

Update on animal models of diabetic retinopathy: from molecular approaches to mice and higher mammals

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Diabetic retinopathy (DR) is the most common microvascular complication of diabetes and one of the major causes of blindness worldwide. The pathogenesis of DR has been investigated using several animal models of diabetes. These models have been generated by pharmacological induction, feeding a galactose diet, and spontaneously by selective inbreeding or genetic modification. Among the available animal models, rodents have been studied most extensively owing to their short generation time and the inherited hyperglycemia and/or obesity that affect certain strains. In particular, mice have proven useful for studying DR and evaluating novel therapies because of their amenability to genetic manipulation. Mouse models suitable for replicating the early, non-proliferative stages of the retinopathy have been characterized, but no animal model has yet been found to demonstrate all of the vascular and neural complications that are associated with the advanced, proliferative stages of DR that occur in humans. In this review, we summarize commonly used animal models of DR, and briefly outline the *in vivo* imaging techniques used for characterization of DR in these models. Through highlighting the ocular pathological findings, clinical implications, advantages and disadvantages of these models, we provide essential information for planning experimental studies of DR that will lead to new strategies for its prevention and treatment.

Introduction

Diabetic retinopathy (DR), a major complication of diabetes mellitus, is one of the leading causes of blindness worldwide. Early diagnosis and prevention of retinopathy in diabetic individuals is crucial for preventing vision loss. Prolonged hyperglycemia causes irreversible pathological changes in the retina, leading to proliferative DR with retinal neovascularization and diabetic macular edema (DME) in some individuals (Mohamed et al., 2007; Cheung et al., 2010).

Treatment of DR can only be achieved through an enhanced understanding of disease pathogenesis; however, because most structural, functional and biochemical studies cannot be carried out in human subjects, animal models are essential for studying DR pathology, and thus for developing new and better treatments.

Clinical features of DR

DR is widely regarded as a microvascular complication of diabetes. Clinically, DR can be classified into non-proliferative DR (NPDR) and proliferative DR (PDR) (Cheung et al., 2010). NPDR is characterized ophthalmoscopically by the presence of microaneurysms and dot and blot hemorrhages (Fig. 1A). NPDR has been further subdivided into progressive stages: mild, moderate and severe. Severe NPDR (also called preproliferative DR) shows increased retinal microvascular damage as evidenced by cotton wool spots, venous beading, venous loops and intra-retinal microvascular abnormalities (IRMAs). Capillary non-perfusion and degeneration of the retina can be detected in individuals with diabetes following intravascular injection of fluorescein. If left untreated, PDR (characterized by abnormal retinal neovascularization) can develop (Fig. 1B). A clinically important outcome of PDR is retinal and vitreous hemorrhage and tractional retinal detachment (Cheung et al., 2010).

DME can occur at any stage (i.e. along with NPDR or PDR) and is now the most common cause of vision loss due to DR (Cheung et al., 2010).

Epidemiology and risk factors

Diabetes affects more than 300 million people worldwide, and is expected to affect an estimated 500 million by 2030 (International Diabetes Federation, 2011). