CHAPTER 3: RESULTS
INHERITANCE OF STRAIN-DEPENDENT DIFFERENCES IN EXPERIMENTAL RETINOPATHY OF PREMATURITY

3.1 ABSTRACT

Oxygen-induced retinopathy (OIR) is a robust animal model of ROP, a potentially blinding condition of premature infants. Different inbred rat strains exhibit differential susceptibility to OIR, a trait which has previously segregated with ocular pigmentation and is inherited in an autosomal dominant fashion in at least one pigmented rat strain. The mode in which susceptibility to OIR is inherited in albino Sprague Dawley (SD) rats has not been determined until now. Using genetic cross and backcross analysis, it was found that susceptibility to OIR loosely follows an autosomal dominant mode of inheritance in albino SD rats, making it likely that genetic modifiers other than ocular pigmentation contribute to disease susceptibility. These modifiers could potentially be involved in the oxygen sensing pathway which is central to the development of OIR and ROP.

3.1a Introduction

Oxygen-induced retinopathy (OIR) is a robust animal model of ROP which mimics the pathology seen in human disease. In rodent models of OIR, neonatal rats or mice are exposed to varying levels of oxygen to recreate the biphasic disease seen in humans [23]. The model used in this study mimics the first phase of ROP, where exposure of neonatal rats to cyclic hyperoxia attenuates normal retinal vascularisation. This exposure to cyclic hyperoxia/relative hypoxia closely reflects the supplemental inspired

oxygen therapy to which premature infants are exposed in the neonatal intensive care unit after birth [96]. The retinal vascular phenotype also mimics the vessel tortuosity observed in human ROP, where increased vascular tortuosity indicates progression towards severe disease [74, 202]. These features make rodents models of ROP suitable surrogates for the study of this disease.

There is increasing evidence from animal models of ROP that there is a genetic component involved in susceptibility to OIR. Studies in different strains of rats and mice show that variation in susceptibility to OIR is associated with differential expression of pro- and anti-angiogenic factors [30, 98, 99]. Gao and colleagues [98] examined two strains of rats, the pigmented Brown Norway (BN) and the albino Sprague Dawley (SD) strains, which varied in their response to OIR. Hyperoxia-exposed pigmented BN rats were more susceptible to OIR and expressed higher levels of the pro-angiogenic factor, vascular endothelial growth factor (VEGF), and lower levels of the anti-angiogenic factor, pigment epithelium-derived factor (PEDF), than albino SD rats. The potential role of ocular pigmentation in susceptibility to OIR in these two strains was not discussed by the authors; however other studies have suggested that ocular pigmentation may play a role [96, 203, 204].

van Wijngaarden and colleagues [96] studied five different inbred, and one outbred strain of rat which differed in their response to exposure to cyclic hyperoxia. Ocular pigmentation in two of these strains, the inbred Dark Agouti (DA) and the outbred Hooded Wistar (HW) rats, was associated with increased susceptibility to OIR, compared with the albino SD strain which was also susceptible to OIR. However, the underlying genetics are not fully understood, similar to uncertainty over the role of ocular pigmentation in susceptibility to ROP in humans.

Evidence derived from animal studies show that susceptibility to OIR is inherited. Using formal backcross analysis, van Wijngaarden and colleagues showed that susceptibility to OIR was inherited in an autosomal dominant mode in pigmented inbred DA rats [203]. The mode of inheritance of susceptibility to OIR in albino rat strains has not previously been determined.

The specific aim of this chapter was to determine how susceptibility to OIR is inherited in two inbred rat strains which differ in their response to cyclic hyperoxia, in the absence of ocular pigmentation.

Inbred albino Fischer 344 (F344, resistant to OIR) and SD (moderately susceptible to OIR) rats were cross-bred to produce an F1 generation. Using formal backcross analysis, randomly selected F1 rats were backcrossed with

either of the parental strains to determine how susceptibility to OIR was inherited.

3.2 RESULTS

3.2.a Strain comparisons of retinal vascularisation in response to cyclic hyperoxia

Neonatal F344 and SD were exposed to cyclic hyperoxia for 14 days after birth. Retinal flat-mounts were assessed for total avascular area as described in section 2.3.c.1. All cyclic hyperoxia-exposed rats showed incomplete retinal neovascularisation at postnatal day 14 with avascular areas observed in the central and peripheral retina, as shown in Figure 3.1. Total retinal avascular area expressed as a percentage of the retina has previously been used to discriminate between rat strains that were resistant or sensitive to OIR. The presence of large total retinal avascular areas indicated that rats were more sensitive to the effects of OIR than rats that had small total retinal avascular areas. At postnatal day 14, mean retinal avascular area was smaller in F344 rats (33.6%; 95% CI: 28.8-38.5), compared to SD rats (57.5%; 95% CI: 50.2-64.8).

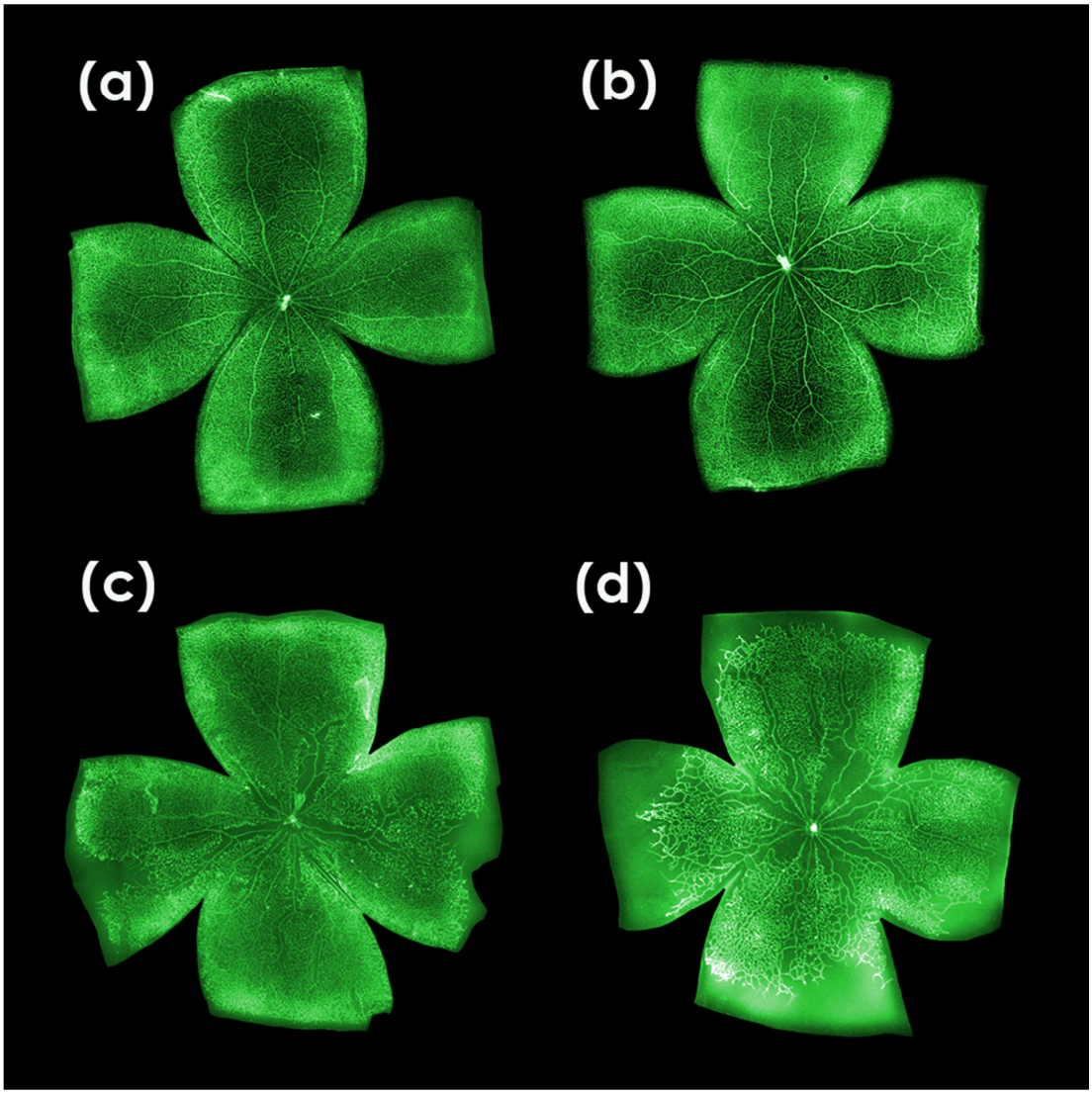


Figure 3.1 Representative montages of room air and cyclic hyperoxia-exposed F344 and SD rats at postnatal day 14. Retinae were flat-mounted and stained with fluorochrome-conjugated GS-IB4 to highlight the vasculature. At postnatal day 14, room air-exposed rats showed near complete retinal neovascularisation, whereas avascular areas were observed in the central and peripheral retinae of cyclic hyperoxia-exposed rats. (a) F344 rat exposed to room air from birth, (b) SD rat exposed to room air from birth, (c) F344 rat exposed to cyclic hyperoxia from birth, (d) SD rat exposed to cyclic hyperoxia from birth. Representative images of room air-exposed F344 and SD rats were kindly provided by Dr Peter van Wijngaarden and have been published previously [205].

3.2.a.1 Retinal vascularisation in F344 x SD rats in response to cyclic hyperoxia

All F344 x SD offspring, referred to as the F1 generation, were albino with white coat colour and red eyes due to the absence of ocular pigmentation (Figure 3.2). Cyclic hyperoxia-exposed F1 rats showed incomplete retinal neovascularisation at postnatal day 14 similar to that of the parental strains with avascular areas observed in the central and peripheral retina as shown in Figure 3.3.

Figure 3.2 Fourteen day-old F1 rat pups. The offspring of the F344 and SD crosses (F1 generation) were albino.

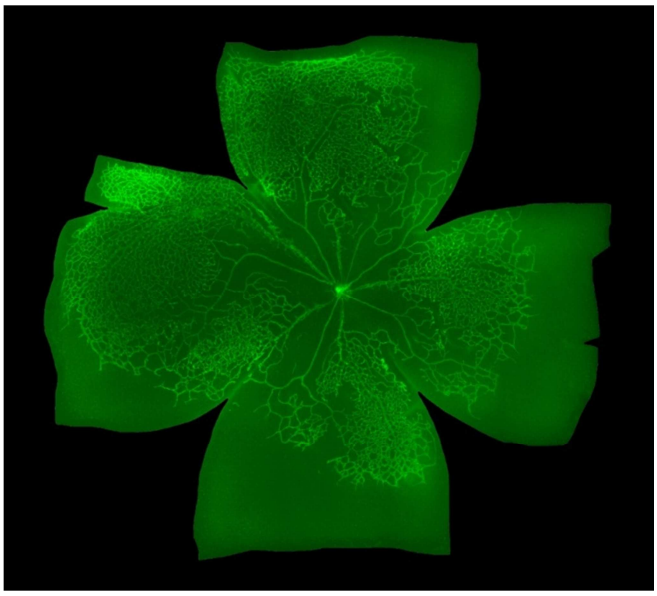


Figure 3.3 Representative montage of a cyclic hyperoxia-exposed F344 x SD rat at postnatal day 14. Retinae were flat-mounted and stained with fluorochrome-conjugated GS-IB4 to highlight the vasculature.

3.2.b Criteria for determining susceptibility to OIR

3.2.b.1 Retinal avascular area

As discussed in 3.2.a, albino F344 and SD strains differed in their susceptibility to OIR as reflected by differences in retinal avascular areas. At postnatal day 14, larger total retinal avascular areas were observed in SD rats than F344 rats, at 57.5% and 33.6%, respectively. The mean retinal avascular area of (F344 x SD) F1 rats was 49.0% (95% CI: 45.4-53.4) as shown in Figure 3.4 and Table 3.1.

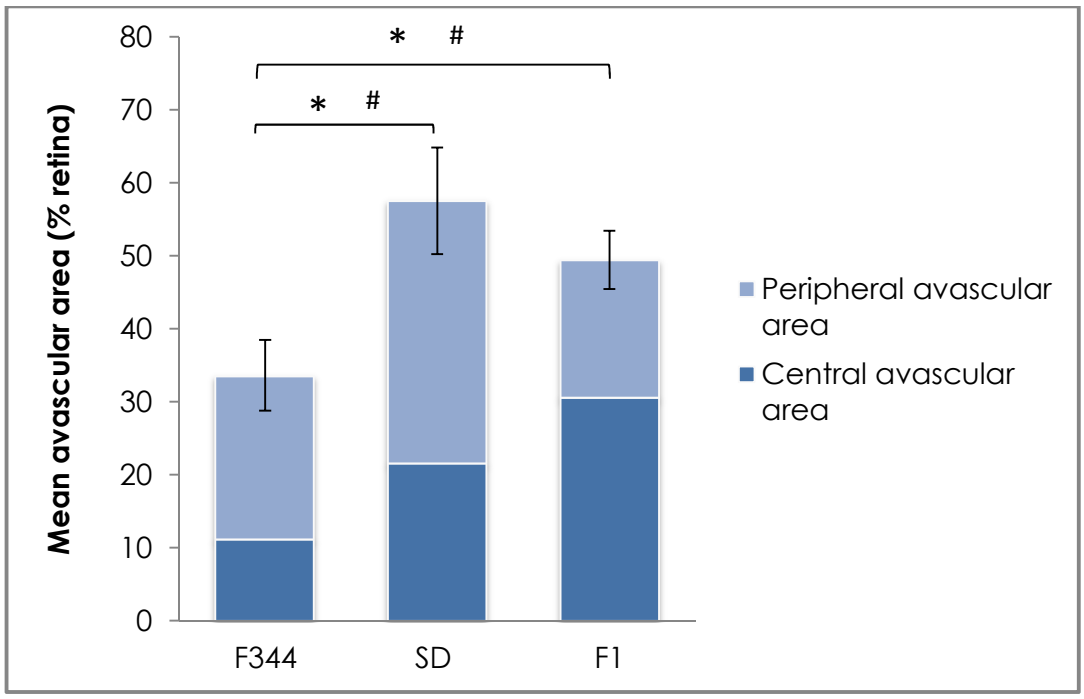


Figure 3.4 Mean avascular area expressed as a percentage of the retina for cyclic hyperoxia-exposed F344, SD and F1 rats at postnatal day 14. Mean total avascular areas were larger in the SD rats than the F344 rats. The mean total avascular area of F1 rats fell between the two parental strains. n(F344) = 8; n(SD) = 7; n(F1) = 32. * $p<0.017$ total avascular area comparison, # $p<0.017$ central avascular area comparison. Error bars: ± 95% CI.

Strain or cross	n	Mean total avascular area (% retina)	Median	Range
F344	8	33.6	32.7	24.3-42.4
SD	7	57.5	61.0	43.1-68.3
F1 (F344 x SD)	32	49.0	50.1	25.7-65.2

Table 3.1 Total avascular area expressed as a percentage of the retina for cyclic hyperoxia-exposed F344, SD and F1 rats at postnatal day 14. The ranges of the total avascular area for the F344 and SD strains were discreet between strains. F1 rats had a range which spanned that of the two parental strains.

Analysis of total avascular areas showed significant differences between the parental strains (F344, n=8; SD, n=7) and the F1 offspring (n=32), x^2 (2, n=47) =15.26, p=0.0005 (Kruskal-Wallis test). Median scores were higher in SD and F1 rats, 58.5% and 50.1% respectively, compared to F344 rats (median=32.7%). Two-tailed Mann-Whitney U-tests were applied and post-hoc Bonferroni adjustment performed, with a new significance level set at 0.017. F344 rats had significantly smaller total retinal avascular areas than SD and F1 rats, both at p=0.001. Total retinal avascular areas did not differ significantly between SD and F1 rats, p=0.085.

Strain-related differences were also observed in the degree of neovascularisation in the central and peripheral retina at postnatal day 14 (Figure 3.4). A closer analysis of central avascular areas showed significant differences between the strains (F344, n=8; SD, n=7) and the F1 offspring (n=32), x^2 (2, n=47) =20.78, p=0.00003 (Kruskal-Wallis test). Median central avascular areas were highest in F1 rats, followed by SD then F344 rats at 32.1%, 21.3% and 12.5% respectively. Two-tailed Mann-Whitney U-tests were applied and post-hoc Bonferroni adjustment performed, with a new significance level set at 0.017. F344 rats had significantly smaller central avascular than SD, p=0.002 and F1 rats, p=0.0001. Comparison of central avascular areas between SD and F1 rats, did not reach statistical significance p=0.019.

During analysis of the total avascular areas of cyclic hyperoxia-exposed F1 rats, it was observed that highly tortuous retinal blood vessels were not necessarily accompanied by large avascular areas (Figure 3.5). Therefore, setting an arbitrary total avascular area cut off point for these two strains to discriminate between rats which were susceptible or resistant to OIR was difficult based on this criterion alone. As a result, retinal blood vessel tortuosity was chosen as a second criterion.

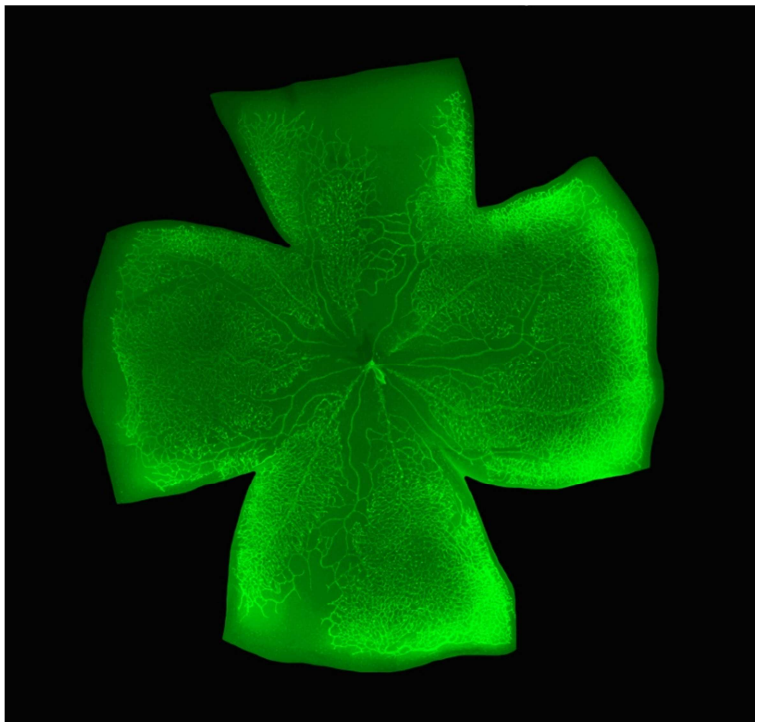


Figure 3.5 Representative montage of a cyclic hyperoxia-exposed F344 x SD rat at postnatal day 14. Retinae were flat-mounted and stained with fluorochrome-conjugated GS-IB4 to highlight the vasculature. Increased vessel tortuosity was not always accompanied by large avascular area.

3.2.b.2 Retinal blood vessel tortuosity

Following assessment of retinal flat-mounts for total avascular area at postnatal day 14, the degree of vessel tortuosity was also determined as described in section 2.3.c.2, to assist in discriminating between rats that were susceptible or resistant to OIR, as dilated, tortuous blood vessels are characteristic of disease progression in human ROP. Retinal blood vessel tortuosity scores for the two parental strains and the F1 generation are summarised in Table 3.2. Discriminatory ranges were observed for the retinal blood vessel tortuosity scores in the two parental strains.

Strain or cross	n	Retinal blood vessel tortuosity scores, median (range)
F344	8	27 (23, 32)
SD	7	37 (35, 46)
F1 (F344 x SD)	32	37 (25, 44)

Table 3.2 Retinal blood vessel tortuosity scores for cyclic hyperoxia-exposed F344, SD and F1 rats at day 14. Retinal clock hours were scored based on the degree of vessel tortuosity in each clock hour and the scores summed. A minimum score of 12 for retinae with no vessel tortuosity, and a maximum score of 48, indicating a high degree of vessel tortuosity, was possible for each retinal flat-mount. n = number of animals.

Analysis of blood vessel tortuosity scores using a Kruskal-Wallis test showed significant differences between the strains (F344, n=8; SD, n=7) and the F1 offspring (n=32), x^2 (2, n=47) =15.71, p=0.0004. Median scores were higher in

SD and F1 rats, both groups having a median score of 37, compared to F344 rats (median=27). Two-tailed Mann-Whitney U-tests with adjustment for multiple comparisons applied post-hoc (significance level of 0.017) were performed. F344 had significantly lower blood vessel tortuosity scores than SD and F1 rats, p=0.001 and 0.0003 respectively. Blood vessel tortuosity scores did not differ significantly between SD and F1 rats (p=0.304).

3.2.b.3 Determining susceptibility to OIR in cyclic hyperoxia-exposed F344 and SD rats

Both retinal avascular area and blood vessel tortuosity scores were used to determine susceptibility to OIR in F344 and SD rats and their F1 offspring. Discriminatory ranges for each criterion were observed for the F344 and SD parental strains (Table 3.3). F344 rats had a total retinal avascular area range of 24-43% whereas the range for SD rats was 43-68%. The ranges for retinal blood vessel tortuosity scores were 23-32 and 35-46 for F344 and SD rats, respectively. F1 rats had a range which crossed that of the parental strains for both criteria.

Strain or Cross	n	Range total avascular area (%)	Range blood vessel tortuosity scoring
F344	8	24-43	23-32
SD	7	43-68	35-46
F1	32	26-65	25-44

Table 3.3 Comparison of retinal total avascular area and blood vessel tortuosity scores for cyclic hyperoxia-exposed F344, SD and F1 rats at day 14. Ranges for each discriminatory criterion are shown. The lower limits of the ranges for total avascular area and retinal blood vessel tortuosity scores in SD rats were chosen as the cut off values for susceptibility to OIR.

As a result of these analyses, the lower limits of the ranges exhibited by the SD strain for both criteria were used as arbitrary cut-off values to determine susceptibility to OIR. It was concluded that rats were susceptible to OIR if they had a total avascular area ≥ 43% **and/or** a retinal blood vessel tortuosity score of ≥ 35. As increased retinal vessel tortuosity is a sign of disease progression, rats resistant to OIR must meet both the following criteria to be considered resistant to OIR; that is they must have a total retinal avascular area ≤ 42% **and** a retinal blood vessel tortuosity score of ≤ 34.

3.2.c Cross-breeding experiments

Heritability of susceptibility to OIR in resistant F344 and susceptible SD albino rats was determined using a series of cross-breeding experiments. Each genetic cross was performed at least twice and with different gender pairings (male F344 crossed with female SD; female F344 crossed with male

SD). All offspring from the crosses were exposed to cyclic hyperoxia for 14 days after birth. Retinal flat-mounts were then assessed for total retinal avascular area and blood vessel tortuosity.

3.2.c.1 Susceptibility of F344 x SD offspring to OIR

A total of 3 matings of F344 x SD rats were performed to determine susceptibility of F1 rats to OIR. All rat pups were exposed to cyclic hyperoxia for 14 days after birth prior to analysis of retinal avascular area and blood vessel tortuosity. Total retinal avascular area and blood vessel tortuosity scores for each of the rats are shown in Figure 3.6. All F1 offspring were susceptible to OIR based on the criteria outlined in section 3.2.b.3. Mean total avascular area and retinal blood vessel tortuosity scores are summarised in sections 3.2.b.1 and 3.2.b.2, respectively.

Two additional F344 x SD matings were also performed. Pups from these litters were allowed to reach maturity for later use in backcross experiments.

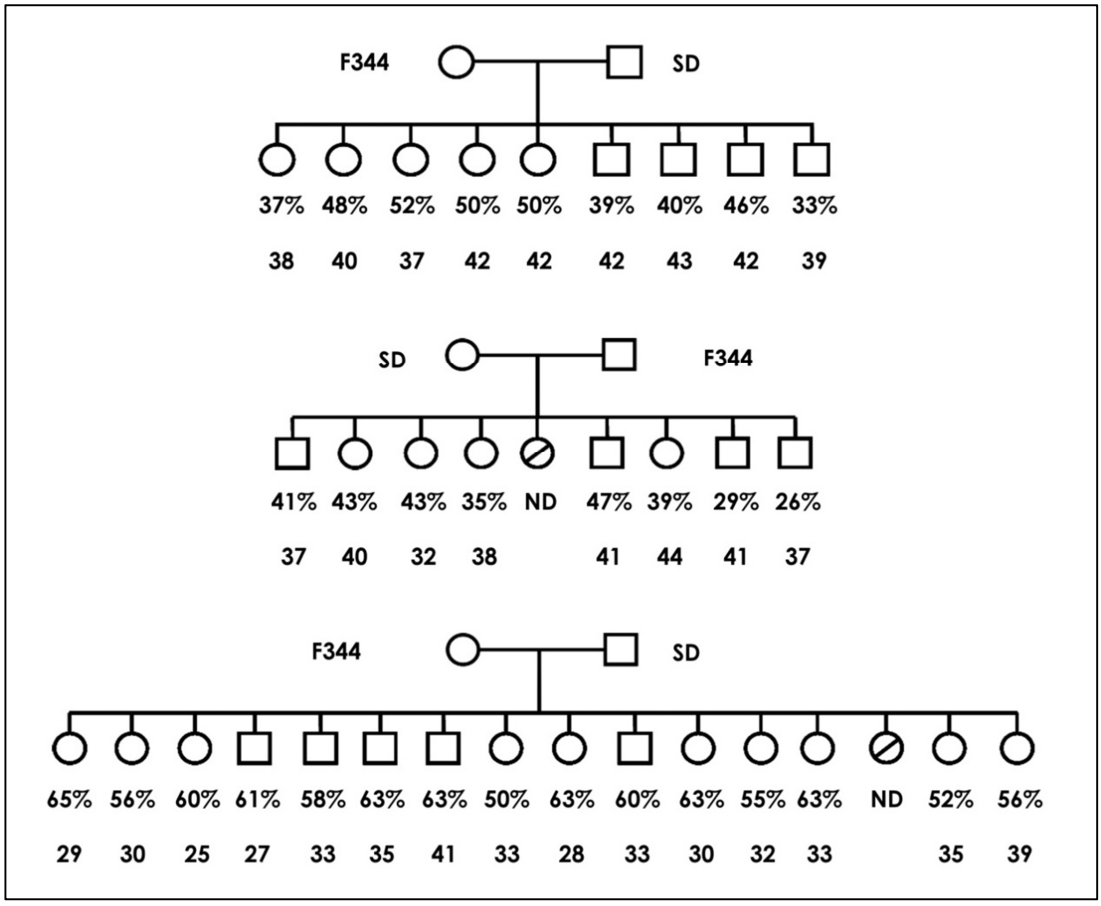


Figure 3.6 Genealogy plots of cyclic-hyperoxia exposed F1 rats. Neonatal rat pups were exposed to cyclic-hyperoxia for 14 days after birth, after which retinal flat-mounts were performed and retinal avascular area (shown as a percentage of the total retinal area) and vessel tortuosity (shown below retinal avascular area) were measured. Unfilled symbols, rats susceptible to OIR; filled symbols, rats resistant to OIR; ND = retinal avascular area and blood vessel tortuosity scores not determined.

3.2.c.2 Susceptibility of backcross offspring to OIR

Adult rats of the F1 generation were mated with F344 and SD rats to produce backcross offspring, all of which had albino colouring. Two matings were performed for each of the backcrosses, each with a different gender pairing. All backcrossed rats were exposed to cyclic hyperoxia for 14 days after birth prior to analysis of retinal avascular area and blood vessel tortuosity.

3.2.c.2.a Susceptibility of F1 x F344 backcross offspring to OIR

The offspring of the F1 x F344 backcrosses varied in their susceptibility to OIR (Figure 3.7). Several rats were deemed unsuitable for analysis due to technical failures in the flat mounting of these retinae for analysis, so that retinal avascular area and tortuosity scores were not able to be determined. Mean total avascular area for F1 x F344 backcrossed rats was 42.7% (95% CI: 37.5-47.8). Two-tailed Mann-Whitney U-test showed total avascular areas were significantly larger in the backcrossed F1 x F344 rats compared to the F344 parental strain, 41.3% and 32.7% respectively, p=0.039. Median vessel tortuosity score for F1 x F344 rats was 31 (95% CI: 30.3-34.9). Blood vessel tortuosity scores were also significantly greater in the F1 x F344 backcrossed rats compared to the F344 strain, at 31 and 27 respectively (p=0.015). Ten of the twenty two backcrossed pups were considered susceptible to OIR. The remaining twelve rats were resistant to OIR.

The offspring of the F1 x F344 backcrosses also varied in their susceptibility to OIR depending on the sex of the F344 rat used in the backcross. When a female F1 rat was mated with a male F344 rat, more offspring were found to be resistant to OIR (ten) than susceptible (two). On the other hand, when a male F1 rat was mated with a female F344 rat, more offspring were found to be susceptible to OIR (seven) than resistant (two). A two-sided Fisher's exact test showed the sex of the F344 rat used in the backcross mating had a

statistically significant effect (p=0.027) on susceptibility of the offspring to OIR. The biological significance of this effect is not yet known.

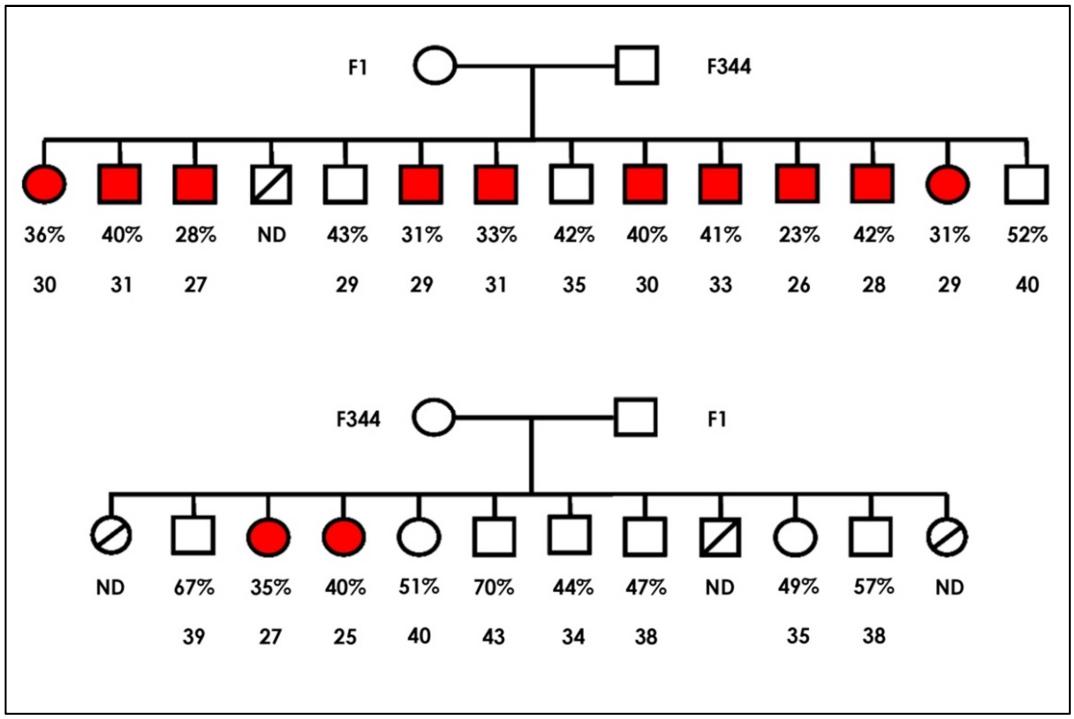


Figure 3.7 Genealogy plots of cyclic-hyperoxia exposed F1 x F344 rats. Neonatal rat pups were exposed to cyclic-hyperoxia for 14 days after birth, after which retinal flat-mounts were performed and retinal avascular area (shown as a percentage of the total retinal area) and vessel tortuosity (shown below retinal avascular area) were measured. Unfilled symbols, rats susceptible to OIR; filled symbols, rats resistant to OIR; ND = retinal avascular area and blood vessel tortuosity scores not determined.

3.2.c.2.b Susceptibility of F1 x SD backcross offspring to OIR

The offspring of the F1 x SD backcrosses also varied in their susceptibility to OIR (Figure 3.8). Again, several rats were deemed unsuitable for analysis with retinal avascular area and tortuosity scores not determined. Mean total avascular area for F1 x SD backcrossed rats was 50.0% (95% CI: 45.3-54.8). Two-tailed Mann-Whitney U-test showed total avascular areas were larger in

the SD rats compared to backcross F1 x SD offspring at 58.5% and 41.2% respectively, although this did not reach statistical significance (p=0.077). Similar blood vessel tortuosity scores were observed in SD and F1 x SD backcross rats (median=37 and 38, respectively; 95% CI: 35.9-41.1; p=0.76). Sixteen out of twenty pups were considered susceptible to OIR and the four remaining pups considered resistant.

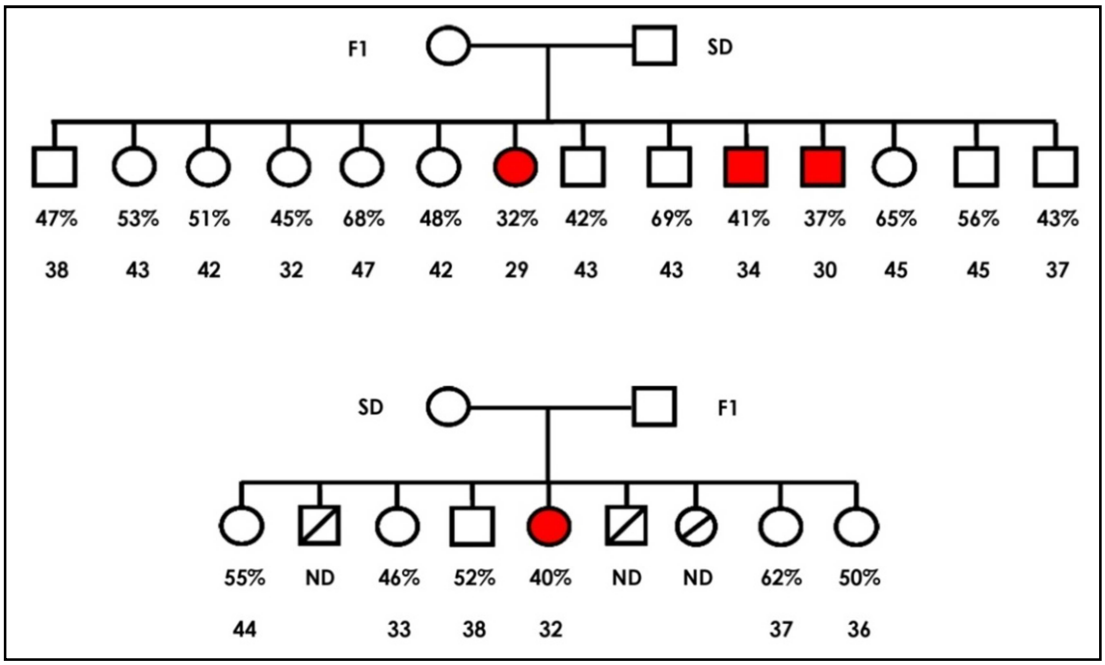
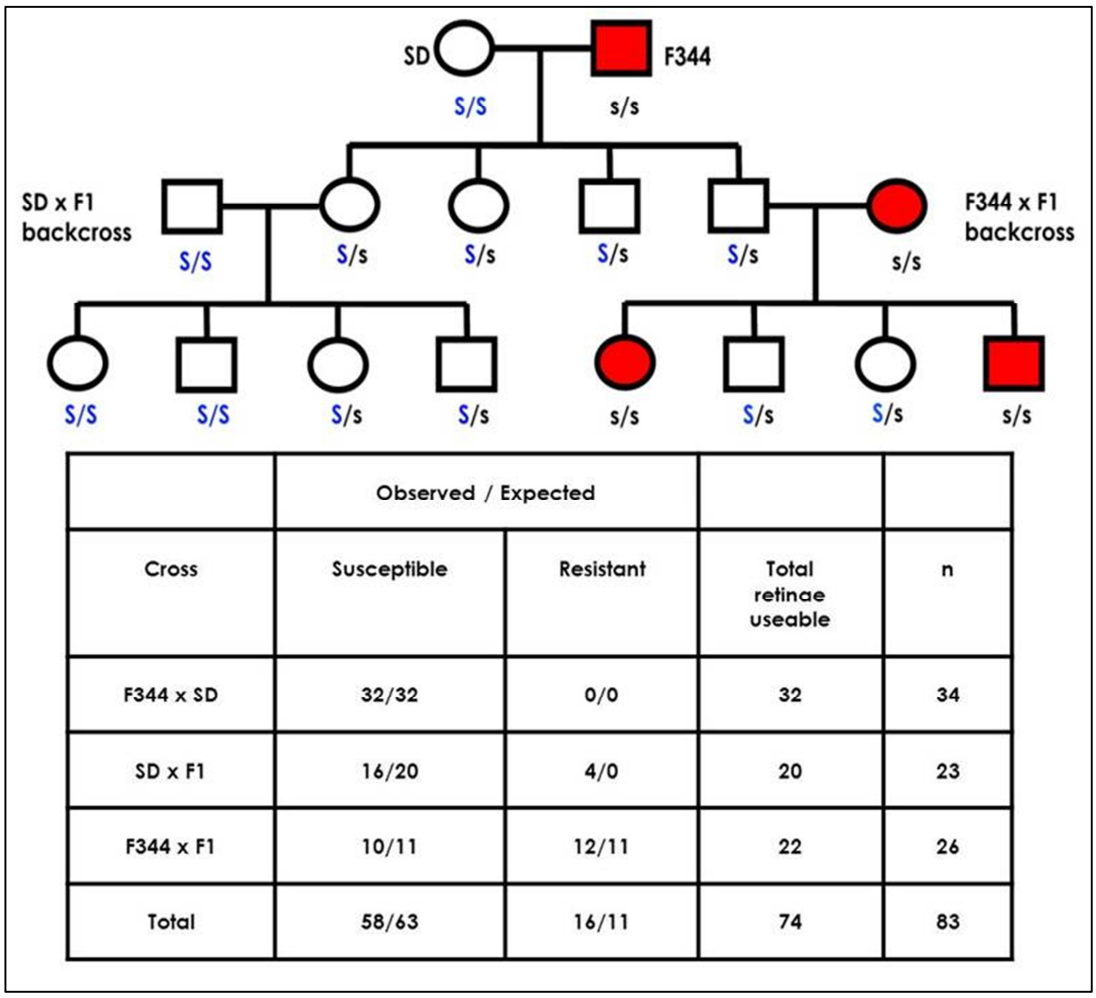


Figure 3.8 Genealogy plots of cyclic-hyperoxia exposed F1 x SD rats. Neonatal rat pups were exposed to cyclic-hyperoxia for 14 days after birth, after which retinal flat-mounts were performed and retinal avascular area (shown as a percentage of the total retinal area) and vessel tortuosity (shown below retinal avascular area) were measured. Unfilled symbols, rats susceptible to OIR; filled symbols, rats resistant to OIR; ND = retinal avascular area and blood vessel tortuosity scores not determined.

3.2.c.3 Genetic modelling of susceptibility to OIR

Predicted genetic modelling of susceptibility to OIR in the absence of ocular pigmentation is shown in Figure 3.9. If susceptibility to oxygen-induced retinopathy is defined as total avascular retinal area ≥ 43% and/or a blood vessel tortuosity score of ≥ 35, then an autosomal dominant inheritance of a monogenic trait is likely. Experimental results loosely match the predicted results, with all offspring of the F344 x SD cross carrying one susceptibility allele and being susceptible to OIR, as predicted. Sixteen out of twenty pups from the F1 x SD backcross rats carry at least one susceptibility allele and express the susceptible phenotype. Ten of the twenty two F1 x F344 backcross pups express the susceptible phenotype and the remaining twelve pups express the resistant phenotype.



	Observed / Expected			
Cross	Susceptible	Resistant	Total retinae useable	n
F344 x SD	32/32	0/0	32	34
SD x F1	16/20	4/0	20	23
F344 x F1	10/11	12/11	22	26
Total	58/63	16/11	74	83

Figure 3.9 Genetic modelling of susceptibility to oxygen-induced retinopathy in albino inbred rat strains. S, dominant susceptibility allele; s, recessive resistance allele; unfilled symbols, rats susceptible to OIR; filled symbols, rats resistant to OIR. If susceptibility to oxygen-induced retinopathy is defined as total avascular retinal area ≥ 43%, and/or a vessel tortuosity score ≥ 35 where each criterion is weighted equally, then autosomal dominant inheritance of a monogenic trait is likely. Observed and expected numbers of cross and backcross offspring with either the susceptible or the resistant retinal phenotype are shown in the table beneath the figure. n= number of rats.

3.3 DISCUSSION

3.3.a Summary of findings

The pattern through which albino rats inherit susceptibility to OIR has not previously been examined. My aim of the work described in this chapter was to address this issue using two inbred albino rat strains which differ in their response to cyclic hyperoxia. Inbred F344 (resistant to OIR) and SD (sensitive to OIR) rats were cross-bred to produce an F1 generation. Using formal backcross analysis, randomly selected F1 rats were backcrossed with each of the parental strains.

F344 x SD (F1) offspring, were albino and had total retinal avascular areas that fell between that of the two parental strains. It was difficult to distinguish between rats that were resistant or susceptible to OIR based on total retinal avascular area alone. It was also noted that some retinae of cyclic hyperoxia-exposed F1 rats exhibited the dilated, tortuous blood vessels which are characteristic of disease progression in OIR [74, 202], however, this did not necessarily correspond with an increase in retinal avascular area. Consequently, retinal blood vessel tortuosity was used as a second criterion to determine susceptibility to OIR.

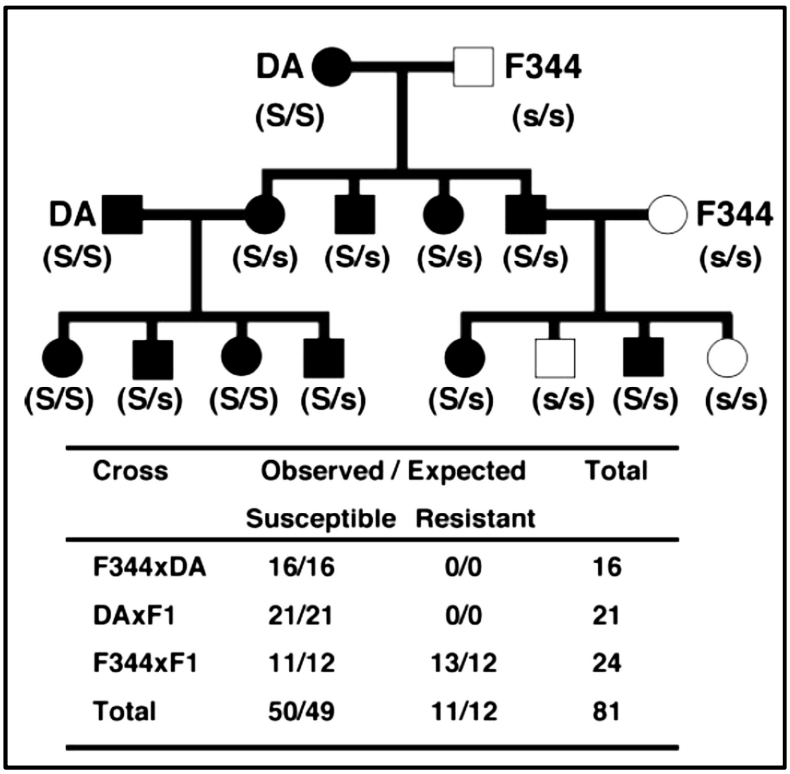
Backcross analysis of F1 rats with the resistant F344 strain showed F1 x F344 offspring had significantly larger total avascular areas and vessel tortuosity scores than the resistant F344 rats. F1 rats crossbred with the susceptible SD

strain resulted in F1 x SD offspring with smaller total avascular areas and blood vessel tortuosity scores than SD rats. Genetic modelling was used to investigate the mode of inheritance of susceptibility to OIR in non-pigmented rats. The results from the backcrosses were similar to those of the predicted results based on an autosomal dominant mode of inheritance, suggesting that susceptibility to OIR in albino SD rats is loosely inherited in this mode. However, the degree of variation in retinal avascular area in F1 and backcross rats, and the presence of 4 resistant pups in the F1 x SD backcross, where all F1 x SD offspring were predicted to be susceptible to OIR, suggests that a single gene is unlikely to be responsible for inheritance of susceptibility to OIR.

3.3.b Ocular pigmentation and susceptibility to OIR

The mode in which inheritance of susceptibility to OIR in inbred albino F344 (resistant to OIR) and pigmented DA (sensitive to OIR) rats has previously been studied by van Wijngaarden and colleagues [203]. F344 and DA rats were cross-bred to produce an F1 generation, the offspring of which had similar pigmentation to the DA strain (brown coat and eye colour) and were universally susceptible to OIR based on total avascular area alone. Using formal backcross analysis, randomly selected F1 rats were backcrossed with either of the parental strains. All offspring of F1 x DA backcrossed rats were pigmented and susceptible to OIR. Offspring of the F1 x F344 backcross showed a range of eye and coat colour, and varied in their susceptibility to

OIR. Pigmented offspring from the F1 x F344 backcrosses were significantly more susceptible to the effects of cyclic hyperoxia exposure than their albino litter mates that lacked ocular pigmentation. This led the authors to consider that inheritance of susceptibility to OIR occurred in an autosomal dominant mode (Figure 3.10).



Cross	Observed / Expected		Total
	Susceptible	Resistant	
F344xDA	16/16	0/0	16
DAxF1	21/21	0/0	21
F344xF1	11/12	13/12	24
Total	50/49	11/12	81

Figure 3.10 Genetic modelling of susceptibility to oxygen-induced retinopathy in albino and pigmented inbred rat strains (adapted from van Wijngaarden [203]). S, dominant susceptibility allele; s, recessive resistance allele; unfilled symbols, non-pigmented rats (F344); filled symbols, pigmented rats (DA). If susceptibility to oxygen-induced retinopathy is defined as total avascular retinal area ≥ 50%, then autosomal dominant inheritance of a monogenic trait is likely. Observed and expected numbers of cross and backcross offspring with either the susceptible or the resistant retinal phenotype are shown beneath the figure.

Results from the backcrosses closely matched the predicted results and it was determined that susceptibility to OIR was inherited in an autosomal dominant mode, segregating with the presence of ocular pigmentation. However, the authors suggested that the degree of variation in retinal avascular areas in cyclic hyperoxia-exposed backcrossed rats argued against a monogenic trait, and that it was likely that other genetic modifiers might contribute to inheritance of susceptibility to OIR. My study supports this argument against a monogenic trait conferring susceptibility to OIR.

Taken together, these data suggest it is likely that inheritance of susceptibility to OIR is modified by at least one other gene in albino and pigmented rats. Genetic modifiers may lead to reduced penetrance of the heritable trait and result in differences in the disease phenotype [206].

3.3.c Genetic modifiers in disease

A majority of the evidence of genetic modifiers affecting disease outcome has been derived from mouse studies, with relatively few examples known in humans [207]. One such example of the latter is the discovery of genetic modifiers present in patients with the haemoglobin disorders, sickle cell disease (SCD) and β-thalassaemia. SCD and β-thalassaemia are common monogenic disorders initially thought to follow a simple Mendelian mode of inheritance. However, vast differences in phenotypes observed in patients with the same mutations were not able to be explained by the involvement of

a single gene. It is now known that elevated expression of foetal haemoglobin during adulthood, and concomitant α-thalassaemia, are strong modifiers of disease severity in patients affected by SCD and β-thalassaemia [208]. It is possible that genetic modifiers are also contributing to disease susceptibility in OIR.

3.3.d Genetic modifiers in ROP and OIR

Whilst evidence derived from rodent studies suggests that ocular pigmentation increases susceptibility to OIR, studies of infants with ROP have shown that ocular pigmentation is associated with a decrease in disease severity [79, 96, 100, 203, 204, 209]. Black infants in a British cohort were less likely to develop any stage of ROP compared to Caucasian infants; however, Asian infants in the same cohort were more likely to develop severe ROP than Caucasian infants [80]. In North American cohorts, severe ROP occurred less frequently in African American infants than in Caucasian infants [79]. Whilst pigmentation appears to be protective in this particular cohort, a separate cohort showed that Alaskan natives had an increased risk of developing severe ROP compared with non-native Alaskans [81]. In Australia and New Zealand, no significant risk for susceptibility to ROP for infants born to mothers of white, Asian, Indigenous Australian, Maori or Pacific Islander descent was observed [210]. These data suggest that while ethnicity is a potential risk factor in susceptibility to ROP, its role remains unclear.

A combination of genetic modifiers and environmental factors may modify disease susceptibility. Differences in cultural and economic approaches in the provision of neonatal intensive care world-wide have made it difficult to identify causative changes that result in differential susceptibility to ROP [69]. In addition to this, ethical considerations limit the extent of studies which can be carried out in humans, therefore animal models of ROP are still able to provide valuable insights into the pathogenesis of ROP.

The exact role ocular pigmentation plays in susceptibility to OIR is unknown; however it is possible that it may act as a genetic modifier in susceptibility to OIR. In the late stages of OIR, pigmented DA and HW rats have been shown to be more susceptible to the effects of cyclic hyperoxia than non-pigmented, Lewis, SD and F344 rats [96]. Brown Norway (BN) rats have also been shown to be more susceptible to OIR than albino SD rats, where susceptibility was associated with increased expression of VEGF and decreased expression of the anti-angiogenic factor PEDF in the pigmented strain during the proliferative, late stages of the disease [98, 100].

PEDF is produced by the retinal pigment epithelium (RPE) cells of the retina and is upregulated in response to hyperoxia [27, 211]. The anti-angiogenic effects of PEDF are mediated by inducing activated endothelial cells to undergo apoptosis and by suppressing VEGF-induced endothelial cell proliferation and migration [29]. PEDF-deficient pigmented mice have been

shown to be more sensitive to the effects of hyperoxia than wild type mice, suggesting PEDF may act to down-regulate VEGF expression under hyperoxic conditions [212].

Imbalances in VEGF and/or PEDF expression in response to oxygen can either stimulate or inhibit angiogenesis, and as such the VEGF/PEDF ratio has previously been used as a marker for retinal angiogenesis [28, 30, 213]. An increased VEGF/PEDF mRNA ratio, determined using mRNA derived from the neural retina without the RPE, has been observed in albino F344 rats resistant to OIR in the early induction phase of OIR when normal retinal vascularisation is attenuated, whereas a decrease in this ratio was observed in the susceptible pigmented DA strain [30]. These results were consistent with observations that retinal vascular development was more advanced in resistant F344 rats than susceptible DA rats. During the later stages OIR, a reversal in the VEGF/PEDF ratio was observed between resistant and susceptible strains. Limited VEGF-mediated angiogenesis during early retinal vascular development and the subsequent increased VEGF/PEDF ratio at the later time points in the susceptible DA strain may render these rats more sensitive to the effects of hypoxia and promote aberrant retinal angiogenesis.

In the absence of hyperoxic stress and ocular pigmentation, normal retinal development is delayed in albino mammals and is associated with abnormal

development of the neural retina [214]. Ocular pigmentation has also been associated with changes in retinal structure and function in response to sustained hyperoxia. Hyperoxia-exposed pigmented Long Evans (LE) rats exhibit more severe functional abnormalities, increased structural changes and larger avascular areas compared with albino SD rats [204].

Melanin, which is expressed in the retinal pigment epithelium (RPE), is critical in regulating normal retinal development in pigmented rats [215]. The TYR gene encodes tyrosinase, the key enzyme in melanin biosynthesis which converts tyrosine to dopaquinone via the intermediate product dihydroxyphenylalanine (DOPA) [216]. Mutations in the TYR gene are responsible for albinism in Wistar rats and ferrets and are associated with type 1 oculocutaneous albinism in humans [217, 218]. Ectopic expression of tyrosinase in retinal pigment epithelium has been shown to rescue retinal abnormalities and restore visual function in albino mice [216].

DOPA is involved in retinal cell production in the RPE and regulates the cell cycle, processes which are affected by oxygen levels [215, 219-221]. The production of oxygen free radicals during melanin biosynthesis is increased when cells are exposed to hyperoxia and this DOPA-mediated oxidative damage may impinge on the survival of RPE and vascular endothelial cells [219, 220]. It has been hypothesized that DOPA-mediated endothelial cell damage may be responsible for increased susceptibility of pigmented rats to

OIR [203]. However, this mechanism is unlikely to be responsible for the susceptibility to OIR observed in albino SD rats.

The association between ocular pigmentation and susceptibility to OIR may be coincidental in light of the differential response to cyclic hyperoxia observed in F344 and SD rats. Despite evidence that susceptibility to OIR appears to segregate with ocular pigmentation, the fact that SD rats are susceptible to OIR in the absence of pigment suggests that different mechanisms may underlie susceptibility to OIR in albino and pigmented rats. Differences in susceptibility to OIR may be associated with the ability or inability of different strains to overcome hyperoxia-mediated vessel obliteration as suggested by Dorfman and colleagues [204].

Oxygen is central to the pathogenesis of ROP and OIR and perturbations in the oxygen sensing pathway may be responsible for the differences in susceptibility to OIR that are exhibited by the two strains examined in this study. Expression of VEGF and PEDF are upregulated by activation of hypoxia-inducible factors (HIFs) [34, 35, 222]. HIF-1α and HIF-2α are required for normal retinal vascular development and have been implicated in a number of oxygen dependent diseases including von Hippel-Lindau disease, diabetic retinopathy and ROP [8, 46, 49]. Genes that are either regulated by oxygen, or are involved in the regulation of HIF-α may contribute to differential susceptibility to OIR.

3.3.e CONCLUSION

Whilst pigment appears play a role in increased susceptibility to OIR, it is likely that other genetic modifiers are involved. The need to identify modifier genes associated with genotypic-phenotypic discrepancies in monogenic diseases is becoming increasingly important, as nucleic acid-based therapies to modify gene expression or compensate for phenotypic abnormalities could potentially be used in the future to treat a range of genetic diseases [223]. Identifying genetic modifiers in susceptibility to OIR would provide valuable insight into disease pathogenesis that may be relevant to human ROP.