• Review •

The progress of prophylactic treatment in retinopathy of prematurity

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Abstract

• Retinopathy of prematurity (ROP) is a retinal vascular disorder frequently found in premature infants. Different therapeutic strategies have been developed to treat ROP. However, there are still many children with ROP suffering by severe limitations in vision or even blindness. Recently, ROP has been suggested to be caused by abnormal development of the retinal vasculature, but not simply resulted by retinal neovascularization which takes about 4 to 6wk after birth in premature infants. Thus, instead of focusing on how to reduce retinal neovascularization, understanding the pathological changes and mechanisms that occur prior to retinal neovascularization is meaningful, which may lead to identify novel target(s) for the development of novel strategy to promote the healthy growth of retinal blood vessels rather than passively waiting for the appearance of retinal neovascularization and removing it by force. In this review, we discussed recent studies about, 1) the pathogenesis prior to retinal neovascularization in oxygen-induced retinopathy (OIR; a ROP in animal model) and in premature infants with ROP; 2) the preclinical and clinical research on preventive treatment of early OIR and ROP. We will not only highlight the importance of the mechanisms and signalling pathways in regulating early stage of ROP but also will provide guidance for actively exploring novel mechanisms and discovering novel treatments for early phase OIR and ROP prior to retinal neovascularization in the future.

• **KEYWORDS:** retinopathy of prematurity; oxygen-induced retinopathy; retinal neovascularization

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INTRODUCTION

Retinopathy of prematurity (ROP) is one of the leading causes of visual loss in children[1]. Although the timely diagnosis and treatment of ROP have resulted in better improvement of retinal structure and visual acuity than before, however, ROP remains as a major cause of blindness in premature infants and the incidence is increased along with the elevated survival of infants born at very early gestational ages[2-6]. ROP can be divided into an early ischemic stage and a late neovascular stage, and early retinal ischemia leads to late retinal neovascularization (RNV)[7-8]. At present, most of the therapeutic treatments are focusing on depressing RNV[9-10]. However, at some circumstance, such as residual anatomical changes, amblyopia and high ametropia, and injury to the vulnerable retina caused by treatments themselves, make it difficult to restore impaired vision function by inhibiting RNV[11-17]. These studies suggest that treatment for ROP should be done prior to RNV. We predict that the optimal timing for ROP treatment should focus on retinopathy during the early ischemic stage but not during the late neovascular stage. Indeed, early treatment of retinopathy of prematurity (ETROP) has been proven to be promising method for rescuing visual function in premature infants[18-25]. However, currently, ETROP still aimed at pre-threshold retinopathy, which still hurt retina, thus, to actively explore the optimal therapeutic strategy for ETROP is necessary. In this review, we summarize the potential mechanisms involved in early ischemic stage ROP, which may contribute to improve ETROP in the future.

OXYGEN AND RETINOPATHY OF PREMATURITY

Oxygen plays a critical role in ROP[26]. It was found that the relatively high levels of oxygen routinely given to premature infants were an important risk factor, and that reducing the level of oxygen given to premature babies reduced the incidence of ROP[27-28]. With advanced technology to monitor the oxygen levels applied to infants, the importance of oxygen as a risk factor has been diminished. However, for understanding the

mechanisms of ROP, oxygen-induced retinopathy (OIR) is still used to generate ROP in animal models[29]. OIR can be divided into an early hyperoxic phase and a late hypoxic phase as in ROP[30]. The morphological changes of retina in the early phase of OIR are more obvious than those in the late phase[31]. To explore the mechanism of hyperoxia-induced vascular loss in the early phase of OIR, which is closely related to primary retinal vascular loss in ROP, may help to develop better therapeutic strategies for treating ROP.

Limited oxygen application is able to reduce the incidences of ROP, whereas it is concomitant to an increase in mortality among preterm infants. Thus, studies of how to avoid the occurrence of ROP while an infant is treated with oxygen are significant[32-33]. Clinical studies indicated that newborn resuscitation should not be conducted with 100% oxygen supplementation and the levels of SaO_2 during the neonatal period in extremely low-birth-weight (LBW) infants should be maintained at between 85% and 93% or possibly between 88% and 95%, but absolutely not exceed 95%, and fluctuations should be avoided[34]. Maintain of SaO_2 values between 83% and 93% in the period immediately following birth combined with the strict control of oxygen fluctuations could prevent the early vaso-obliterative phase and the subsequent development of severe ROP in very LBW premature infants[35]. It had also been observed that the hyperoxia of 85%-93% versus 90%-99% was beneficial for the development of the immature retinal vasculature and decreased the incidence of ROP in preterm infants with body weights ≤1000 g[36].

Lower oxygen (85% to 92% SaO_2) at early gestational ages (<34wk) and higher oxygen (92% to 97% SaO_2) at older gestational ages (>34wk) induced normal retinal development and decreased the severity and the incidence of ROP[37-38]. However, there is a debate of whether partially decreasing SaO_2 increased the rate of mortality or disability in premature infants. It was reported that SaO_2 of less than 90% in extremely preterm infants was associated with an increased risk of death, whereas one study found SaO_2 between 85%-89% versus 91%-95% resulted in no significant effects on the mortality rate or the disability rate in extremely preterm 18mo infants, while recently, another study denied this opinion once again[32,39-40]. In addition, oxygen used with 90%-99% versus 85%-93% has the similar clinical effects on the development of the early and late type 1 ROP[41]. It is also observed that hyperoxia treatment (40%-75% SaO_2) initiated on P14 during the pre-proliferative phase of ischemic retinopathy was effective in accelerating the process of retinal revascularization and preventing the development of RNV[42]. Thus, hyperoxia is not always harmful to premature infants, and in some circumstance, it may be beneficial for the development of retinal vessels and reduce the incidence of ROP. Thus, to what extent that hyperoxia should be lowered which benefit to the development of retinal vasculature and simultaneously do not harm to the fate of premature infants still need be further investigated in clinical studies.

HYPOXIA INDUCIBLE FACTOR AND EARLY RETINOPATHY OF PREMATURITY

Hypoxia inducible factor-1 (HIF-1), including an oxygen-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit, regulates gene transcription by binding to the hypoxia-response elements in gene promoters. Some of the HIF-1 target genes are involved in adapting to insufficient oxygen or hypoxia[43-47]. HIF-1α is regulated by prolyl hydroxylases (PHD1, PHD2, and PHD3) and asparagine hydroxylase factor inhibiting HIF (FIH-1)[48-50]. During normoxia and hyperoxia, HIF-1α is unstable due to its hydroxylation and subsequent ubiquitination and proteasomal degradation[51-53], whereas during hypoxia, HIF-1α can be stabilized by PHDs *via* post-translational modifications[54-55]. It has been reported that increasing HIF-1α expression or decreasing HIF-1α degradation during the early stage of OIR can markedly reduce the avascular area and prevent hyperoxia-induced vessel loss, and recently, the role of liver-specific HIF-1α in promoting retinal vasculature in hyperoxia has been further proved[56-57]. However, the expression of HIF-1α in the late phase of OIR results in an inevitable increase in vascular endothelial growth factor (VEGF) expression, which accelerates neovascularization[58]. In addition, the systemic administration of dimethyloxalylglycine, a PHD inhibitor, during the early phase of OIR stabilizes HIF activity in the retina, and prevent oxygen-induced central-vessel loss and subsequent vascular tortuosity and tufting, which reduces subsequent RNV[59-60]. These studies suggest that HIF-1α may be a drug target and inhibition of PHDs during the early ischemic stage may be an effective treatment for ROP.

VEGF AND EARLY RETINOPATHY OF PREMATURITY

VEGF stimulates vasculogenesis and angiogenesis and is primarily regulated by HIF-1 at the transcriptional level under the condition of hypoxia[61]. VEGF can bind with its two membrane-bound receptors: VEGFR-1 and VEGFR-2, on endothelial cells[62]. The primary role of VEGFR-1 after binding by VEGF is to negatively regulate the bioactivity of VEGFR-2[63-64]. VEGFR-2 is essential for endothelial physiology and pathology during development, including the processes of angiogenesis and neovascularization[64-66]. In a mouse model of ischemia-induced retinal revascularization, an increased level of VEGFR-2 was noted in the vessels near the avascular area, whereas VEGFR-1 expression in the hypoxic retina was almost the same compared to that in control animals[67]. A critical role of VEGFR-1 in maintaining the vasculature of the neonatal retina has been reported and activation of VEGFR-1 by placental growth factor-1 has been proposed as an alternative strategy for preventing OIR without provoking RNV[68]. It has been noted that the levels of serum VEGF were lower at birth in infants who developed ROP

than infants without ROP, and it remained low in children with ROP who required treatment[69-70], which suggested that addition of VEGF-A during the early stage might be beneficial to the development of the retinal vasculature in preterm infants. However, it is also reported that exogenous VEGF administration on P14 was not sufficient to induce RNV in hyperoxia treatment mice, whereas injection of the VEGF antagonist VEGFR1/Fc blocked both pathologic and physiological angiogenesis but did not rescue astrocytes[42]. In addition, administration of a neutralizing antibody targeting VEGF decreased the phosphorylation of VEGFR-2 within the retina and around the blood vessels, and increased levels of VEGF in the free intraretinal space during the early stage of OIR, leading to significantly and sustainably reduce RNV without interfering in the ongoing retinal vascularization[71]. Thus, early administration of VEGF may be not enough to promote retinal vascular development in premature infants, and it is necessary to investigate the role of VEGFR1 and VEGFR2 in this process.

NORRIN AND EARLY RETINOPATHY OF PREMATURITY

Norrin is constitutively expressed in the retina and involved in retinal angiogenesis. Together with its receptor (Frizzled-4, FZD4), Norrin activates the Wnt-signalling pathway and controls the formation of the retinal vasculature during eye development[72-73]. It was reported that Norrin could significantly reduce vascular loss in transgenic mice with OIR and could increase the anatomically accurate regrowth of vessels while suppressing RNV[74]. Abnormal Norrin production led to premature retinal vascular invasion, resulting in characteristic defects in the intraretinal vascular architecture[75]. In addition, an antagonist of FZD4 not only inhibited physiological and pathologic sprouting angiogenesis within the retina but also induced the upregulation of plasma lemma vesicle-associated protein. Thus, FZD4 is required for physiological and pathologic angiogenesis in the retina and for the regulation of retinal endothelial cell differentiation[76]. These studies suggest the role of Norrin in ROP and its therapeutic potential in the treatment of the early stage ROP.

INSULIN-LIKE GROWTH FACTORS AND EARLY RETINOPATHY OF PREMATURITY

Insulin-like growth factor (IGFs) constitute a large family of insulin-related peptides that include IGF-I and IGF-II, their cell surface receptors (IGF-IR and IGF-IIR), and IGF binding proteins (IGFBP-1 through -6) as well as their proteases and interacting molecules, which work together to regulate cell proliferation, differentiation and apoptosis[77-78]. The majority of circulating IGF-I and IGF-II binds to IGFBPs, whereas IGFBPs also regulate their biological activities and modulate cellular activity *via* IGF independent pathways[79-80]. It has been found that IGF-IR and insulin receptor (IR) are predominantly expressed in photoreceptors and blood vessels, in which the expression of IGF-IR is 100-fold more than that of IR[81]. IGFBP-3 expression in neovascular tufts of OIR was increased more than 5-fold during hypoxia, whereas IGFBP2, IGFBP4 and IGFBP5 expression remains unchanged. In addition, it also found that neonatal mice from larger litters showed lower body weights and lower levels of circulating IGF-I than mice from smaller litters and that they were more susceptible to developing more severe OIR[82]. The early administration of IGF-I at postnatal day 4 increased body weight and resulted in more rapid maturation and less OIR[82]. The early short-term systemic administration of JB1, an IGF-I analog, increased soluble VEGFR-1 and decreased retinal OIR pathology more effectively than long-term treatment with JB1[83]. It has also been reported that the IGF-I levels are deficient after premature birth and that, at birth, non-ROP neonates show higher IGF-I levels than neonates with ROP. The severity and duration of low-serum IGF-I are correlated with the development of ROP[84-86]. The serum levels of IGF-I during the third week post partum provide a sufficient and reliable prognostic marker for the identification of patients at high risk to develop ROP[87]. IGFBP-3 regulates the elevated expression of endothelial NO synthase in human endothelial progenitor cells (EPCs) and promotes NO generation, thereby facilitating EPC migration into the ischemic retina. IGFBP-3 exposure also led to the redistribution of vasodilator-stimulated phosphoprotein, an NO-regulated protein critical for cell migration. Furthermore, IGFBP-3 increased pericyte ensheathment and reduced pericyte apoptosis, activated microglia and induced the apoptosis of neuronal cells in the developing retina with OIR, resulting in a more stable retinal vascular bed[88-89]. Fresh-frozen plasma (FFP) from adult donors contains higher concentrations of IGF-I and IGFBP-3, and two or more transfusions of FFP during the first week of life decreases the risk of developing any grade of ROP in preterm infants with a gestational age of less than 29wk[90-91]. Direct and continuous intravenous infusion of IGF-I/IGFBP-3 was effectively and safely to increase the serum concentrations of IGF-I and IGFBP-3 in preterm infants[92]. The prolonged administration of IGF-I/IGFBP-3 did not show any negative impact on blood-glucose levels and was beneficial for the total body growth of neonatal mice[93]. These studies suggest that IGF-I and IGFBP-3 may be safely used for ETROP.

OXIDATIVE STRESS AND EARLY RETINOPATHY OF PREMATURITY

Oxidative stress plays an important role in angiogenesis and neovascularization[94-95] and is also crucial for ROP. Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), in which cyclo-oxygenase (COX), NADPH oxidase and NO synthase are their important sources[95-97]. It has been reported that the administration of indomethacin and ibuprofen, improved OIR during the hyperoxia phase in newborn C57BL/6J mice without affecting the normal retinal

development. Administration of high-dose ibuprofen at birth decreased retinal VEGF levels and VEGFR-2 transcripts in a rat OIR model at postnatal day 14, whereas indomethacin only suppressed retinal VEGF164 transcripts with no effects on the expression of VEGF receptors[98-99]. Furthermore, ibuprofen is more effective than indomethacin in suppressing retinal VEGF signaling. Newborn rats treated with high-dose ibuprofen at birth showed significantly less somatic growth and higher serum and vitreous IGF-I levels than indomethacin treated rats[100]. However, early administration of indomethacin exerted more potent suppressive effects than ibuprofen on growth hormone binding protein, somatic growth, renal COX-2 and vasodilator prostanoids[101-102]. In addition, both of ibuprofen and indomethacin could increase serum IGF-I[101-102]. Although the administration of ibuprofen and indomethacin during the hyperoxia phase was able to improve OIR in the neovascular phase, their roles in ameliorating early ROP and their adverse effects on the development of newborn infants needs to be further investigated.

It has also been shown that treatment with apocynin, a NAPDH oxidase inhibitor, reduced avascularity and apoptosis in the OIR model *via* pathways triggered by the generation of ROS[30,103]. In addition, glutathione peroxidase-1, Nrf2, epicatechin, the thiol donor N-acetylcysteine and vitamin E have all been shown to protect against retinal vascular cell death and to reduce the avascular OIR area in hyperoxia, and additionally, Nrf2 can protects against oxidative stress-mediated damage to glia of OIR retina in hyperoxia[104-109].

RNS also plays an important role in early ROP besides ROS. It has been reported that in OIR, hyperoxia-induced vascular injury is mediated by dysfunction of endothelial NO synthase, which results in peroxynitrite formation. Treatment with arginase inhibitor 2 or deletion of the arginase 2 gene can normalize NOS activity and reduce peroxynitrite formation, thus prevents hyperoxia-induced retinal vascular injury[110].

Furthermore, the effect of superoxide dismutase 1 and 2 and vitamin E on the prophylactic treatment of ROP has also been shown in the clinic, although complications of high rates of sepsis and necrotizing enterocolitis resulting from vitamin E therapy have made it difficult to use routinely for the prophylaxis of ROP[111-114]. In sum, these studies suggest that oxidative stress plays a crucial role in the formation of the avascular area during the hyperoxia phase of OIR and ROP.

NEURAL RETINA AND EARLY RETINOPATHY OF PREMATURITY

The sensitivity of photoreceptor and postreceptor cells in a rat OIR model at early ages was associated with vascular tortuosity[115]. Following the cessation of oxygen exposure, the recovery of postreceptor neural retinal-cell sensitivity and the decrease of vascular tortuosity occurred in parallel. Furthermore, mRNA expression of VEGF(164) and semaphorin IIIA (Sema3A), the neuronal guidance cue proapoptotic/repulsive factor, was elevated early and decreased with age. Low sensitivity of rod photoreceptors and postreceptor cells were significantly associated with high VEGF(164) and Sema3A expression[115-116]. Sema3A can be secreted by hypoxic neurons in the avascular retina in response to the proinflammatory cytokine interleukin-1β (IL-1β)[117]. Sema3A contributes to vascular decay and the later formation of a chemical barrier that repels neo-vessels toward the vitreous through interfering with the actions of the IL-1 receptor[117]. Sema3A can enhance normal vascular regeneration within the ischemic retina, thereby diminishing aberrant neovascularization and preserving neuroretinal function[117-118]. The reduction in astrocyte density induced by hyperoxia led to a reduced astrocytic network in hypoxia[119]. Astrocytes provide important guidance for RNV. Protection of the retinal astrocytes and microglia was correlated directly with accelerated revascularization of the normal retinal plexuses and a reduction in RNV, which are normally associated with OIR[119-120]. Müller cell-derived and astrocyte-derived VEGF played a minor role in the development of the normal retinal vasculature, whereas it played an important role in hyperoxia-induced vaso-obliteration and RNV[121-123]. The ablation of the expression of neural cell adhesion molecule (N-CAM) from Müller cells and astrocytes, resulted in reduced vascular tuft formation in OIR, whereas retinal developmental angiogenesis remained unaffected[124]. These studies suggest that the neural retina appears to mediate the vascular abnormalities in OIR, and early treatment that targets the neural retina by decrease of Sema3A and N-CAM may be beneficial to retinal vasculature of ROP in hyperoxia.

NEUROPEPTIDES AND EARLY RETINOPATHY OF PREMATURITY

Neuropeptides and their receptors are widely distributed throughout the central and peripheral nervous systems and the peripheral organs including the retina[125-126] and their roles in ROP have been explored.

Somatostatin and Early Retinopathy of Prematurity

Somatostatin inhibits the secretion of many hormones by binding to G-protein-coupled somatostatin receptors (Sstr) and then activating cellar signaling pathways, such as adenylate cyclase/cAMP and MAPK signaling[127]. It has been showed that in OIR, somatostatin and Sstr2 levels were reduced. Sstr2 was decreased in the neuroretina but increased in capillaries. Octreotide, a Sstr2 agonist, caused a notable reduction in the hypoxia-induced increase in VEGF and its receptors and inhibited apoptotic signals from retinal cells, resulting in recovery of the a- and b-waves of electroretinograms[128-130]. In the ischemic retina, VEGF was released by damaged neurons and reached the retinal capillaries, whereas the activation of Sstr2 protected neurons from ischemic damage by decrease of the VEGF release and response[131-132]. Growing evidence indicate that retinal neurodegeneration is an early event in the

pathogenesis of diabetic retinopathy (DR) and administration of somatostatin has been contemplated as an appropriate therapeutic approach for DR[133]. As DR initiates with neurodegeneration as that occurs in ROP, thus, it is worth to test whether administration of somatostatin is an option for the prophylaxis of ROP.

Neuropeptide Y and Early Retinopathy of Prematurity Neuropeptide Y (NPY) is located primarily in the majority of sympathetic nerve fibers and regulates hormone release from the pineal glands of mammals[134]. It has been showed[135] that retinal NPY and NPY-Y2 receptor expression was altered during the development of OIR in a mouse model and this alteration might provide a target for potential modification during the development of retinopathy. However, the change in NPY levels without the presence of NPY-Y2 receptors in the immature retina, indicate that NPY may not be involved in the physiological vascularization of the retina. A recent study further showed[136] that NPY decreased only to a slight extent during hyperoxia, and a more pronounced decrease in NPY was significantly delayed during relative hypoxia. Therefore, whether NPY is involved in early ROP is uncertain and needs to be determined.

Other Neuropeptides and Early Retinopathy of Prematurity In addition to somatostatin and NPY, vasoactive intestinal peptide, opioid peptides, angiotensin II (Ang II) and other peptides have been explored in the ischemic retina[137]. However, their roles in early ROP are also uncertain. Based on neuropeptides being neuroprotective for the retina and on the intimate relationship between the retinal neurons and the retinal vasculature, it would be valuable to actively explore the role of neuropeptides in the prophylaxis of early ROP.

ENDOCRINE HORMONES AND EARLY RETINOPATHY OF PREMATURITY

Erythropoietin and Early Retinopathy of Prematurity Erythropoietin (Epo) is effective in maintaining the erythrocyte mass in the circulation and shows marked neuroprotective and neotrophic effects[138-139]. Although early administration of high-dose recombinant human Epo to very preterm infants did not markedly improve brain injury or ROP [140], the low plasma levels of Epo in preterm infants provided a rationale for the use of Epo to prevent or treat anemia. It was found that local retinal Epo levels were suppressed during the vessel-loss phase and the early administration of exogenous Epo not only prevented both vessel dropout and subsequent RNV but also protected against hypoxia-induced retinal neuronal apoptosis[141]. In contrast, retinal Epo mRNA levels were highly elevated during the neovascular phase of retinopathy, resulting in that late exogenous Epo treatment did not protect the retina but instead enhanced RNV. However, it has been reported that early Epo treatment significantly increased the overall risk of ROP (any grade) compared with late Epo administration (initiated at 8 to 28 days of age)[142], and early Epo+Fe administration could induce the appearance of grade 1 ROP[143]. Therefore, understanding the change of Epo levels during ROP is critical for determining the timing for treating ROP.

Glucocorticoids and Early Retinopathy of Prematurity Early treatment with triamcinolone acetonide reduced neovascularization and subsequent endostatin presence in an OIR model, and late treatment limited pathological vascular sprouting but did not interfere with normal vascularization of the retina[144]. Ng *et al*[145] reported that the stage of ROP was significantly associated with the basal and peak plasma levels of adreno-cortico-tropic-hormone (ACTH) and with peak serum cortisol levels at P7. It was also reported that antenatal corticosteroids (ACS) reduced the need for exogenous surfactant endotracheal tube insertion at birth in very LBW premature infants, whereas the development of ROP did not differ between groups administered one dose or multiple doses of ACS or between a betamethasone-treated group and a dexamethasone-treated group[146]. In addition, low-dose dexamethasone therapy in 4-7 days old preterm infants with surfactant-pretreated respiratory distress syndrome facilitated weaning from mechanical ventilation and shortened the duration of oxygen supplementation, although the incidence of ROP was not different at P28[147]. Thus, whether application of glucocorticoids works in the prophylaxis of ROP cannot be concluded at present.

Estrogen and Early Retinopathy of Prematurity The role of estrogen in ROP has been explored, particularly, with regard to angiogenesis[148]. The serum levels of estradiol were low in premature infants, which suggested that estradiol might play a role in ROP. An inhibiting effect of 17-alpha- and 17-beta-estradiol on RNV in OIR was reported[149-153]. However, due to the adverse effects, it is unfeasible to treat premature infants with estradiol. In addition, since 17-beta- and 17-alpha-estradiol are not highly selective for the estrogen receptor and can combine with the alpha, beta and other estrogen receptors[154-155], studies aim to explore which estrogen receptor plays the primary role in the prophylaxis of ROP are necessary.

Ghrelin and Early Retinopathy of Prematurity Ghrelin, a gastrointestinal endocrine peptide and predominantly generated in the gut, which also expressed in the rodent eye with the highest expression levels occurring in the retina and iris[156-157]. It was reported that OIR pups with poor weight gain showed high levels of ghrelin during the early post-OIR phase[158]. Ghrelin was produced locally in the retina and its level decreased during the vaso-obliterative phase but increased during the proliferative phase of OI [159]. Intravitreal delivery of ghrelin significantly reduced retinal vessel loss during the hyperoxic phase of OIR whereas ghrelin promoted pathologic angiogenesis during the neovascular phase[159]. These findings suggest that early supplementation with ghrelin might contribute to the retinal vasculature in hyperoxia, thus reducing RNV. Whether ghrelin plays a role in the prophylaxis of ROP need to be investigated.

Insulin and Early Retinopathy of Prematurity Hyperglycemia has been associated with the development of ROP in premature infants[160-161]. Early insulin therapy could decrease blood-glucose and increase IGF-I bioactivities, resulting in decrease of morbidity associated with hyperglycemia and IGF-I levels[162]. Although insulin treatment in premature infants has been suggested to increase the risk of ROP, another study showed that insulin infusions for hyperglycemia were safe and resulted in infrequent episodes of hypoglycemia with no increased risk of ROP[163-164]. In addition, the results from randomized trials also could not provide sufficient evidence to determine the effects of insulin administration in treating or preventing neonatal hyperglycemia in very LBW infants with ROP or other adverse effects[165-166]. Thus, whether insulin treatment for hyperglycemia is beneficial for the prophylaxis of ROP needs to be further investigated.

Angiopoietin and Early Retinopathy of Prematurity Ang is an important modulator of angiogenesis. Together with Tie receptors, Ang is essential for embryonic vessel assembly and maturation and functions as a key regulator of adult vascular homeostasis[167]. Ang-1 recruits pericytes and smooth muscle cells during vascular remodeling and integrates them during neovascularization[168-170], whereas Ang-2 is a natural antagonist of Ang-1 and Tie-2[171]. There was a negative correlation between the Ang-1 and Ang-2 levels in moderately and mildly vascular-active ROP eyes[172]. Ang-1 was found to play a substantial role in the formation of the retinal vascular network during postnatal development. It was reported that Ang-1 supplementation rescued vascular retinopathies by simultaneously promoting healthy vascular network formation and inhibiting subsequent abnormal angiogenesis and neuronal dysfunction in the retinas of an OIR model[173]. The functions of Ang-1 might be related to a dual signalling pathway of Tie-2 signaling in the vascular region and integrin αvβ5 signaling in astrocytes[173]. Oliner *et al*[174] observed that AMG 386 (a selective Ang-1/2-neutralizing peptibody) prevented RNV in OIR when administered from P8 to P16, but it transiently impeded regression of these abnormal vessels when administered from P17 to P23. Combining AMG 386 with VEGF inhibition also led to cooperative prevention of retinal angiogenesis in this model. Whether Ang-1 supplementation is beneficial for ROP prophylaxis needs to be further investigated.

Adrenergic System and Early Retinopathy of Prematurity The adrenergic system involves the primary ligands epinephrine and norepinephrine and their adrenergic receptor (AR) families: α_1, α_2, α_3, β_1, β_2 and β_3 receptors[175-176]. It has been reported that in OIR, hypoxia did not influence β-AR expression beyond increasing β_3-AR expression in engorged retinal tufts[177]. Propranolol dose-dependently reduced the upregulation of VEGF and decreased hypoxic levels of IGF-I mRNA and HIF-1α, thereby protecting against retinal angiogenesis and ameliorating blood-retinal barrier dysfunction in OIR[177]. It was also reported that a β_2-AR antagonist, ICI 118 551, regulated Müller-cell release of VEGF in an OIR model, indicating that β_2-AR activity might play a role in RO[178]. Although propranolol could retard the progression of stage 2 ROP to stages 3 and 4 ROP as well as reduce laser and intravitreal Lucentis treatment, however, it is controversial whether it can induce serious systemic adverse effects[179-180]. By the way, 2% of topical propranolol could significantly reduce VEGF and IGF-1 upregulation following hypoxia and profoundly reduced HIF-1α accumulation and STAT3 phosphorylation, leading to reduce RNV in the superficial, but not the deep, vascular plexus in OIR[181]. Thus, it is hopeful that the agents of adrenergic system may be used in the early stage of ROP in the future.

Renin-angiotensin-aldosterone System and Retinopathy of Prematurity It has been reported[182] that renin-angiotensin-aldosterone system (RAS) components are localized to blood vessels and cells in the retinal ganglion cell (RGC) layer of newborn rats, where they may stimulate the growing vasculature to extend into the peripheral retina and increase the vascular density in the periphery. It has also been reported[183-184] that Ang II *via* its type 1 and type 2 receptors regulates the generation of ROS by NADPH oxidase, which is crucial for the vasculature, including retinal angiogenesis. In addition, aldosterone might be through reducing glucose-6-phosphate dehydrogenase to exacerbate angiogenesis in early OIR, whereas an antagonist of the mineralocorticoid receptor (MR) reversed this effect of aldosterone[185]. Furthermore, the fact that the 32-gestational-week twin girls who were exposed to blockage of the RAS during gestation and received very little additional oxygen following birth showed severely reduced retinal vasculature and developed severe ROP later[186], suggested that RAS might play an important role in ROP development. Whether RAS is crucial for retinal vasculature and early administration Ang II is beneficial for the prophylaxis of ROP need to be further investigated.

EARLY NUTRITIONAL SUPPLEMENT AND RETINOPATHY OF PREMATURITY

Vitamin A and Early Retinopathy of Prematurity Vitamin A refers to a group of compounds, including retinol, retinaldehyde, and retinoic acid. In the retina, reversible oxidation of vitamin A produces retinaldehyde, which is an essential constituent of the visual pigment rhodopsin[187]. It has been reported that in hyperoxia, retinoic acid promoted VEGF expression in OIR retina, supported retinal vascular development and counteracted vaso-obliteration in OIR mice[188]. Premature infants were prone to subclinical vitamin A deficiencies during the first week of life, and these deficiencies could be treated with adequate enteral feeding and routine multivitamin supplementation, suggesting that a high dose of vitamin A was not necessary for healthy premature infants[189-190]. Mactier *et al*[191-192] showed that early high-dose

intramuscular vitamin A supplementation in infants at risk for ROP improved retinal function at 36wk of postmenstrual age. Thus, early vitamin A supplementation may be beneficial to premature infants at risk for ROP. However, the optimal dose and most appropriate route of administration of vitamin A in preterm infants needs to be determined in the future.

Early Nutrition and Weight Gain and Early Retinopathy of Prematurity Premature infants often have low weights, and early nutrition plans and weight gain have been speculated to be beneficial to the treatment of ROP. However, only limited data regarding the administration of early parenteral and enteral nutrition to very LBW infants are available[193]. It has been reported that newborn OIR mice with poor postnatal nutrition and poor weight gain exhibit a prolonged phase of proliferative retinopathy, prolonged overexpression of VEGF, low serum non-fasting levels of glucose, insulin, and IGF-I and high levels of ghrelin during the early post-OIR phase[158]. In addition, early and aggressive introduction of total parenteral nutrition and enteral feeding could benefit weight, length and head circumference measurements, reduce nutritional deficits in very LBW infants, increase the levels of IGF-I and IGFBP3 and reduce the risk of ROP, while other study found that feeding with human milk and vitamin, rather than parenteral nutrition, reduced the rate of severe ROP[194-198]. The risk of developing severe ROP in extremely premature infants could also be reduced by providing nutritional support *via* special delivery of lipids and total calories to increase weight gain, and recently, a fish-oil based lipid emulsion (LE) and a newer LE from alternative lipid sources with reduced polyunsaturated fatty acid (PUFA) content compared to the conventional soybean oil based LE has been preliminarily proved to be more effective in decreasing the early stages (1-2) of ROP[199-201]. It has also been suggested that monitoring postnatal longitudinal systemic factors, such as weight gain and IGF-I and IGFBP3 levels, may enhance the clinician's ability to identify the patients who would require treatment for ROP[202]. Studies further indicated that WINROP, could accurately predict when the rate of weight gain would be decreased to a specific threshold, and enabled early detection in 100% of infants who developed ROP and required treatment[203-205]. ROP typically occurs 4-6wk after birth in premature infants, which provides sufficient time to supply premature infants with adequate and reasonable nutrition to reduce the incidence and severity of ROP, and it has been proved that poor postnatal weight gain in the first two weeks is an independent risk factor for ROP requiring treatment[206], so it is meaningful to actively explore the components and methods that should be used for nutritive treatment for premature infants.

Iron Supplementation and Early Retinopathy of Prematurity Iron is an essential micronutrient that plays an important role in cellular function. It has been found that premature infants showed reduced iron stores, compared with full-term infants[207]. Early iron supplementation in preterm very LBW infants could improve serum ferritin and hemoglobin levels but has less effect on the incidences of ROP[208-209]. Whether iron supplementation benefits to preterm or LBW infants with regard to the development of the retinal vasculature needs to be further investigated.

Polyunsaturated Fatty Acids and Early Retinopathy of Prematurity Omega-3 and omega-6 PUFAs are essential components of cell membrane phospholipids and substrates of various enzymes. It has been reported that omega-3 PUFAs decrease the avascular area of the OIR retina by increasing vessel regrowth under hyperoxic conditions, thereby reducing the hypoxic stimulus of neovascularization[210]. Bioactive omega-3-PUFA-derived mediators also potently protected against neovascularization by suppressing tumor necrosis factor-alpha[211]. Sapieha *et al*[212] found that 5-lipoxygenase (LOX) played a pivotal role in the protection of dietary omega-3 PUFAs against OIR and that COX inhibitors, might be used without losing the beneficial effects of dietary omega-3 PUFAs. Recently study suggested that, in addition to anti-angiogenic metabolites of COX and LOX, cytochrome P450 epoxygenases (CYP2C8) metabolized omega-3 PUFAs and produced bioactive epoxides that were inactivated by soluble epoxide hydrolase (sEH) into transdihydrodiols[212]. In an OIR model, CYP2C8 is upregulated, whereas sEH is suppressed, resulting in an increased retinal epoxide-to-diol ratio. Overexpression of CYP2C8 or sEH in mice does not affect normal retinal vascular development. The proangiogenic role of CYP2C8 in the retina on both omega-3 LCPUFAs and omega-6 LCPUFAs and the anti-angiogenic role of sEH on omega-3 LCPUFA metabolism could influence RNV[212]. And recently, It has been reported that early administration parenteral omega-3 FAs in the form of fish-oil lipid emulsions markedly reduced the incidence of severe ROP or need for laser therapy in preterm infants[213]. Thus, early supplementation of omega-3 LCPUFAs and omega-6 LCPUFAs may contribute to retinal vasculature of premature infants and reduce the risk of ROP.

Sepsis and Early Retinopathy of Prematurity Sepsis is a potentially fatal whole-body inflammatory state that is caused by severe infection. Early-onset sepsis was associated with severe ROP, and sepsis also associated with the onset of posterior ROP[214-216]. In addition, perinatal inflammatory stress induced a significant increase in retinal vascular density, as well as a pronounced increase in activated microglial cells in RGC layer and in the outer plexiform layer immediately prior to their vascularization[217]. At maturity, perinatal inflammatory stress led to depleted retinal vascular beds and significantly decreased retinal function, resulting in abnormal retinal vascular development and increased vessel anastomosis and, finally, impairment in retinal function associated with microglial activation[217]. Therefore, management of sepsis may be beneficial in reducing the incidence and severity of ROP.

Hypercapnia and Early Retinopathy of Prematurity Hypercapnia is generally defined as an abnormally high level of CO_2 (*e.g.* more than 45 mm Hg) in the arterial blood. Permissive hypercapnia has been recognized, as a method to reduce lung injury and other adverse effects and to improve survival in preterm neonates[218]. However, hypercapnia might be a risk factor for ROP in clinical setting[219]. It has been reported that increase of CO_2 levels was associated with retardation of normal retinal vascular development and increase of peripheral avascular area in neonatal OIR rats, which is a critical step preceding RNV. The following findings suggest that hypercapnia may hinder efficient neovascularization and contribute to ROP[220-223]. Hypercapnia is able to induce an early increase in endothelial NO synthase and RNS. *In vivo* RNS is associated with retinal vaso-obliteration and leads to the nitration of arachidonic acids (AAs) into trans-AAs (TAAs). TAAs can act as mediators of nitrative stress by: 1) causing microvascular degeneration through inducing the expression of the antiangiogenic factor thrombospondin-1, which is associated with astrocyte impairment and endothelial cell death; 2) downregulation of the proangiogenic prostaglandin E2 receptor EP3. Thus, management of hypercapnia may ameliorate early ROP.

Early Light Reduction and Early Retinopathy of Prematurity Recent study suggested that continuous light radiation caused a time-dependent decrease in RGC-5 response and resulted in photo-damage within 10h due to the depletion of adenosine 5'-triphosphate and an increase in ROS levels, similar to photo-damage *in vivo*[224]. Thus, it is reasonable to postulate that an early reduction in light exposure in premature infants might decrease the incidence of acute ROP. However, this hypothesis was not supported by studies that decreased exposure of the retina to light in premature infants did not reduce the incidence of ROP[225]. Whether light contributes to ROP needs to be further investigated.

Stem Cells and Early Retinopathy of Prematurity Stem cells (SCs) are pluripotent cells with self-renewing capability. Recent studies have shown that SCs play important roles in RNV[226-227], which suggests a role of SCs in ROP. It was found that a deviation in the functional bioactivities of bone marrow-derived EPCs (BM-derived EPCs) enabled intact vascular development under abnormal oxygen dynamics[228]. EPCs were increased significantly in the peripheral blood and bone marrow of mice with OIR and a decrease in circulating EPCs might arrest vessel growth during normal retinal development in OIR rats[229-230]. Early EPCs and very small embryonic-like SCs were also significantly increased in preterm infants with ROP, which suggested that EPCs and circulating SCs may play a role in ROP[231]. In addition, BM-derived monocyte lineage cells (BM-MLCs) could differentiate into endothelial cell (EC)-like cells and function as EC progenitors that acquire the ability to adhere to injured endothelium in a MCP-1-dependent manner[232]. The reduction of BM-MLCs infiltrating into the OIR retina is associated with an increase in the avascular area and preretinal neovascular tufts, which suggests that recruitment of BM-MLCs to the hypoxic retina may be used to promote intraretinal revascularization, thereby preventing RNV[233]. Furthermore, it has been reported that a number of adult BM-derived myeloid progenitor cells could migrate to avascular regions of the OIR retina and then differentiated into microglia to facilitate normalization of the vasculature[234]. These studies suggest a role of SCs in regulating vascular regeneration in ROP, which also provides a rationale for stem cell therapy in ROP in the future.

Gene Expression Profiles and Retinal Proteome Changes During Early OIR In the past years, studies are also focusing on analysing gene expression profiles and retinal proteomic alterations during OIR. It has been shown that the expression of 83 genes, which are associated with development, metabolism, transport, stress response, cell adhesion, inflammation or vision, are significantly altered in hyperoxic P12 retinas. In particular, genes associated with retinal growth and vascular development, such as Pdgfb and Robo4, were downregulated[235]. In a mouse OIR model, enriched genes associated with cytoskeletal formation were identified at P8, whereas the enriched genes associated with various pathological processes, including the modulation of RNV were identified at P13[236]. Furthermore, in the iTRAQ study, upregulation of 25 proteins and downregulation of 14 proteins were identified in OIR retinas at P12 compared with the control retina[237]. These genes and proteins identified in OIR may be potential novel therapeutic targets for treating ROP.

CONCLUSION

Current treatments of ROP are focusing on RNV, which have caused new problems and debates over the current management protocols[238]. Since it takes approximately 4 to 6wk from birth to the RNV of ROP, the optimal preventive treatments for ROP should also be applied during these periods[239]. In Figure 1, we summarized the molecular regulators and signalling pathways that may contribute to the pathogenesis prior to RNV in OIR and in premature infants with ROP, which includes signalling regulators of HIF-1α, VEGF, IGF and Norrin, oxidative stress, neuroretina and its communication with the retinal vasculature, neuropeptides, endocrine hormones, early nutrition supplementation, sepsis and SCs as well as blood SaO_2 and hypercapnia. We also discussed the preclinical and clinical studies on preventive treatment of early OIR and ROP, which includes: 1) supplementation of HIF-1α, Norrin, IGF-I or IGFBP-3, somatostatin, NPY or Epo, vitamin A, iron, PUFAs or other nutrients; 2) administration of VEGF, VEGFR-1 agonist or VEGFR-2 antagonist, glucocorticoids, highly selective estrogen receptor agonist, ghrelin, insulin, Ang-1, Ang-2 inhibitor, β-AR antagonist or Ang-II; 3) suppression of oxidative stress; 4) protection of neural cells in

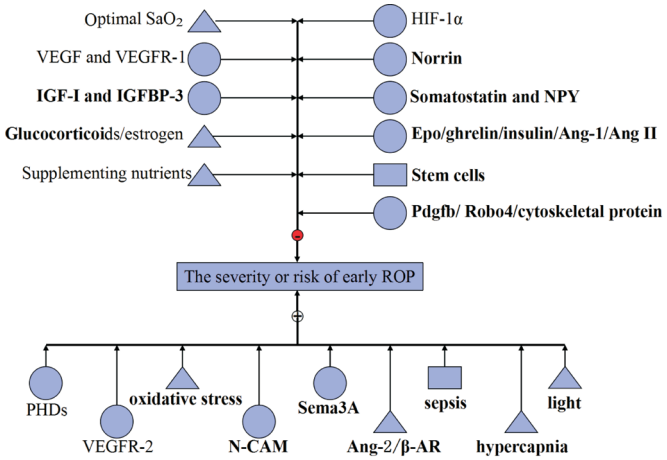

Figure 1 The factors related to early ROP We summarize the molecular regulators and signalling pathways that may contribute to the pathogenesis in OIR and ROP, which includes HIF-1α, VEGF, IGF and Norrin, oxidative stress, neuroretina and its communication with the retinal vasculature, neuropeptides, endocrine hormones, early nutrition supplementation, sepsis and stem cells as well as blood SaO_2 and hypercapnia. Targeting these molecular regulators and pathways may reduce the risk and severity of ROP.

retina and decrease of Sema3A and N-CAM; 5) mitigation and even elimination of sepsis; 6) management of hypercapnia; 7) decrease of light exposure; 8) transfusion of stem cells; 9) increase of the expression of Pdgfb, Robo4 and cytoskeletal formation genes. We wish to not only highlight the importance of the mechanisms or signalling pathways in regulating early stage of ROP but also provide guidance for exploring novel treatments for early phase OIR and ROP prior to RNV in the future.

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