance was used to compare the number of nuclei in the endothelial cells of new blood vessels that broke through the internal limiting membrane among the groups. P<0.05 was considered to indicate a statisti-cally significant difference.

Results

Vascular morphological changes following E_2 treatment. No retinal vascular abnormalities were observed at P17 in the normal mice that received various doses of E_2 or NS between P7 and 16. All blood vessels were mature, and the larger vessels had more branches with consistently large lumen. The surrounding small vessels were well developed, forming a vascular arch at the edge of the retina. The branching structure of the superficial vascular network and the polygonal mesh-like structure of the deep vascular network

Table I. Experimental grouping for E_2 therapy.

Group	Number of mice	Drug	Dose	Normal air or oxygen	Time of injection
1	6	E_2	0.5 μg/0.05 ml	Normal air	P7-16
2	18	E_2	0.5 μg/0.05 ml	Oxygen inhalation	P7-11
3	6	E_2	0.5 μg/0.05 ml	Oxygen inhalation	P7-16
4	6	E_2	0.5 μg/0.05 ml	Oxygen inhalation	P12-16
5	6	E_2	1.0 μg/0.05 ml	Normal air	P7-16
6	18	E_2	1.0 μg/0.05 ml	Oxygen inhalation	P7-11
7	6	E_2	1.0 μg/0.05 ml	Oxygen inhalation	P7-16
8	6	E_2	1.0 μg/0.05 ml	Oxygen inhalation	P12-16
9	6	E_2	1.5 μg/0.05 ml	Normal air	P7-16
10	18	E_2	1.5 μg/0.05 ml	Oxygen inhalation	P7-11
11	6	E_2	1.5 μg/0.05 ml	Oxygen inhalation	P7-16
12	6	E_2	1.5 μg/0.05 ml	Oxygen inhalation	P12-16
13	6	NS	0.05 ml	Normal air	P7-16
14	6	NS	0.05 ml	Oxygen inhalation	P7-16

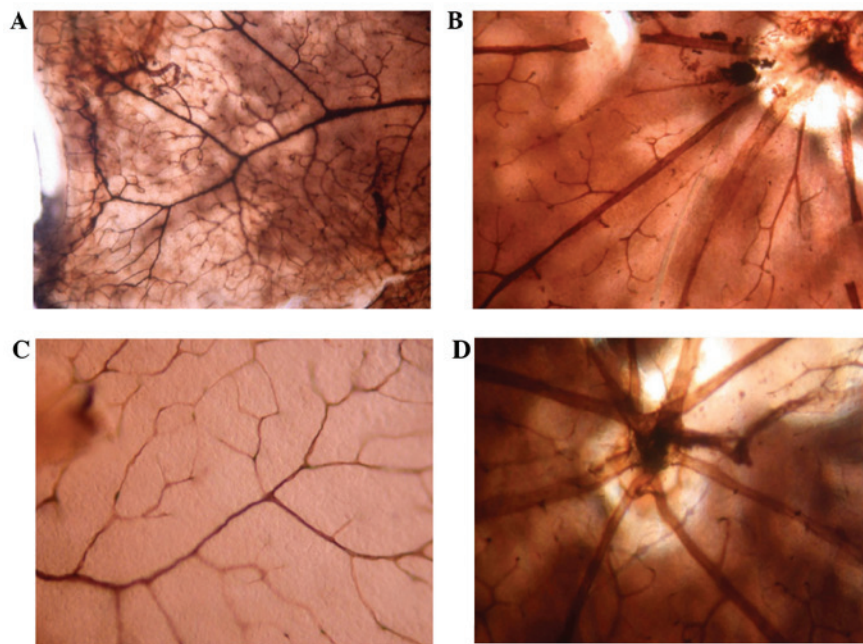E_2, estrogen; NS, normal saline; P, postnatal day.

Figure 1. No retinal vascular abnormalities were observed at postnatal day 17 (P17) in the normal mice that received (A) 0.5 μg estrogen (E_2), (B) 1 μg E_2, (C) 1.5 μg E_2 or (D) 0.05 ml normal saline between P7 and P16 (magnification, x100-200).

could be clearly noted, and no blood vessel obstruction was observed (Fig. 1).

