THE ROLE OF FLUCTUATING OXYGEN AND CARBON DIOXIDE IN DEVELOPMENT OF RETINOPATHY OF PREMATURITY

Ph.D.Thesis

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Abbreviations

ANOVA	Analysis of Variance
C	group raised in room air
CI	confidence interval
DAB	diaminobenzidine
DNA	deoxyribonucleic acid
FITC	fluorescein isothiocyanate
IQR	interquartile range
NEC	necrotising enterocolitis
NO	nitric oxide
PBS	phosphate-buffered saline
PCO_2	partial pressure of carbon dioxide
PEDF	pigment epithelium-derived factor
PFA	parformaldehyde
PO_2	partial pressure of oxygen
RNA	ribonucleic acid
ROP	retinopathy of prematurity
SD	standard deviation
SpO_2	arterial pulse oxygen saturation
SPSS	SigmaPlot SigmaStat (Statistical Software)
TBS	Tris-buffered saline
$TcPCO_2$	transcutaneous carbon dioxide pressure
$TcPO_2$	transcutaneous oxygen pressure
TESPA	3'aminopropyltriethoxysilane
TGF-β	transforming growth factor-beta
V	variable oxygen group
V/CO_2	variable oxygen with continuous 5% carbon dioxide
VEGF	vascular endothelial growth factor

Summary

ROP is a potentially blinding disease of preterm infants. This thesis focuses on the role of CO_2 and fluctuating O_2 levels in the development of ROP. In our clinical cohort study the aim was to determine whether hypercarbia or hypocarbia or the variability of CO_2 in the first 2 weeks of life in extremely preterm infants were associated with the development of ROP. In contrast to the previous clinical studies using intermittent arterial blood gas analysis to associate PCO_2 with pathology, we have analysed continuous $tcPCO_2$ data of infants. The daily mean and SD of $tcPCO_2$ was compared between infants who had stage 1 or 2 ROP and stage 3 ROP. The total time spent hypocarbic and/or hypercarbic was also compared between these groups. Intermittent PCO_2 was also measured and compared with the simultaneous $tcPCO_2$ data. There were no significant differences in CO_2 variability or time spent hypocarbic and/or hypercarbic between the ROP groups on any day. 86 % of transcutaneous values were within 1.5 kPa of the simultaneous arterial values. Our findings proved that $tcPCO_2$ measurement can be a very useful management technique but in our cohort neither variable blood CO_2 tension nor duration of hypercarbia or hypocarbia in the first two weeks of life was associated with the development or severity of ROP. Recently, using a unique animal model of O_2-induced retinopathy we showed that newborn rats raised in a relatively hypoxic but variable O_2 environment (fluctuated around 17 % mean O_2) develop less severe retinal abnormalities than those raised in variable O_2 around higher (24 %) or normal (21 %) O_2 means. In our study we used the Edinburgh-model to investigate O_2 variability compared with the combination of O_2 variability and hypercarbia on the development of ROP. Neonatal rats were raised for 2 weeks in a fluctuating O_2 environment (around 21 % mean inspired O_2) with or without (5 %) CO_2. The small but frequent changes in O_2 were able to induce peripheral avascularity of the retina and abnormal terminal vessels. When combined with continuous hypercarbia the rat pups developed larger peripheral avascular area of the retina and the vasculature was immature and unremodelled. Abnormal, dilated vessels were present throughout the retina. At the vascular/avascular interface of the retina terminal buds that stained with endothelial cell specific lectin represented endothelial cell proliferations. Continuous hypercarbia combined with O_2 fluctuation around a normoxic mean resulted in similar changes in retinal vasculature than fluctuation around mildly hyperoxic mean. Hypercarbia increased the severity of fluctuating O_2-induced retinopathy in newborn rats.

1. INTRODUCTION

1.1. History of the disease

Retinopathy of prematurity (ROP) was first described in 1942 in infants who were born 8 weeks preterm[1]. ROP was later associated with the introduction of high concentrations (80%) of supplemental oxygen, given to preterm infants without arterial monitoring, for up to 8 weeks. A reduction in inspired oxygen concentration in subsequent decades resulted in a dramatically reduced incidence of the disease, but this was accompanied by a dramatic increase in preterm mortality and handicap from hypoxia[2].

In the 1970s and 1980s, the development of techniques to continuously monitor oxygen (both with intra-arterial and transcutaneous probes), together with improved ventilators with better control of the inspired oxygen content, enabled the arterial oxygen to be maintained within 'safe' limits. However, the increased number of extremely immature infants (up to 16 weeks preterm) who were able to survive because of these and other technological advances may be the reason that the incidence of retinopathy of prematurity has once again begun to increase and become the most common acquired retinal disease in premature babies[3].

1.2. Normal retinal blood vessel development

The retina has two blood supplies, the choroidal vessels and the inner retinal vessels. The choroidal vasculature develops early (20-29 weeks' gestation), lies on the outer surface of the retina, and is the sole supplier of nourishment to the thin, undifferentiated immature retina. The inner retinal vasculature takes longer to develop (16-40 weeks' gestation), lies within the inner surface of the retina, and nourishes the inner portions of the thick, maturing retina[4]. One stimulus for the normal formation of the inner retinal blood vessels is the thickening of the maturing retina that displaces the inner retina further from the choroidal vessels, presumably increasing the levels of hypoxia in these tissues. Another stimulus is that as photoreceptors mature their metabolic rate increases, and they utilise more oxygen that cannot be supplied by the non-vasoconstricting choroidal vasculature alone[4].

The inner retinal vasculature develops from spindle cells which are the mesenchymal precursors of vascular endothelial cells[4,5]. These cells are apparent at 14 to 15 weeks' gestation and migrate from the optic disc centrally and in a superficial plane toward the retinal

periphery, reaching the ora serrata at 29 weeks' gestation[4,5]. The spindle cells proliferate and aggregate to produce cords of endothelial cells. This *de novo* formation of blood vessels by the differentiation of endothelial precursor cells is called vasculogenesis[6].

The early, primordial vessels of the human retina are formed by vasculogenesis[5]. However, angiogenesis, the formation of vessels by budding and sprouting from existing vessels[5], is responsible for increasing the vascular density of the early vessels and peripheral vascularisation in the inner retina.

Lastly, remodelling – the process of matching the vascular tree to the tissue oxygen requirements, comprises two distinct processes. Some capillaries coalesce into larger vessels and others, particularly those in proximity to arteries undergo retraction. As a result of their collapse, the vascular tree becomes less exuberant, and capillary-free zones are formed along the flanks of arteries[5]. The process of remodelling and maturation of the inner retinal vasculature is complete at 40 weeks' gestation[4,5]. This development is regulated first of all by oxygen levels in retinal tissue and vascular endothelial growth factor (VEGF) has a key-role during this procedure.

VEGF is a potent angiogenic glycoprotein that is increased, and its mRNA stabilised, by hypoxia[7,8]. VEGF induces endothelial cell proliferation, promotes cell migration and inhibits apoptosis[7,8]. In the retina it is secreted by the retinal microglial cells[9]. During the formation and maturation of the retina a "physiological hypoxia" develops because of the increase in retinal neuronal and metabolic activity and the rise in oxygen demands of retinal tissue[10,11]. Hypoxia induces VEGF expression[9,10] and the secreted VEGF causes the formation of the retinal vessels by its endothelial cell-specific mitogen and chemoattractant effects[7]. VEGF therefore reduces the hypoxic stimulus for retinal vessels formation, thus balancing the oxygen supply and demand of the retina.

Although VEGF plays a major role in retinal vascularization, it is likely that other molecules, both angiogenic and angiostatic, are involved. Numerous proangiogenic molecules have been proposed to play a role, including growth hormone and the insulin-like growth factors[12], basic fibroblast growth factor[13], and hepatocyte growth factor[14]. TGF-β[15] has been proposed as an inhibitor of retinal neovascularization and several systemic inhibitors of angiogenesis have been characterized, including angiostatin[16], antithrombin III[17], endostatin[18], thrombospondin[19]

and platelet factor-4[20], however the role of these molecules in the process of retinal vascularization is unclear.

Perhaps the most potent natural inhibitor of angiogenesis is pigment epithelium-derived factor (PEDF)[21]. It is an endogenous protein that is secreted by retinal epithelial cells into the inner photoreceptor matrix of the retina[22] and promotes apoptosis of cells within ocular neovascular lesions[23]. PEDF is also an essential factor for normal retinal development. Recent data indicate that elevated concentrations of PEDF inhibit VEGF-induced retinal endothelial cell growth and migration and thus retinal neovascularization[24]. PEDF has photoreceptor and neuroprotective activity as well[25]. It has been found to be downregulated by hypoxia; in contrast, hyperoxia increased the expression of PEDF[23,24]. Therefore, like VEGF, its regulation by oxygen makes it potentially important in the development of ROP. However, in the literature there is a debate on the role of PEDF in ROP. Recently some animal models[26,27] suggested that over-expression of PEDF, or no expression of PEDF produced no abnormalities in the retinal vasculature. Therefore the importance of PEDF in the pathogenesis of ROP is still to be fully understood.

1.3. Oxygen and carbon dioxide

Oxygen has proven therapeutic value in newborn infants to correct, and prevent the many complications of hypoxia. Oxygen decreases the incidence and severity of apnoea in premature infants[28], dilates the pulmonary arteries and increases pulmonary blood flow[28], and constricts the ductus arteriosus[28]. Although its essential role in life is well known, oxygen is also potentially toxic. Breathing a higher than normal oxygen concentration can cause pulmonary epithelial cell injury and has been implicated as a principal cause of chronic lung disease and bronchopulmonary dysplasia in preterm infants with respiratory distress syndrome[28]. Within, and adjacent to, cells throughout the body, oxygen can react with prooxidants to produce reactive oxygen species that then produce inflammation and even cell death. We have recently demonstrated that severe vasomotor changes during hypoxia (ischaemia/reperfusion) may result in mesenteric endothelial dysfunction implicated in the development of necrotizing enterocolitis (NEC), which is the most common acquired gastrointestinal emergency in neonates[29]. Oxygen is also important in the regulation of

cerebral vessels. Generally, higher blood oxygen levels constrict certain blood vessels, such as those in the retina[28].

The physiological effect of carbon dioxide in newborn infants is also very important, particularly as a potent respiratory stimulator and its effect on cerebral vessels. Hypocarbia and hypercarbia have opposite effects on the cerebral vasculature. Hypercarbia increases cerebral blood flow, hypocarbia reduces it[30]. Retinal blood flow also increases during hypercarbia[31]. Some earlier studies showed that hypercarbia could be highly protective in experimental models of acute ischaemic myocardial, lung or brain injury[30,32,33]. However, clinical investigation suggests that premature infants who require mechanical ventilation for respiratory distress syndrome are at an increased risk for periventricular leucomalacia if hypocapnia occurs during respiratory management[30]. A series of studies also investigated whether hypercapnia or hypocapnia might be a risk factor for ROP.

1.4. Pathogenesis of ROP

Retinal neovascularisation is a pathological condition in which abnormal angiogenesis can lead to severe consequences such as tractional retinal detachment, vitreal bleeding or sometimes to blindness. ROP begins to develop between 32 and 34 weeks after conception, regardless of gestational age at delivery[34].

Historically, ROP has been shown to have two distinct pathophysiological phases during the progression of the disease[34].

There have been several theories in the past, which have sought to explain the pathogenesis of retinopathy of prematurity. All of these concur that oxygen is implicated either directly or indirectly in the initiation of ROP, but the management of premature infants and our understanding about ROP have changed in many ways over the last 10-15 years so most of these explanations are out of date.

The first acute phase of ROP is the vasoattenuation, the occlusion and the obliteration of the existing retinal blood vessels. The normal vasculogenesis of the retina is disturbed by the relative hyperoxia of the extrauterine environment. This causes vaso-obliteration and non-vascularisation of some areas of the anterior retina. The resulting vascular insufficiency ultimately produces inner retinal hypoxia, leading to the second chronic phase, the vasoproliferation. This vasoproliferation is a poorly controlled process of retinal

vascularization, characterised by the proliferation of new blood vessels which are unstable and leaky and arteriovenous shunt formation, occasionally leading to involution or permanent cicatrial changes and visual impairment[10].

In the first classic theory[35,36] the retinal vessels constrict and their endothelial cells are damaged due to raised partial pressure of oxygen (PO_2) above normal for the foetus. This leads to ischaemia and the production of angiogenic factors and subsequent vasoproliferation. *Kretzer et al*[4] in their theory also postulate the production of angiogenic factors but propose that they are not induced by vasoconstriction but direct oxidative insult to the mesenchymal precursor cells (spindle cells). These cells then synthesise and secrete angiogenic factors generating the vasoproliferative response by the retinal vessels. After premature birth oxygen can diffuse easily from the non-vasoconstricting choroidal vasculature, can cause physiological vasoconstriction of the inner retinal vasculature, and create an abnormal hyperoxic environment for spindle cells.

Other investigators[21,37] suggest a theory implicating the balance of growth factors and inhibitors, and apoptosis. The maintenance of the retinal vascular tree depends on a continuous supply of survival factors, which can be downregulated by oxygen. Thus hyperoxia induces a shutdown of VEGF and markedly increased expression of PEDF, the major natural inhibitor of angiogenesis, which causes endothelial apoptosis and leads to vaso-obliteration of newly formed retinal capillaries. The ischaemia that follows generates an upregulation of VEGF that induces angiogenesis and the vasoproliferative response of ROP.

Brooks et al[38] hypothesised that oxygen-induced retinal vaso-obliteration was mediated in part by endothelial nitric oxide (NO)-derived oxidants such as peroxynitrite. Evidence for peroxynitrite mediated protein modification and cellular injury has been reported in cerebral ischaemia[39], myocardial ischaemia-reperfusion[40] and experimental models of retinal ischaemia[41]. These important in situ mediators of oxidative injury may also have a pathogenic role in oxygen-induced retinopathy in developing retina by protein modification and direct cytotoxicity.

1.5. Risk factors

ROP is a multifactorial disease[42] with gestational age[34,42,43,44] and low birth weight[4,34,42] being the most powerful predictors of progression to disease.

1.5.1. *Oxygen and the development of ROP*

Although in the early 40s and 50s it was postulated that high arterial oxygen levels[45,46] were the cause, more recently data has shown that fluctuations in arterial blood oxygen tension[47] are more closely related to the development and severity of the disease.

Johns et al[48] showed that premature infants with cyanotic congenital heart disease can develop ROP despite persistent hypoxia. In their study the infant population was persistently hypoxic, but generally they had normal arterial carbon dioxide levels and pH values.

Previous data suggested that infants with a partial pressure of oxygen (PO_2) more than or equal to 80 mmHg, which correlates with an arterial pulse oxygen saturation (SpO_2) of 97-100 %, in the first two weeks of life are at an increased risk for developing severe ROP[49]. There was lower prevalence of severe ROP when the maximum SpO_2 was less than or equal to 92 % after the first two weeks of life[49].

Continuous transcutaneous monitoring of blood gases with intermittent confirmatory analysis of arterial gases assists clinical staff in maintaining PO_2 and partial pressure of carbon dioxide (PCO_2) within acceptable ranges. The most recent theory[47] is based on the current neonatal intensive care treatment strategy. Clinical staff at the Neonatal Units in Edinburgh attempt to maintain the transcutaneous oxygen levels of babies between the conventional alarm limits of 6 and 10 kPa (45 – 75 mmHg) to guard against hyperoxia. However, *Cunningham et al*[47] demonstrated that maintaining the arterial oxygen levels within these clinically accepted 'safe' limits did not guard against the development of ROP. The variability of PO_2 within these limits in the first two weeks of life was a significant predictor of severe ROP[47]. They hypothesised that the frequent small changes in PO_2 in the blood may cause retinopathy by frequent interruption of the development of the retinal vasculature and its stabilisation, which is controlled by relative hypoxia and hyperoxia in response to blood supply and metabolic demands. The extremely preterm infants tend to fluctuate their PO_2 within conventional alarm limits because of pathological and iatrogenic causes. These are small but frequent changes in the arterial oxygen, which may affect VEGF induction and suppression and prevent the production of stable new retinal blood vessels. Whilst these findings have major implications for neonatal care, it has not been possible to define a concentration, or duration of oxygen,

which is, or is not associated with ROP. The current consensus is that hyperoxia can be important in ROP initiation.

1.5.2. *CO_2 and the development of ROP*

There are contradictory data in the literature on whether high or low levels of carbon dioxide (CO_2) are associated with or contribute to the development of ROP.

Clinical studies

Shoat et al[49] reported a highly significant association between episodes of hypocarbia and the development ROP, but no significant association to either episodes of hypercarbia or hyperoxia. In agreement with this was the cohort study of *Brown et al*[50] that showed that the mean PCO_2 values were consistently lower for the group of infants with scarring retinopathy of prematurity but there was no association with elevated PCO_2. In contrast *Bauer et al*[59] noted a correlation between hypercarbia and ROP using discriminant analysis for statistical evaluation. In their study the maximum PCO_2 measured, and hypercarbia associated with simultaneous hyperoxia, were the best discriminating variables. They suggested that retinal vasoconstriction due to hyperoxia may be overcome by vasodilatation from hypercarbia which overall facilitates the delivery of highly oxygen saturated blood to the retinal tissues.

Animal models

Flower et al[52] support this association using an animal model. Newborn puppies made hypercarbic had both an increased incidence and severity of ROP. In their study the most significant predictor of ROP was the maximum PCO_2 measured that occurred simultaneously with elevated PO_2. They found that the tendency of blood vessels to dilate in response to elevated PCO_2 was greater than their tendency to constrict in response to elevated PO_2. *Yu et al*[53] in a rat model investigated the intraretinal oxygen distribution with graded systemic hyperoxia with or without hypercarbia. Hyperoxia resulted in a significant increase in oxygen level in all layers of rat retina, which was augmented by hypercarbia. *Holmes et al*[54] in their neonatal rat model found that hypercarbia alone, followed by room air recovery, could result in preretinal neovascularisation In their study newborn rat pups were exposed to room air oxygen levels (21 % O_2) and 10 % CO_2 ("high-inspired CO_2 group") or to 12.5 % oxygen levels and 10 % CO_2 ("pure hypercarbia group") for 7 days and then the animals were placed in room air for five days. They found that neovascularisation occurred at the junction of the

vascular and avascular retina in 19 % of the animals in the "high-inspired CO_2 group" and 14 % in the "pure hypercarbia group".

1.6. Aims

Our aim was to investigate the role of oxygen fluctuation and carbon dioxide in retinopathy of prematurity.

1.7. Clinical studies

Previous clinical studies used intermittent arterial blood gas analysis to associate PCO_2 with pathology[49,50,51]. Frequent blood sampling is invasive and unless performed from indwelling arterial lines may rapidly alter the PCO_2 as the infant either cries or holds its breath[55]. This would not permit analysis of variability of blood CO_2 whereas non-invasive transcutaneous measurement, as performed in this study does.

A computerised cotside monitoring system at the Neonatal Unit of Simpsons Memorial Maternity Pavilion (Edinburgh, UK) continually stores physiological data (including PO_2, PCO_2, pH) from all intensive care neonates. It is a personal computer based cotside network system with a central server, which accesses information each second from standard neonatal monitors. Up to 32 channels of physiological data can be monitored at any time. The technique allows the review of corresponding physiological graphs and the use of notes to indicate changes in therapy and patient interventions[56]. We analysed continuous transcutaneous carbon dioxide data from this computerised monitoring system calibrating this with intermittent arterial samples.

Our aim was to determine whether hypercarbia or hypocarbia or the variability of CO_2 in the first two weeks of life in extremely preterm infants were associated with the development of ROP.

1.8. Animal models

A newborn rat retina is not fully vascularised and the process takes about 14 days to complete. This makes a rat at birth roughly equivalent to a 24-week gestation human preterm infant and a useful tool for investigating retinal vascularisation.

Inducing retinopathy in the rat has not been totally successful. Exposure to 80% oxygen followed by a period in room air, results in the vasoattenuation but not the vasoproliferative stage of the disease[57]. Other studies have used large swings of 80 % to 40 % oxygen over 12 hour periods[57], going from 80 % to room air[58] or from 50 % to 10 %[59]. These studies result in the acute oxygen injury, which is typically seen in preterm babies developing severe ROP, but not the later retinal detachment stage. Whilst they have helped to investigate how large swings in oxygen contribute to the development and severity of the disease, they only partially mimic what is experienced by an extremely preterm infant currently.

Investigation of elevated carbon dioxide in rats has shown that both alone and in combination with high oxygen it increases the severity of disease[54]. In this study the role of combined carbon dioxide and variable oxygen in an animal model of ROP was investigated. A previously developed computer controlled oxygen delivery system has allowed a more representative model of newborn arterial oxygen to be developed. It enables the simulation in rats of transcutaneous arterialised oxygen values recorded from an infant who developed severe retinopathy of prematurity[60]. It is suitable to investigate the role of clinically relevant fluctuations in oxygen with or without the combination of hypercarbia on the development and severity of ROP.

2. MATERIALS AND METHODS

2.1. Clinical study

2.1.1. *Patients*

This was a retrospective cohort study of infants who were admitted to the Neonatal Intensive Care Unit, Edinburgh, between 1996 and 1998. Inclusion criteria were 14 days of continuous monitored data of transcutaneous CO_2, at least stage 1 ROP and less than 1001 gram birth weight or less than 30 weeks of gestation.

2.1.2. *Data collection*

A computerised neonatal cot monitoring system in routine clinical use recorded physiological data including transcutaneous carbon dioxide pressure ($tcPCO_2$) from *Hewlett Packard*

combined oxygen and carbon dioxide probes and *78344A* neonatal monitors. The data were recorded every second and later stored as a one-minute average of 60 one second data points. After removal of obvious artefact due to probe calibration[56] the mean and standard deviation were calculated for each day of the first 14 days of life. For each period it was also noted the number of minutes that the $tcPCO_2$ was under 3 kPa, was over 10 kPa and was over 12 kPa. All values were further aggregated over a week or 2-week period to produce a single mean for each statistic for each baby. Arterial carbon dioxide tension (PCO_2) was also measured intermittently by umbilical or peripheral arterial catheter sample and compared to the simultaneous transcutaneous CO_2 data.

2.1.3. *Classification of ROP*

Retinopathy of prematurity was diagnosed using binocular indirect ophthalmoscopy with a speculum and scleral indentation. Careful 360° examination of the peripheral retina up to the ora serrata was performed in every instance, by one of two experienced paediatric ophthalmologists. The first examination occurred at 4-6 weeks post delivery and was repeated weekly until the retina was fully vascularised. ROP was determined using the international classification for ROP[61].

2.1.4. *Clinical stages*

Active ROP. Clinically, ROP is classified by the International Classification of Retinopathy of Prematurity[61]. The severity of active ROP is classified by stage, but its location within the retina is also important.

Stage 1 (demarcation line). The development of a thin tortuous line, which runs roughly parallel with the ora serrata, is more prominent in the temporal periphery, lying within the plane of the retina at the junction of the vascularized and the peripheral avascular retina. The demarcation is the accumulation of the mesenchymal precursors of the retinal vessels.

Stage 2 (ridge). The demarcation line develops into a ridge of tissue, which extends out of the plane of the retina. The ridge represents a mesenchymal shunt, which joins veins with arteries.

Stage 3 (ridge with extraretinal fibrovascular proliferation). It is the development of fibrovascular proliferation along the surface of the retina into the vitreous. These findings are often associated with dilatation and tortuosity of the retinal blood vessels and retinal, vitreous

haemorrhage. The highest incidence of this stage is around the postconceptual age of 35 weeks.

Stage 4 (subtotal retinal detachment). The progression of fibrovascular proliferation gives rise to a tractional retinal detachment, which starts in the extreme periphery and then spreads centrally.

Stage 5 (total retinal detachment). Typically develops when the infant is about 10 weeks old.

'Plus' disease. It is characterized by dilatation of veins and tortuosity of the arterioles in the posterior pole (fundus). When these changes are present, a 'plus' sign is added to the stage number.

Regressed ROP. In about 80% of infants, the ROP will regress spontaneously, leaving few if any residua. Complete regression is the rule for all stage 1 and 2.

Severe ROP. Some babies with stage 3 also undergo resolution, but at this stage the likelihood of significant sequel is very high and some babies become blind, hence the use of the term severe to describe stage 3 and above. Although retinal detachment and blindness is rare, its development frequently preceded by progression of 'plus' disease, development of fresh vitreous haze, increasing preretinal and vitreous haemorrhage, gross vascular engorgement of the iris and failure of the pupil to dilate.

2.1.5. ***Statistical analysis***

Data from the infants who were in ROP1,2 group was compared with those who were in ROP3 group by repeated measures analysis of variance (SPSS).

For each baby on each day there was a mean value of $tcPCO_2$ (with artefacts excluded) and a standard deviation (SD). The SDs were then averaged as a measure of variability. The daily means and the daily standard deviations of the babies in the ROP1,2 and ROP3 groups were compared on a daily basis by *t test* and throughout the study using a *repeated measures ANOVA* (Analysis of Variance). A *Bonferroni correction* (with significance defined as $p \leq 0.05$) was used because there were a large number of comparisons for the *t test*. The time in minutes that the $tcPCO_2$ was <3 kPa, >10 kPa and >12 kPa was calculated for each infant during week 1 and again during week 2. The values in ROP1,2 group were compared with ROP3 group using a *Student t test.*

2.2. Laboratory study

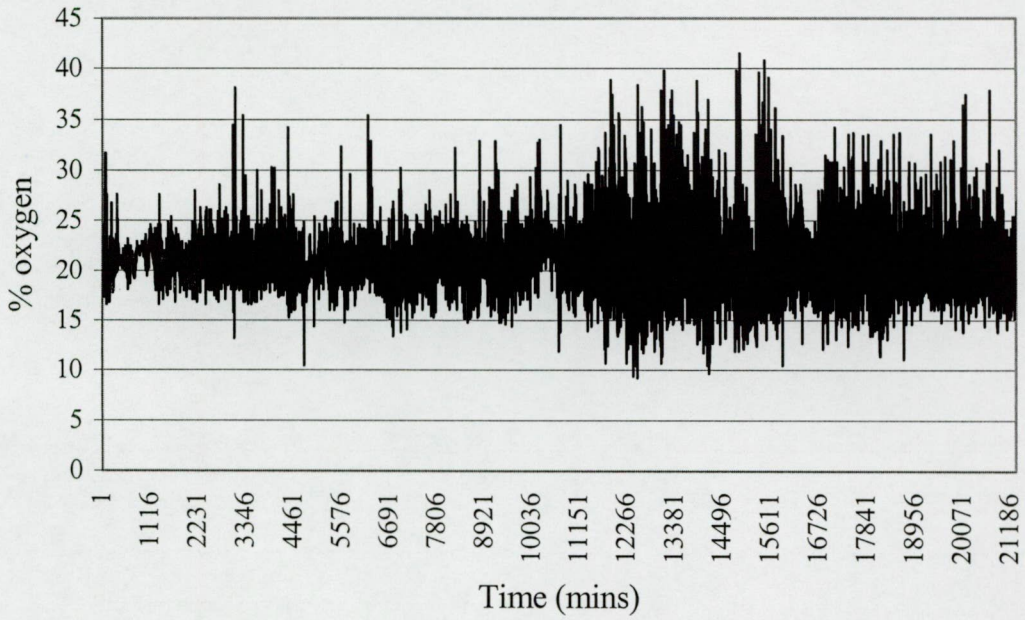
This was a group-controlled study with masking of samples prior to assessment. Approval for this study was given by the UK Home Office, and all animals were cared for in accordance with UK Home Office legislation.

2.2.1. *Experimental methods*

Derivation of rat oxygen profile

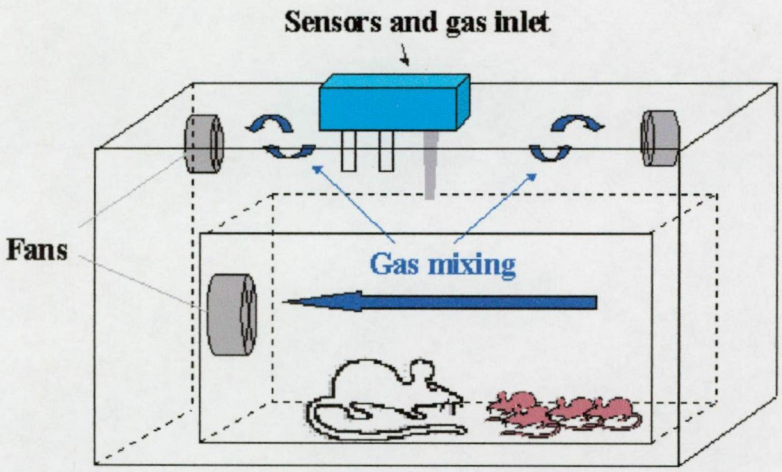
The transcutaneous oxygen values from an infant who developed severe retinopathy of prematurity was recorded using a computerised cot monitoring system[60]. These values were averaged every minute for 14 days and artefacts (from calibration or when air was trapped under the sensors) were removed manually. This resulted in a stream of 21326 transcutaneous oxygen values, representing partial pressure of arterial oxygen, one value per minute. The values were translated from the transcutaneous oxygen values to percentage inspired oxygen for the rat - as this relationship is linear[57] *(Figure 1.).*

Figure 1. Percentage inspired oxygen values for the rat



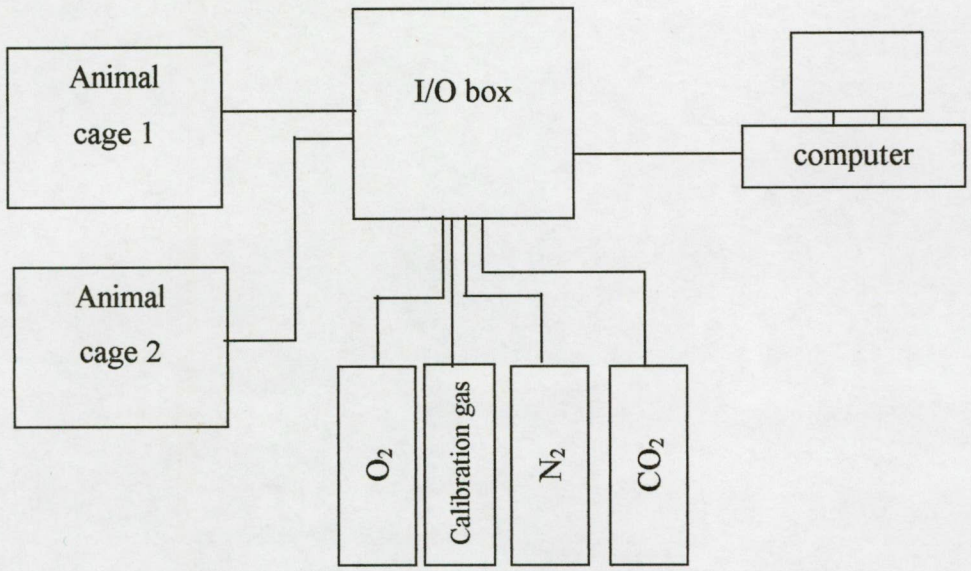
Staff in neonatal unit attempt to maintain the PO_2 in preterm infants - between 6 and 10 kPa (45 – 75 mmHg) with a mean of 8 kPa (60 mmHg). However a newborn rat breathing 21% oxygen has an arterial value of 12.9 kPa (96.8 mmHg) and therefore we had to translate the values to give an equivalent rat for the preterm arterial value. This was achieved by adding 4.9 kPa (36.8 mmHg) to every preterm value. From this set of values we derived the inspired oxygen in the rat that would produce the equivalent arterial oxygen as the relationship between arterial and inspired oxygen in the rat is linear[57]. The rat pups were then raised for 14 days in a specially built animal cage in which a computer controlled the atmosphere to mimic the fluctuating oxygen profile[60]. The system was designed and built by BioSpherix (Redfield, NY, USA) *(Figure 2).*

Figure 2. Animal cage



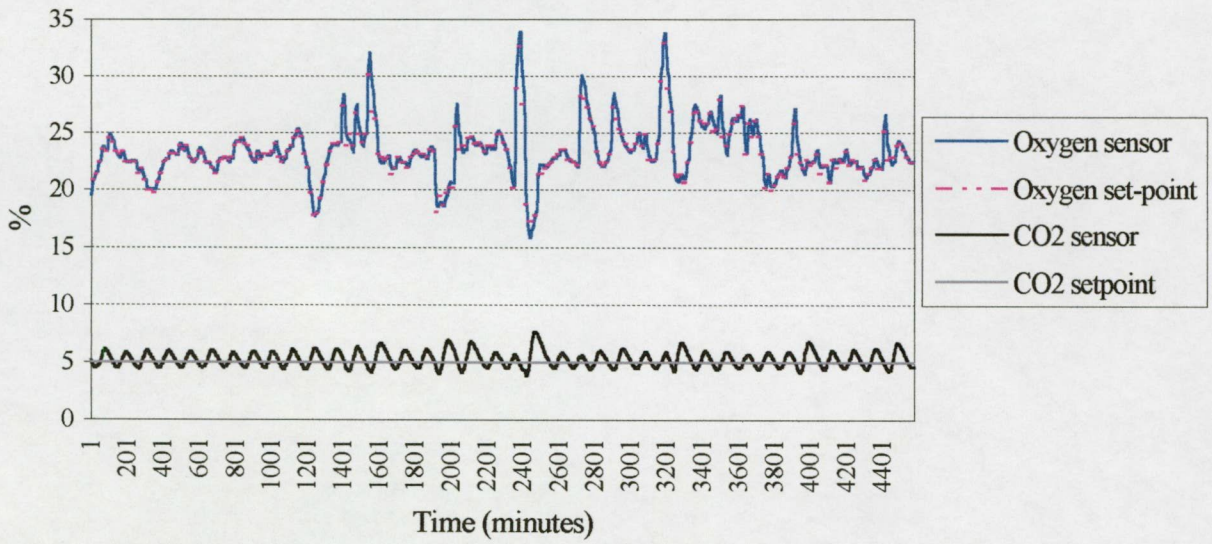
The system delivers either oxygen or nitrogen to effect the required atmospheric change in oxygen. It is capable of producing up to a ± 50 % change in atmospheric oxygen within one minute and proved at testing to be sufficiently precise[60] *(Figure 3).*

Figure 3. Diagrammatic overview of the system



The median difference between required and monitored value of oxygen was 0.3 %, with an interquartile range (IQR) of 0.2 - 0.7 % (n= 17456). 85 % of all monitored readings were within 1% oxygen either side of the set point; 95 % were within ± 2 % oxygen. The computer system was also capable of preserving continuous carbon dioxide concentration in the animal cage during 14 days using CO_2 gas cylinders simultaneously working with the minute oxygen variable profile *(Figure 4)*.

Figure 4. Carbon dioxide and oxygen profile (values represent 3 days)



2.2.2. ***Animal groups***

Newborn Sprague-Dawley rat pups were used in our animal model. Three groups of animals were studied, each for 14 days *(Figure 5)*:

- control group raised in room air (C)
- the variable oxygen group (V)
- the variable oxygen group combined with continuous, simultaneous 5% carbon dioxide(V/CO_2)

Figure 5. Groups of rats

Room air controls	**Variable O_2 group**	**Variable O_2 with continuous 5% CO_2 group**
		
n = 30	n = 30	n = 28

At **DAYS 14** rat pups were:

1. weighed
2. anaesthetised
3. perfused cardially
4. euthanised
5. retinas analysed

Pregnant animals were acclimatised to isolators at least 24 hours prior to delivery. A minimum of 12 pups was required per litter, which all rat mothers produced in these experiments. Experiments were begun within 4 hours of the delivery of the final pup in each litter. Bedding was changed every 7 days at which point the profile was paused and restarted a few minutes later. No other interruption to the profile was required.

At 14 days the rat pups were weighed and anaesthetised by intraperitoneal injection of ketamine (2.5 mg/kg) and xylazine (1 mg/kg). Paraformaldehyde (PFA) was then directly perfused (0.4 mls 0.5 %) into the left ventricle, prior to euthanasia by intracardiac injection of pentobarbitone (80 mg/kg). Both eyes were enucleated. Left eyes were used for retinal flatmounts and lectin staining. Right eyes were frozen and later cryosectioned for VEGF in-situ hybridisation.

2.2.3. *The preparation of tissues*

Retinal wholemounts

The retinas were dissected using a modification of the method of *Chan-Ling*[62]. Enucleated eyeballs were fixed whole in 2 % PFA for 2 hours before being washed in 1 M phosphate-buffered saline (PBS) pH 7.4. Under a dissecting microscope an incision was made at the limbus between the cornea and sclera. Scissors were then used to cut round the junction between the cornea and sclera until the cornea could be removed. The lens was gently removed, taking care not to remove the retina. The eyecup was transferred to 1 M PBS for further dissection. The retina was gently eased from the sclera using fine forceps, taking care to leave the ora serrata intact as it defines the edge of the retina. The retina was then placed onto a TESPA (3'aminopropyltriethoxysilane) coated slide and flattened by making four incisions perpendicular to its outer edge. At this stage as much vitreous as possible was removed using cellulose sponges and scissors.

Lectin stain

Endothelial cells were visualized by incubation of *Griffonia simplicifolia* (Bandeiraea) isolectin B4 as follows. The flattened wholemounted retinas were permeabilised in 70 % v/v ethanol (kept at –20 °C) for 20 minutes, and then in 1 M PBS/1 % Triton X-100 for 30 minutes. The retinas were then incubated with biotinylated *G. simplicifolia* (Bandeiraea) isolectin B4 (ICN Pharmaceuticals, UK) at 5 μg/ml in 1M PBS overnight at 4 °C. They were

rinsed in 1 M PBS/1 % Triton X-100 for 10 minutes, then in 1 M PBS for 10 minutes twice. Streptavidin-conjugated flourescein isothiocyanate (FITC) (Sigma Chemical, USA) at 25 μg/ml in 1 M PBS was added for 4 hours at room temperature and the slides were rinsed twice in 1 M PBS for 10 minutes each. The retinas were mounted in PBS:glycerol (2:1) and the coverslip was sealed with nail varnish.

Immunohistochemistry

The whole enucleated eyeball was pierced by a needle and fixed in 4 % (PFA) for 2 hours. It was washed twice in 1 M PBS (pH 7.4) before being embedded in agarose (1.5 % in 1 M PBS pH 7.4 supplemented with 5 % sucrose). The solid agarose blocks were trimmed and left in 30 % sucrose overnight at 4 °C or until the agarose block sank. The blocks were then frozen slowly and stored at –70 °C until sectioning. Serial cryosections 10 μm thick were made from whole frozen eyes and incubated for 1 hour using biotinylated *G. simplicifolia* (Bandeiraea) isolectin B4 (ICN Pharmaceuticals, UK) at 12.5 μg/ml in Tris-buffered saline (TBS). After successive washes in TBS the slides were then incubated with peroxidase-labelled streptavidin (Dako, UK) at 8.75 μg/ml for 1 hour. After washing, diaminobenzidine (DAB) was applied for 5 minutes, the slides were rinsed in tap water and counterstained in haematoxylin before being mounted in Depex (BDH Chemicals, UK) and viewed under a light microscope. Slides were analysed for pre-retinal vessels that grow out from the surface of the retina.

Analysis

The stained, flatmounted retinas were viewed using argon/krypton laser confocal microscope (Leica, Germany), which allowed low- and high-powered images to be taken and digitally stored for later analysis of peripheral avascular areas, central capillary density and neovascularisation of the retina.

2.2.4. ***Capillary density***

Capillary bed sample areas were chosen in the central retina with no major vessels present in the fields analysed. As each retina has four quadrants, to allow flattening onto the slide, one area from the centre of each quadrant was imaged, making four areas in total. Images were taken at x20 magnification and stored later analysis. All stored files were assigned a random number to mask the observers and counts of the number of branches were made. One observer

counted all files and a second observer counted a sub-set to compare results. There was a statistically significant correlation ($p<0.05$) between the two observers using a Pearson correlation, and no evidence of bias assessed using a Bland-Altmann plot.

2.2.5. *Avascular areas*

Digitised images of the total retinal area and peripheral avascular area were measured using Scion Image Software (Scion Corporation, MD, USA) and the avascular area was expressed as a percentage of the total retinal area.

2.2.6. *Neovascularisation of the retina*

Digitised images of the total retinal area were analysed and the presence or absence of neovascularisation was recorded by two, masked, independent ophthalmologist[61].

The different groups were compared using multilevel analysis.

2.2.7. *In-situ hybridisation*

10 μm sections were cut from frozen eyes (as above) using a Leica CM 1300 cryostat and transferred to TESPA coated slides. Slides were air-dried for at least 30 minutes before storage at -20 °C until required.

VEGF probes

DNA from exons 1-4 (392 b.p.) were cloned into a pBluescript II KS (Stratagene, USA) at the Sac II site (gift from Dr Steve Charnock-Jones, University of Cambridge, UK). The plasmid was digested with *Sac*I restriction endonuclease and end filled using T4 DNA polymerase. A DIG labelled sense probe was generated using T3 RNA polymerase (Roche Molecular Biochemicals). The antisense probe was generated by digesting the plasmid with *Bam*H1 restriction endonuclease. After purification using Elutip columns (Schleicher and Schuell, USA), the DNA was transcribed in vitro with T7 polymerase in the presence of DIG-UTP (Roche Molecular Biochemicals). The size of both probes was confirmed using Agarose gel electrophoresis.

Method for VEGF identification

Frozen sections were allowed to defrost at room temperature for at least 1 hour, and hybridised with probe overnight at 65 °C. Slides were washed at 65 °C in SSC buffer (3 M

sodium chloride, 0.3 M sodium citrate, pH 7) with 50 % formamide, 0.1 % Triton X-100 and then at room temperature in TBST (0.14 M NaCl, 2.7 mM KCl, 0.025 M Tris HCl pH 7.5, 1 % Triton X-100). Slides were then blocked in 10 % heat-inactivated sheep serum (in TBST) for > 1 hour at room temperature. Anti-DIG AP-Fab fragment (in 10 % heat-inactivated sheep serum in TBST) was then added to each section and incubated overnight in a humidified chamber at 4 °C. Slides were washed in TBST at room temperature, then in NTMT (100 mM NaCl, 100 mM Tris HCI pH 9.5, 50 mM $MgCl_2$, 0.1 % Triton X-100). Staining was performed in the dark with nitroblue tetrazolium (NBT + 3.5 % 5-Bromo-4-chloro-3-indolyl phosphate /BCIP/ in NTMT). After a few hours staining reaction was checked though colour development could take up to 24 hours at 4 °C. The reaction was stopped using distilled water and the sections fixed in 4 % PFA/0.1 % gluteraldehyde for 20 minutes. The sections were dehydrated through a series of alcohols then counterstained with filtered 0.1 % eosin in 95 % ethanol for 20-30 seconds. Rinses were made in 95 % then 100 % ethanol before transferral to histoclear and mounting in Vecta mount (Vector Laboratories, UK).

VEGF concentration in retinal sections

The presence or absence of VEGF mRNA assessed by *in-situ hybridisation* in retinal sections was qualitatively scored by two masked, independent observers.

2.3. Statistics

Summary statistics are presented as means (± standard deviation) or median and interquartile range. Correlation between groups was by Pearson correlation and correlation of between-group differences was made by Mann-Whitney U. The capillary branching was compared between groups by multilevel analysis using the software package MLWin (Institute of Education, University of London).

3. RESULTS

3.1. Clinical study

Over the 2-year period 50 infants were diagnosed with any stage of ROP and 25 of these babies met the requirements of the inclusion criteria. The others failed mainly because of a lack of the 2 weeks of continuous monitoring data, which is the strength of the study. Infants enrolled on the study had a mean (range) birth weight of 691 g (530-1245 g) and gestational age 25.2 weeks (24-29). 10 were in the ROP1,2 group and 15 were in the ROP3 group.

There was no statistical difference between mean $tcPCO_2$ values or in the variability of $tcPCO_2$ during the first 14 days of life between the two groups *(Table 1)*.

Table 1. Mean (standard deviation) and the variability of $tcPCO_2$ (in kPa) during the first 14 days of life in the ROP1,2 and the ROP3 group. Significance was defined as $p \leq 0.05$ however there were no significant differences between the groups on any day.

	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 14**
ROP1,2 Mean $tcPCO_2$	5.3 (1.0)	5.9 (0.7)	6.1 (0.8)	6.0 (1.1)	5.9 (0.8)	6.2 (0.9)	6.6 (0.9)	6.4 (1.0)	6.1 (1.1)	6.6 (1.3)	6.8 (1.5)	6.5 (1.9)	6.8 (1.4)	7.1 (1.4)
ROP3 Mean $tcPCO_2$	5.7 (1.0)	6.1 (0.9)	6.2 (0.8)	6.4 (0.9)	6.2 (1.1)	6.2 (1.3)	6.7 (1.4)	6.8 (1.1)	6.8 (1.1)	6.9 (1.2)	7.1 (1.4)	6.7 (1.4)	6.6 (1.6)	7.1 (1.6)
ROP1,2 Variability of $tcPCO_2$	1.0 (0.3)	1.0 (0.3)	0.9 (0.4)	1.0 (0.2)	1.0 (0.4)	0.9 (0.2)	1.0 (0.3)	1.0 (0.4)	1.0 (0.2)	1.0 (0.4)	0.9 (0.4)	0.8 (0.1)	0.9 (0.2)	1.0 (0.5)
ROP3 Variability of $tcPCO_2$	1.3 (0.5)	1.2 (0.5)	0.9 (0.3)	0.9 (0.3)	0.8 (0.2)	0.9 (0.2)	1.0 (0.5)	1.0 (0.4)	0.9 (0.3)	1.0 (0.3)	1.0 (0.5)	0.9 (0.4)	1.0 (0.2)	0.9 (0.4)

The length of time that the $tcPCO_2$ was under 3 kPa, was over 10 kPa or was over 12 kPa was also not significantly different between the ROP1,2 and ROP3 group in either the first or the second week of life *(Figure 6)*.

Figure 6. The median (interquartile range /IQR/) of the duration of $tcPCO_2$ levels <3 kPa, >10 kPa and >12 kPa during the first and second week of life in the ROP1,2 and ROP3 group.

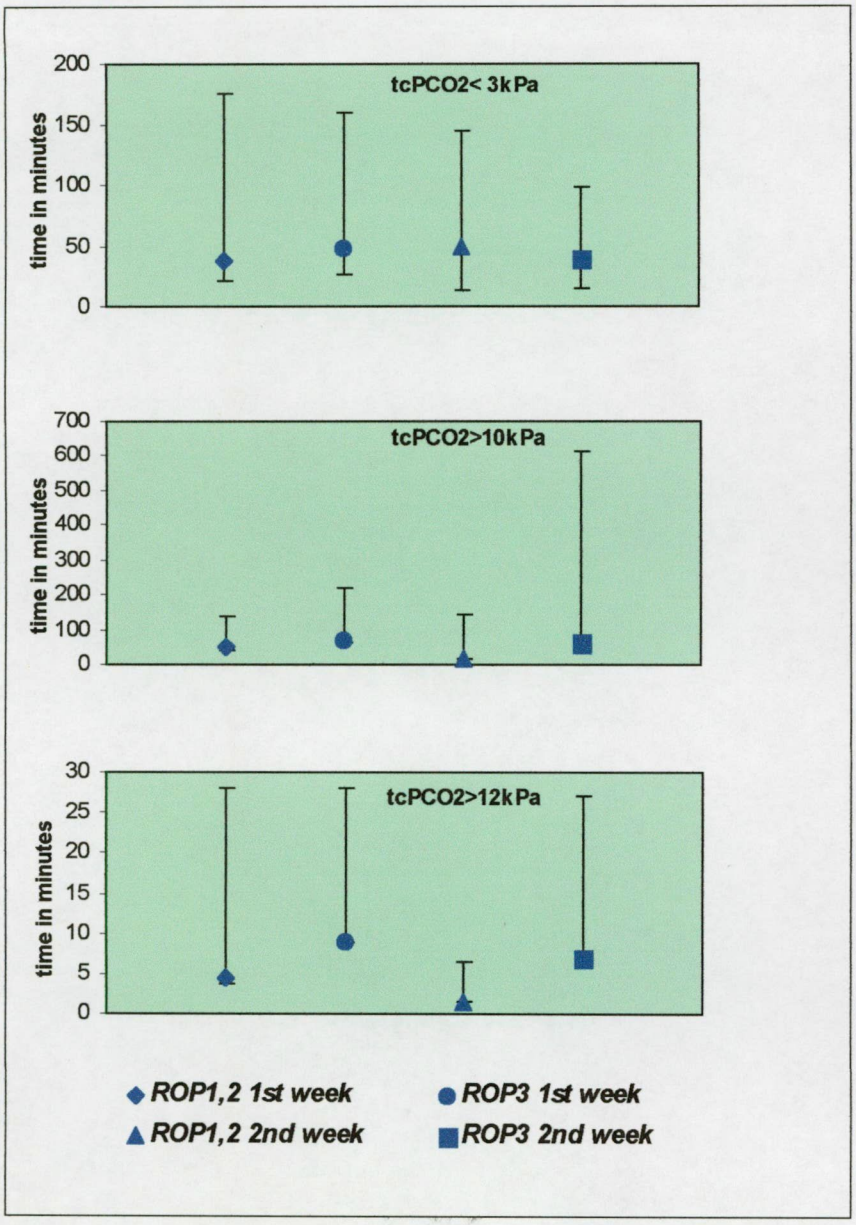
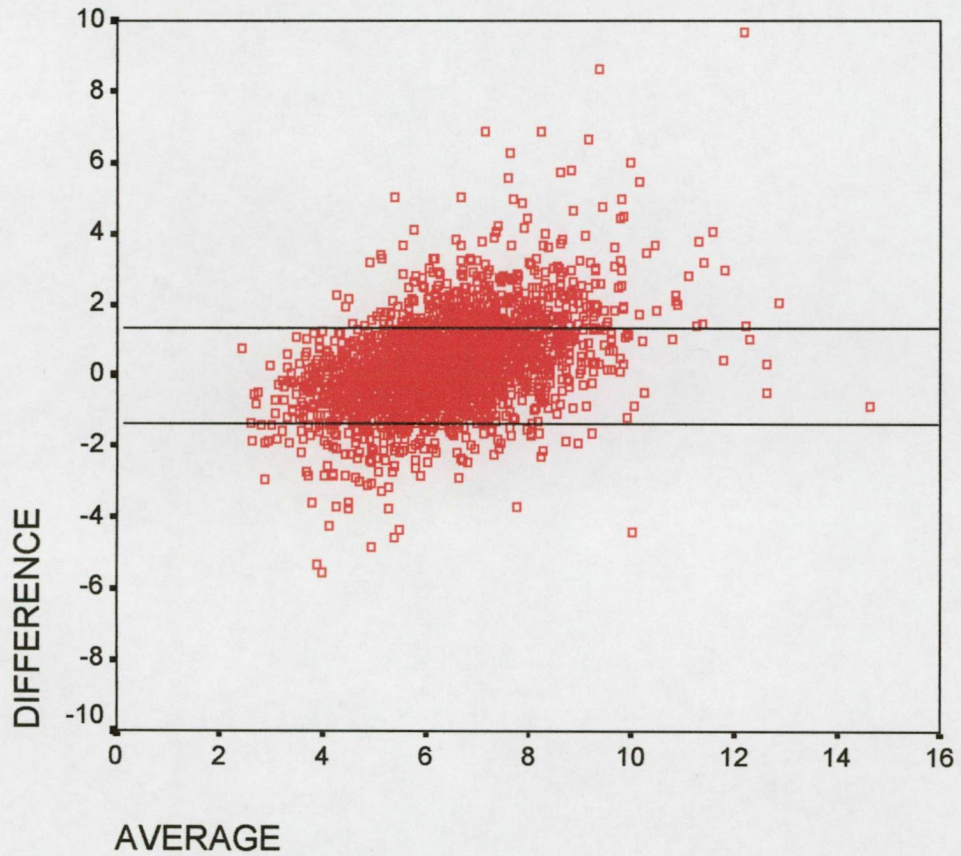


Figure 7 is a Bland-Altmann plot of the intermittently measured PCO_2 versus the simultaneous $tcPCO_2$ data and the differences were not significant. 85.8 % of transcutaneous values were within 1.5 kPa of the simultaneous arterial value, and a difference of ≤1.5 kPa between $tcPCO_2$ and PCO_2 was accepted in this study as a satisfactory agreement.

Figure 7. The comparison of transcutaneous and simultaneous arterial PCO_2.
DIFFERENCE = difference between PCO_2 and $tcPCO_2$ (in kPa) at the time of each arterial blood gas measurement.
AVERAGE = mean of PCO_2 and $tcPCO_2$ (in kPa) at the time of each arterial blood gas measurement.



3.2. Laboratory study

3.2.1. *Assessment of vascular injury*

Three methods of assessing changes in the vasculature are described, two quantitative measures of the vasculature from an assessment of lectin stained retinal flatmounts (radial extent of retinal vasculature and capillary concentration) and a semi-quantitative assessment of retinal sections by in-situ hybridisation for VEGF. All samples were randomised once processed for analysis and counts.

3.2.2. *Retinal wholemounts*

Peripheral avascularity

30 animals were assessed in the minute variable oxygen and control groups and 25 in the minute variable oxygen combined with continuous CO_2 group at 14 days postnatal age. Results are given in *table 2*.

Table 2. Analysis of lectin stained whole flatmounted retinas for each experimental group. Comparison made using multilevel analysis.

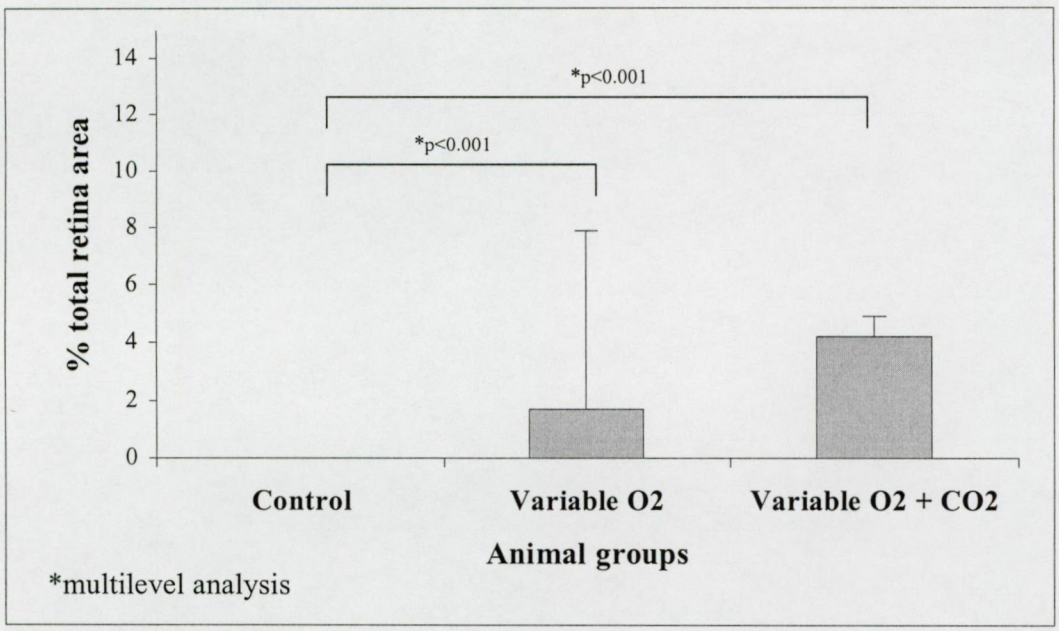
[a]p< 0.05, [b]p< 0.01, [c]p< 0.001 compared to Control

*[d]p< 0.001 V/CO_2 versus V. *n=16*

	n	Number of branches/mm^2 (median)	Peripheral avascularity (% total retina area)	% Retinas with abnormal terminal vessels	Mean weights (g) (95% CI)
Control(C)	30	277 (253 - 311)	0 (0 - 0)	0	29.2 (28.8 - 31.9)
Variable O_2(V)	30	[a]261 (215 - 290)	[c]1.7 (0 - 7.9)	21	[c]23.7 (23.5 - 23.9)
Variable O_2 &5%CO_2 (V/CO_2)	28	[c,d]346 (339 - 354)	[c]4.2 (3.1 - 4.9)	44*	[b]25.4 (23.2 - 25.7)

Control animals had fully vascularised retinas, however both the experimental groups had a significantly larger median peripheral avascular area. The variable oxygen group had median 1.7%, the CO_2–variable oxygen group had median 4.2 % peripheral avascularity *(Table 2).* Between the two variable groups was a subtle but not statistically significant difference in the degree of avascularity *(Graph 1).*

Graph 1. The degree of peripheral avascularity of retina



Edge of retina taken from a representative image in each experimental group showing the degree of avascularity.

Figure 8, 9 and 10 demonstrates lectin stained retinal flat mounts that shows typical pictures of the relative degree of avascularity in the two variable groups and the fully vascularised retina in room air controls.

Figure 8. Control
fully vascularized edge of retina
(magnification, 10x)

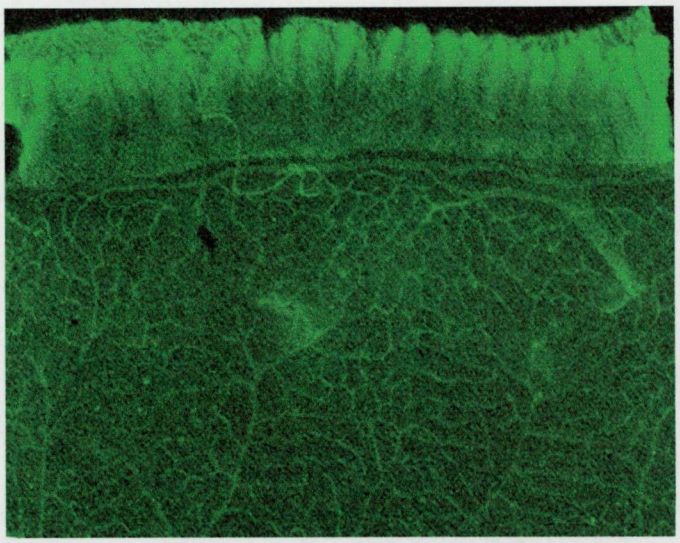


Figure 9. Variable O_2
peripheral avascular area
(magnification, 10x)

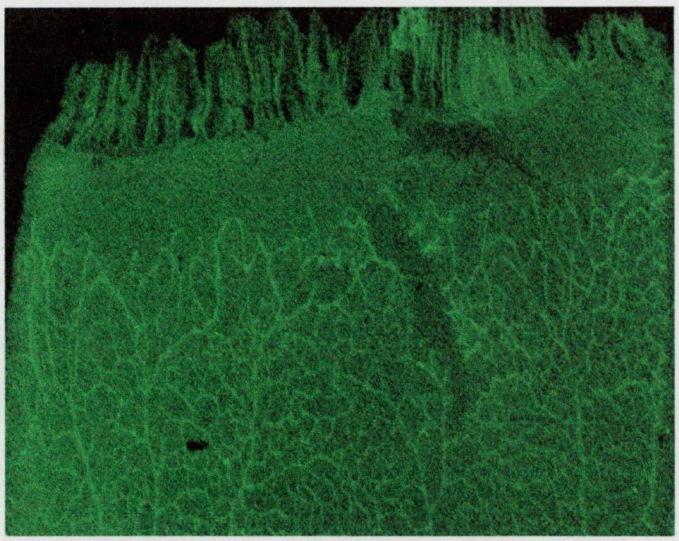
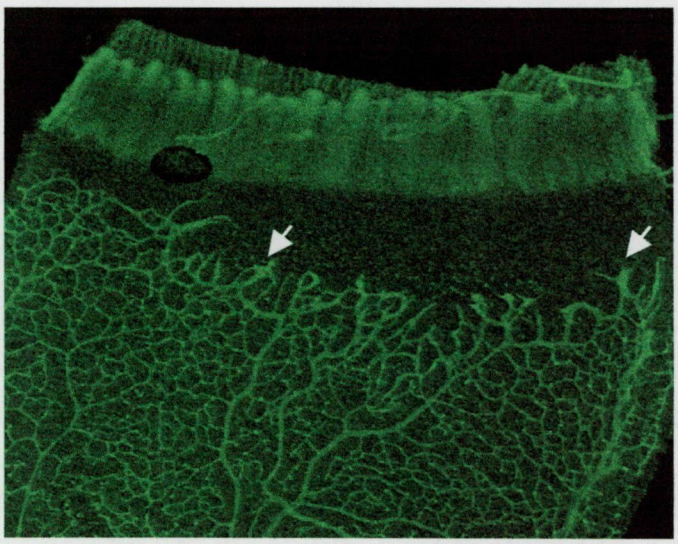


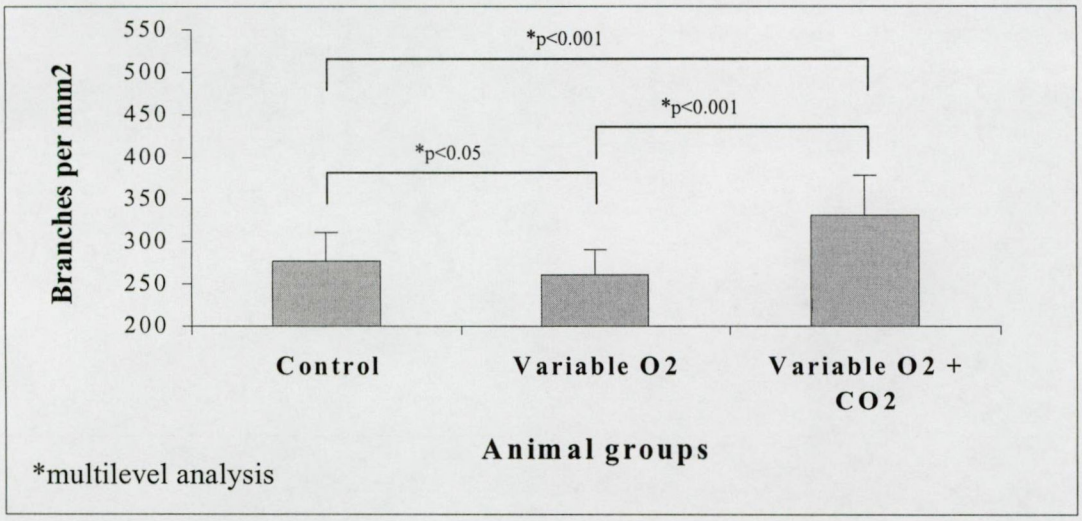
Figure 10. VariableO_2 + CO_2
larger peripheral avascular area, abnormal dilated vessels on vascular/avascular interface (*white arrows*)
(magnification, 10x)



Capillary density

Graph 2 demonstrates the number (Median and IQR) of capillary branches per mm^2 of retina in the three experimental group. There was slightly, but significantly lower capillary density in the variable oxygen group compared to control ($p<0.05$) using multilevel analysis. The comparison between the variable oxygen group and the CO_2-variable oxygen group showed a highly significant difference ($p<0.001$) in the number of capillary branches. In the CO_2-variable oxygen group were more capillary branches compare with the two other groups and the vasculature was qualitatively different as well.

Graph 2. Number of capillary branches of retina (Median, IQR)



During maturation of retinal arteries retraction of capillary connections contributes to the formation of a peri-arterial capillary-free zone.

Figure 11, 12 and 13 shows images captured from one typical retinal capillary bed from each experimental group. The vasculature of the retina in the control group showed a normal remodelled vasculature. The capillary bed in the CO_2-variable oxygen group was qualitatively different to that of the controls and the variable oxygen group. The appearance suggesting an immature, unremodelled vasculature with dilated capillaries, with more capillary branches and large vessels without a peri-arterial capillary-free zone.

Figure 11. Control
normal remodelled vasculature;
absence of abnormal dilated vessels,
capillary free zones
(marked with *asterisk*)
(magnification 20x)

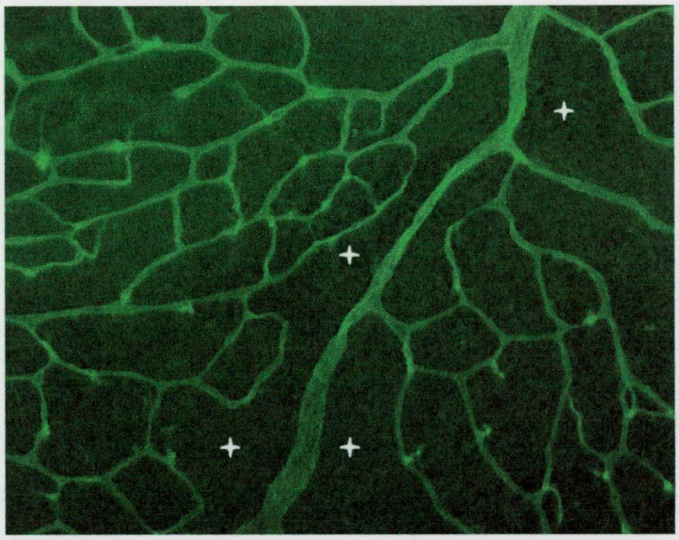


Figure 12. Variable O_2
similar capillary density as in control,
with some abnormal dilated vessels
(*white arrow*),
less capillary free zone
(magnification 20x)

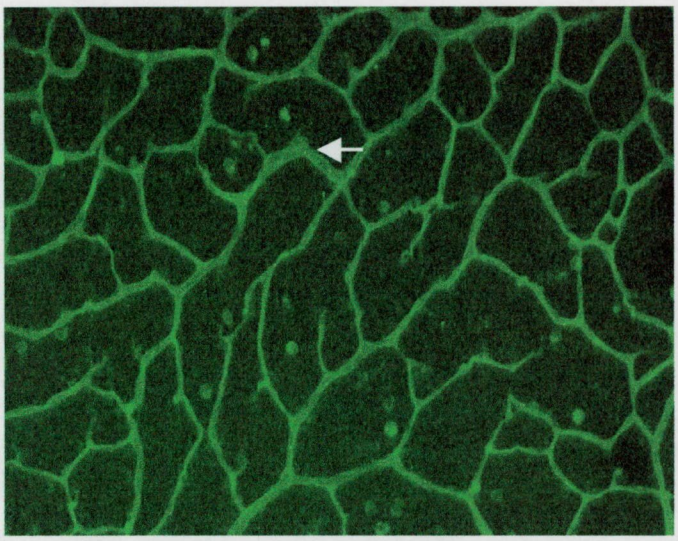
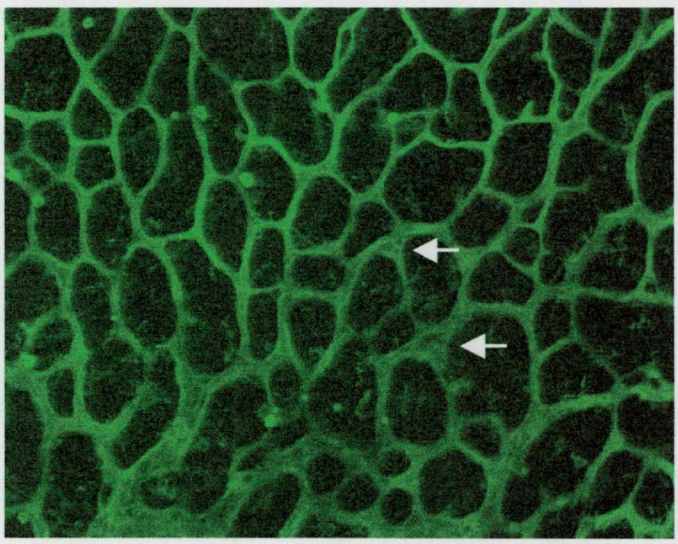


Figure 13. Variable O_2 +CO_2
unremodelled homogenous,
immature vasculature;
dilated vessels, resembling
sinusoid-like embryonal capillaries
(*white arrows*), more capillary branches,
higher capillary density,
absence of capillary-free zones
(magnification 20x)



Abnormal vessels

Observers noted no extra-retinal neovascularisation on the flatmounts though these are often difficult to distinguish in these preparations.

Although, there was no neovascularisation seen in any group, two masked observers noted abnormal terminal dilatations present at the vascular/avascular interface of 21 % of retinas from the variable oxygen group and 44 % of the retinas *(Graph 3)* in CO_2-variable oxygen group.

Graph 3. % Retinas with abnormal terminal vessels

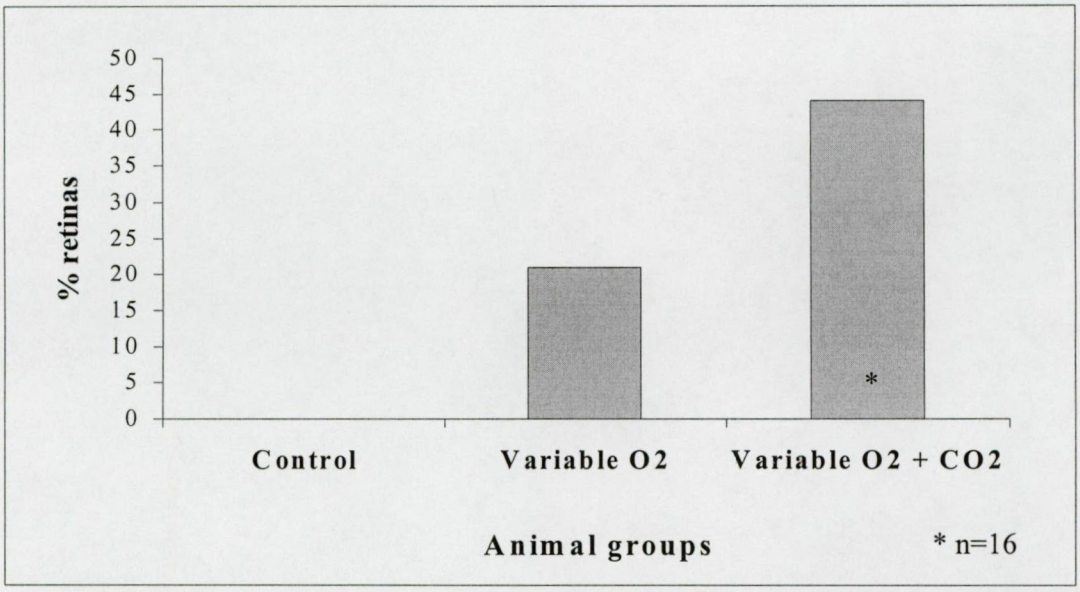
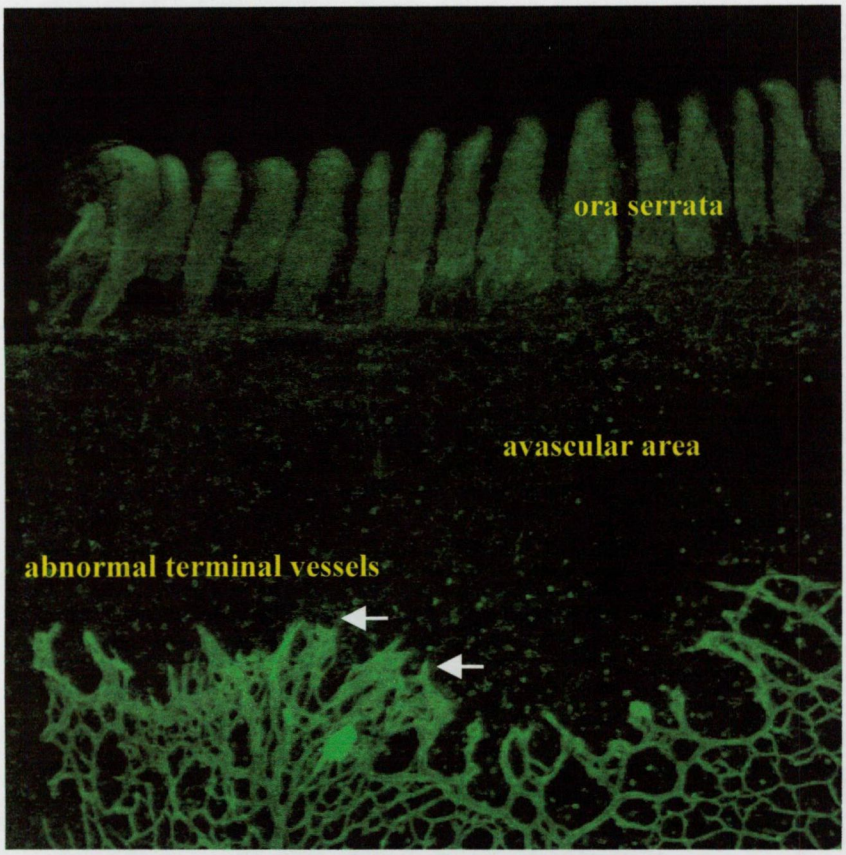


Figure 14 represents the capillary bed from the CO_2-variable oxygen group which has the appearance of a 14 days old rat pup's unremodelled immature vasculature with more capillary branches and the presence of abnormal terminal dilated vessels on the vascular/avascular interface. This picture (*white arrows*) shows an endothelial cell sheet that is part of the abnormal vascular development. *Figure 14* also demonstrates a typical picture of the large peripheral avascular zone.