Mice receiving oxygen supplement consistently showed retinal neovascularization following NS injection or injection of E_2 at varying doses during different periods. On P12, non-perfusion areas at the center of the retina, poorly-branched large vessels with a narrow lumen and naïve surrounding small vessels were observed. On P14, neovascularization was found at the interface between the vascularized zone and the non-vascularized zone. On P17, the number of new vessels had increased, and the two-tier vascular network had lost its normal structure, with accompanying vascular obstruction. The number of blood vessels or the size of the central non-perfusion area could not be compared morphologically among the different groups.

Quantification of angiogenesis following treatment. The effect of E_2 treatment on angiogenesis was quantified by counting the endothelial cell nuclei of the blood vessels that broke through the internal limiting membrane of the retina. It was found that, following both E_2 and NS injection, the number of nuclei in the normal mice was low, and pair-wise comparison revealed

Table II. Pair-wise comparison of the results of the mean (P-value).

|	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9	Group 10	Group 11	Group 12	Group 13
Group 2	<0.001	-											
Group 3	<0.001	<0.001	-										
Group 4	<0.001	0.052	0.001	-									
Group 5	0.973	<0.001	<0.001	<0.001	-								
Group 6	<0.001	0.071	<0.001	0.897	<0.001	-							
Group 7	<0.001	<0.001	0.669	<0.001	<0.001	<0.001	-						
Group 8	<0.001	0.517	<0.001	0.010	<0.001	0.014	<0.001	-					
Group 9	0.901	<0.001	<0.001	<0.001	0.928	<0.001	<0.001	<0.001	-				
Group 10	<0.001	0.039	<0.001	<0.001	<0.001	<0.001	<0.001	0.155	<0.001	-			
Group 11	<0.001	<0.001	0.389	0.010	<0.001	0.007	0.198	<0.001	<0.001	<0.001	-		
Group 12	<0.001	0.048	0.001	0.978	0.536	0.875	<0.001	0.009	0.597	<0.001	0.011	-	
Group 13	0.514	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	-
Group 14	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
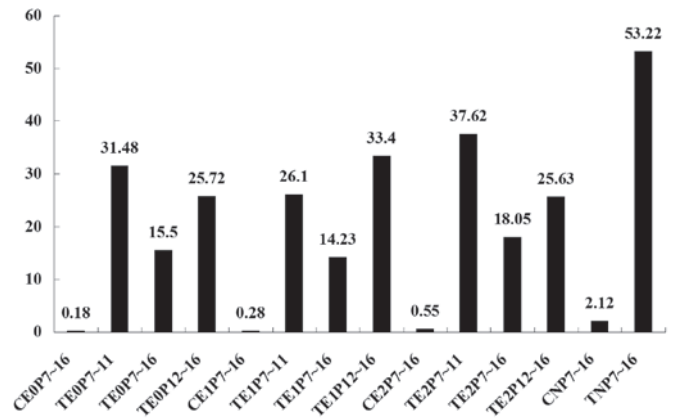
Figure 2. Endothelial cell nucleus count of new blood vessels on each slice prepared from the eyes of the mice following E_2 treatment in each group. Each group is shown on the x-axis. C indicates the control group and T indicates the hyperoxia group. E0, E1 and E2 stand for E_2 at 0.5, 1.0 and 1.5 μg/0.05 ml, respectively. N represents normal saline. P7-16, P7-11 and P12-16 represent the dosing periods. The number of cell nuclei is shown on the y-axis. E_2, estrogen; P, postnatal day.

no significant difference among the four groups (P>0.05). By contrast, a highly significant difference was found in the number of nuclei between the normal mice and the mice receiving oxygen inhalation (P<0.0001). The number of nuclei decreased in the mice receiving oxygen inhalation following injection of E_2 at all doses and time-points. Following NS injection, the number of nuclei in the mice receiving oxygen inhalation was significantly higher than that in the remaining groups (P<0.0001; Table II and Fig. 2).

The number of nuclei differed significantly among the different dose groups due to the difference in the time-period of drug administration. The number of nuclei was significantly reduced in the three groups in which the drug was administered during P7-16, when compared with the number of nuclei in the groups in which the drug was administered during the two other time-periods (P<0.05). The effect of different doses administered during the same time-period was also compared by counting the number of endothelial cell nuclei in the newly formed blood vessels. For P7-11, the doses could be ranked as follows: 1.0 μg <0.5 μg <1.5 μg. No significant difference in the number of endothelial cell nuclei was found between the 1.0- and 0.5-μg groups, but the results for both these groups were significantly different from the result for the 1.5-μg group (1.0 μg vs. 1.5 μg, P=0.039; 0.5 μg vs. 1.5 μg, P<0.0001). For P7-16, the order was 1.0 g <0.5 g <1.5 g and no significant difference could be found among the three groups. For P12-16, the order was 1.5 μg <0.5 μg <1.0 μg; no significant difference in the number of endothelial cell nuclei was found between the 1.5- and 0.5-μg groups, but both were significantly different from the 1.0-μg group (1.5 μg vs. 1.0 μg, P=0.009; 0.5 μg vs. 1.0 μg, P=0.01).

Discussion

In addition to its effect on the reproductive organs, E_2 acts on the vascular cells and neurons and plays varying roles under different physiological and pathological conditions (26,27). E_2 may protect the cardiovascular system against cardiovas-

cular diseases, such as hypertension and atherosclerosis, by dilating blood vessels and increasing blood flow (28). E_2 may increase the activity of endothelial cells, inhibit endothelial cell apoptosis, promote endothelial cell proliferation, facilitate angiogenesis and the growth of blood vessels and play an important role in retinal vascular development (29,30,33). *In vivo*, E_2 shows its specific effects only subsequent to binding to ER (31,32). Basic endothelial cell-derived nitric oxide may dilate blood vessels and prevent thrombosis by inhibiting platelet aggregation, a process that may be regulated by ER (34). In a previous study, we found that ER-mediated E_2 plays an important role in normal retinal vascular development and ROP pathogenesis (35). Maternal E_2 levels during pregnancy may increase by ~100-fold (33,35-39). Fetal E_2 levels increase with gestational age, with the E_2 concentration peaking between 33 and 36 weeks of pregnancy. E_2 levels drop rapidly after birth and may reach normal neonatal levels at 24 h; therefore, since preterm infants are delivered early, their E_2 levels are lower than those of full-term infants. E_2 levels drop more rapidly and fluctuate more in preterm infants than those in full-term neonates, which may explain why such infants are prone to ROP (36). Theoretically, early supplementation of exogenous E_2 would facilitate vascular development and thereby prevent severe retinal hypoxia and subsequent cascade reactions.

In the present study it was found that the number of endothelial cell nuclei in the newly formed blood vessels was reduced significantly in all the three groups receiving E_2 treatment compared with those receiving NS ($P<0.0001$), suggesting that E_2 replacement therapy did have beneficial effects; however, E_2 did not inhibit angiogenesis completely, and it was difficult to compare angiogenesis in the flat-mounted retinas among the different groups based on morphology alone. In the tissue sections in which nuclei could be counted, the nucleus count was significantly different from that of the normal mice ($P<0.0001$), indicating that, to some extent, E_2 plays a role in the development and progression of ROP. E_2 may be involved in this process by promoting the expression of the *VEGF* gene (39), resulting in the formation of a large number of new blood vessels. Miyamoto *et al* (30) found that retinal angiogenesis was reduced in ROP mice following intraperitoneal injection of E_2 during P7-11. They hypothesized that E_2 supplementation at the hyperoxia stage would partially restore the expression of the *VEGF* gene, promoting the growth of retinal blood vessels and thereby alleviating hyperoxia-induced retinal injury. Miyamoto *et al* observed that in the subsequent hypoxia phase, E_2 supplementation at the same dose on P12-16 also reduced angiogenesis. Furthermore, when the drug was administered during P7-16, the number of endothelial cell nuclei in the new blood vessels was the lowest, suggesting that E_2 may inhibit angiogenesis via other mechanisms. Transient blocking of the middle cerebral artery can cause retinal ischemia in adult female Sprague Dawley rats, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining showed a significantly increased number of apoptotic cells in the ganglion cell layer at 1 h after ischemia (40). E_2 treatment can completely reverse such apoptosis (40), suggesting that E_2 has neuroprotective effects when the retina is in a low oxygenation state. This may explain the reduced angiogenesis following E_2 administration during P12-16 and P7-16.

In the present study, the experimental animals were divided into 14 groups according to dosage and dosing period. The dosing periods were the same as those in the study by Miyamoto *et al* (30), as dosing in those periods can exactly reflect the effect of dosing simultaneously or separately in the two phases of ROP development and progression, determine the treatment window and provide a preclinical basis for potential clinical application in the future. Furthermore, elucidating the roles of E_2 and its receptor ER at different stages of ROP pathogenesis may reveal the optimal mode of treatment. Three dosage groups, 0.5, 1.0 and 1.5 μg, were selected. The doses of 1.0 and 1.5 μg represent the upper and lower limits of the dosage for E_2 replacement therapy in menopausal women (0.25-0.5 mg/day), respectively, and were determined by dose conversion between different species (41). While the two higher doses are within the physiological range, the lower dose of 0.5 μg is below the physiological range. It was found that, for each dose, maximal inhibitory effects were achieved when the drug was administered during P7-16, which was consistent with the results of Miyamoto *et al* (30). The results support a potential therapeutic benefit of E_2 at the two stages of ROP pathogenesis. The incidence of blindness in diabetic men aged <45 years is higher than that in diabetic women of the same age, and the incidence is similar in men and women aged >45 years, suggesting that E_2 may improve the prognosis of diabetic retinopathy (42). Diabetic retinopathy and ROP have a similar pathogenesis, which may further support our view that E_2 plays an important role in retinal development and ROP pathogenesis in premature infants and holds promise as a potential treatment for ROP.

The present study additionally compared the effects of different doses of E_2 administered at different time-periods. It was found that the number of nuclei in the high-dose group (1.5 μg) was greatest when the drug was administered at P7-11; at this time-period, the difference from the other two dose groups was highly significant. The results did not demonstrate the superiority of a high dose. The number of nuclei did not differ significantly between the 1.0- and 0.5-μg groups when the drug was administered during P7-16, although the number in the 1.0-μg hypoxia group was slightly lower. It is therefore suggested that E_2 replacement therapy may achieve the maximum effect even at a low dose (1.0 μg). E_2 has a long history of clinical application. It has been used widely in menopausal and postmenopausal women, since it may improve menopausal symptoms and osteoporosis and provide skin care effects; however, misuse and abuse of E_2, particularly overdose of E_2, may increase the incidence of uterine and breast cancer (28,43). E_2 should therefore be appropriately used under the supervision of healthcare professionals and with the close monitoring of blood levels.

A number of clinical trials have evaluated E_2 replacement therapy in premature infants (29,31-33). Extremely low birth weight neonates with an average gestational age of 26.6 weeks and a birth weight of 675 g received E_2 supplementation at an intravenous dose of 2.3 mg/kg/day. If the intravenous infusion could not be maintained, transepidermal administration was used instead. The total duration of treatment was 6 weeks to maintain the plasma E_2 at the intrauterine level (33). The neonates were followed-up until 15 months of corrected gestational age (34). Following drug administration, the uterine volume,

breast, weight, length and head circumference of the neonates showed a transient increase and the bone mineral deposition rate trended towards an increase (33,35). The incidence of chronic lung diseases decreased and complications of the central nervous system were reduced (33,35). Although the administered dose was very high and no long-term follow-up or any multi-center clinical trial is yet available, the results remain encouraging.

The present study demonstrated that E_2 is important in the normal development of retinal blood vessels and the pathogenesis of ROP and has evident efficacy in the prevention and treatment of ROP. Although E_2 may not completely prevent the occurrence of ROP, it may at least alleviate ROP and thereby reduce the incidence of long-term complications, such as blindness and refractive errors. This effect of E_2 is of great significance to individuals and society and may provide significant economic benefits. In addition, due to the special metabolism of E_2 in preterm infants, E_2 supplementation not only facilitates retinal development but also improves the development of several other systems, which may result in a significant improvement in the prognosis of preterm infants and their future quality of life. Despite this, a number of steps are still required to translate these results from animal studies into eventual clinical application, including rigorous clinical trials and long-term follow-up studies, in order to determine the pharmacokinetics, dose, dosing regimen and schedule and assess the efficacy, safety and side effects.

In conclusion, E_2 plays an important role in retinal vascular development and the pathogenesis of ROP in mice. E_2 replacement therapy may alleviate retinal disease in neonatal mice, and intraperitoneal injection of E_2 at a dose of 1.0 μg during P7-16 achieved the best efficacy in mice; however, the long-term efficacy and side effects of E_2 require further study.

Acknowledgements

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