Figure 14. Peripheral avascularity and abnormal terminal vessels in V/CO_2 group *(magnification, 10x)*



3.2.3. *Immunohistochemistry*

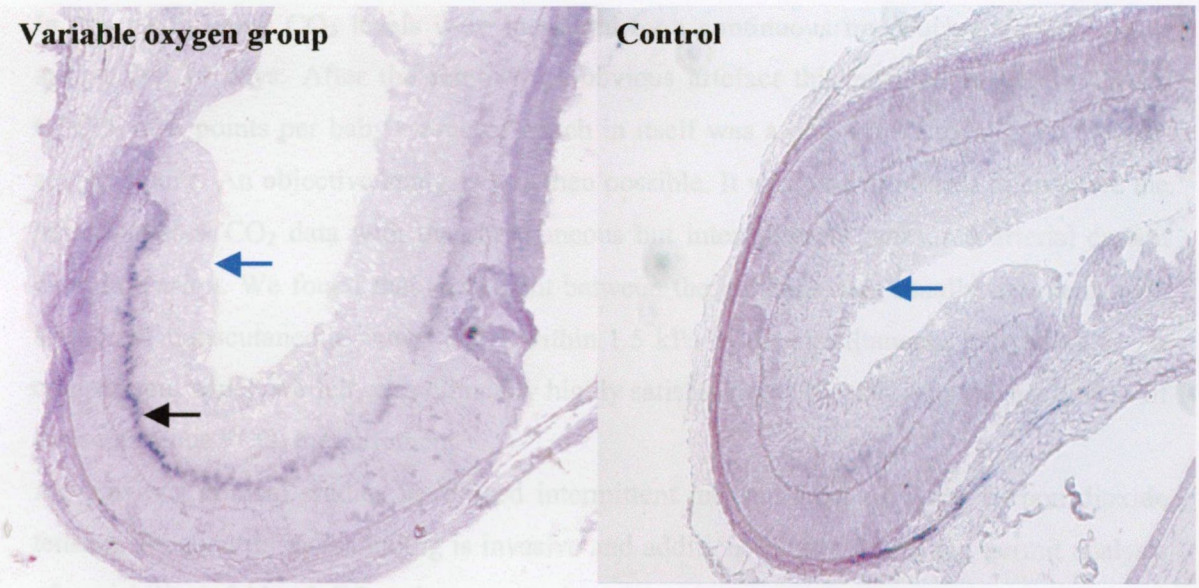
Immunohistochemistry to stain endothelial cells was performed on serial cryosections from each group but no evidence of nuclei above the inner limiting membrane i.e. extraretinal neovascularisation was seen.

3.2.4. *Assessment of VEGF by in-situ hybridisation*

VEGF mRNA was demonstrated in retinas using *in-situ hybridisation* and scored semi-quantitatively. VEGF was found in both the anterior retina and the retina as a whole, though staining was more intense in the anterior retina in most specimens. Supposing a change evoked by variable PO_2, we have analysed VEGF by *in-situ hybridisation* in control and

variable oxygen group. There was an increasing strength of staining present in the variable oxygen group compared with room air controls (*Figure 15*).

Figure 15 demonstrates a cryosection with VEGF mRNA stained in dark purple from a retina in variable oxygen group. Cryosections through the centre of the eye from 14-day-old rats. VEGF is located in the inner nuclear layer (*black arrow*) as previously reported[37], but in control group had little if any VEGF presented (*blue arrows* – retinal surface).
(magnification, 50x)



4. DISCUSSION

4.1. Clinical study

Our present clinical study does not support the view that either increased variability of blood CO_2 or a particular duration of hypercarbia or hypocarbia in the first two weeks of life is related to the development or severity of ROP[63].

Some studies have reported a relationship between hypercarbia and the development of ROP[52,64]. In contrast others have noted a significant association between hypocarbia and ROP[49,50]. In addition there is a body of animal studies suggesting that elevated PCO_2 alone, followed by room air recovery, contributes to the development of ROP[54].

In this study blood CO_2 levels were measured by a continuous transcutaneous monitoring system for 14 days. After the removal of obvious artefact this resulted in nearly 20,000 $tcPCO_2$ data points per baby - each of which in itself was a one-minute average of 60 one-second points. An objective analysis was then possible. It was also important to compare the transcutaneous CO_2 data with the simultaneous but intermittently measured arterial carbon dioxide tension. We found that agreement between the methods was usually excellent, with 85.8 % of transcutaneous values being within 1.5 kPa of the simultaneous arterial value - a comparison, which we felt, was clinically highly satisfactory. This confirms the usefulness of transcutaneous PCO_2 measurement.

All previous clinical studies have used intermittent measurement of blood carbon dioxide tension. Frequent blood sampling is invasive and additionally this would not permit analysis of variability of blood CO_2 whereas non-invasive transcutaneous measurement does. The difference in the method of monitoring blood CO_2 may also explain some of the differences of our results compared with some previous studies[49,50,51]. Some studies were performed before agreement on an International Classification of ROP was reached[61] and the use of older classification systems in these studies makes direct comparison with our study difficult. The survival of the extremely immature neonate has dramatically increased during the last 20 years and our study population may behave differently from those older studies.

The number of infants enrolled in the study was small but the confidence intervals of the results suggest that the lack of difference between groups is unlikely to be related to small numbers creating a type II error. It would certainly be preferable to involve more babies, but

during the 2-year period of investigation only 25 infants met the requirements of the inclusion criteria. A larger multicentre study would probably be impossible because of absence of similar monitoring equipment in other units.

4.2. Animal study

The Edinburgh group has developed an animal model of ROP based on clinically relevant fluctuations in oxygen[60,65]. It demonstrated that small, frequent fluctuations in oxygen, mimicking those experienced by a human preterm could induce features of retinopathy in neonatal rats.

Although previous groups have induced retinopathy using large fluctuations of 40 %-70 % oxygen[59,66,67], 80 % to 40 % oxygen[57], 80 % to room air[58] or from 50 % to 10 %[59] over periods of 6-48 hours, our model produced a change in oxygen each minute.

The development of models for identifying the pathogenesis of ROP has predominantly concentrated on extreme oxygen injury[68]. Although these models are able to induce retinopathy with neovascularization[69,70], they have not closely represented the arterial oxygen of the preterm infant who might in current times develop the disease, and this has been considered a potential weakness in understanding the precise pathogenesis of the disease in extremely preterm infants. Therefore, whether the oxygen injuries induced in previous animal models of ROP are representative of human disease, especially in terms of ROP in 21st century, is debatable.

The Edinburgh animal model, based on the arterial oxygen data derived from an infant with ROP, is able to more closely represent the retinal oxygenation of a preterm infant developing ROP than has previously been possible. Subtle changes in inspired oxygen and clinically relevant profiles contrasts with previous animal models of ROP, which, although they may characterize severe neovascularization, do not reflect the present-day clinical management of oxygen therapy.

The known effects of CO_2 tension on small vessel calibre make it inappropriate to discard CO_2 as an important factor from our clinical study alone. It may be that specific interaction of CO_2 with the oxygen is more important[51,52,53].

Our model is capable of preserving continuous carbon dioxide simultaneously with the minute variable oxygen concentration in a special animal cage. We used this model to investigate the

oxygen variability compared with the combination of oxygen variability and hypercarbia on the development and severity of ROP[71]. In the oxygen variable group the neonatal rats were raised in a fluctuating oxygen environment, where 50 % of the time was spent above and below 21 % (mean 21.3 %) inspired oxygen. There was slightly lower capillary density in the variable oxygen group compare to room air control. Their peripheral retina was avascular, suggesting that the retinal vascular development had been retarded. The small but frequent changes in oxygen were able to significantly induce peripheral avascularity of the retina when compared with room-air controls. Abnormal terminal vessels were present at the vascular/avascular interface of 21 % of retinas from the variable oxygen group.

In the CO_2-variable oxygen group there also developed a peripheral avascularity of the retina and it was slightly larger than in the other experimental group. The central capillary density in this group was much higher than in the other two groups and showed smaller capillary free zones around larger, dilated vessels. The vasculature was immature and unremodelled. Although, there was no neovascularisation seen in any group, the CO_2-variable oxygen group had abnormal, dilated, terminal buds present at the vascular/avascular interface of 44 % of the retinas, which may have resulted by the absence of apoptosis. They were stained with endothelial cell specific lectin and represent endothelial cell proliferations that could be precursors to pathologic neovascularisation.

Apoptosis is the genetic control of cell death and viability and preserves tissue and organ homeostasis by eliminating senescent, damaged, or abnormal cells[72]. This process involves different gene families of inhibitors and stimulators of cell death. The endothelium is one of the most critical sites for the control of apoptosis in vascular remodelling. Endothelial cell-specific mitogens, including VEGF, transduce survival signals critically maintaining endothelial cell viability. Inhibition of apoptosis may also be required during retinal endothelial cell proliferations and new blood vessels formation[6].

VEGF has been implicated in several studies in vascular development and in proliferative retinopathies. In part the goal of our study was to examine the localisation and qualitative analysis of VEGF. The presence or absence of VEGF mRNA was assessed by in-situ hybridisation in retinal sections.

VEGF mRNA was present in the inner nuclear layer of the retina in variable oxygen group, whereas room air controls had little if any VEGF present.

In our study the differences in body weight between controls and experimental groups are similar to those in other investigators' model of oxygen-induced retinopathy[54,57]. In all our groups there was no difference between litter sizes nor in the time the pups spent with their dams. We do not rule out the possibility that mothers raised in the variable oxygen with or without continuous CO_2 environments were affected and may have passed this on to their pups indirectly, however, we did not directly measure this.

Recently, we demonstrated that the oxygen–induced retinopathy could be influenced by small shifts in the mean inspired oxygen concentration. Rat pups raised in a relatively hypoxic but variable oxygen environment develop less severe retinal vascular abnormalities than those raised in variable oxygen around higher oxygen means[71]. Those results would suggest that variability around mildly hypoxic mean enables normal growth of the retina and that nursing infants in slightly lower oxygen may reduce the incidence and severity of ROP. In contrast, fluctuation around a mildly hyperoxic mean resulted in a delay of vascularization and an immature retinal vasculature[71]. Continuous hypercarbia combined with oxygen fluctuation around a normoxic mean resulted in very similar, abnormal changes in retinal vascularization. Hypercarbia increased the severity of fluctuating oxygen-induced retinopathy in newborn rats.

4.3. Conclusions

In our clinical study we confirmed the usefulness of transcutaneous PCO_2 measurement and demonstrated that the variability of $tcPCO_2$ in the first 14 days of life is not associated with the development or severity of ROP, whereas fluctuation of $tcPO_2$ has been shown to be significant[47,63]. However, our laboratory findings in animal study showed that the pathological effects of oxygen in the development of ROP might be augmented by hypercarbia. The exact pathomechanism is not yet known. It is possible that the elevated blood PCO_2 may influence the normal autoregulation of retinal and choroidal vessels, and that the vasodilatation due to hypercarbia perhaps facilitates the delivery of high oxygen saturated blood to the retinal/choroidal tissues enlarging the toxic effect of oxygen.

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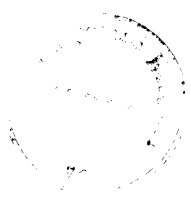

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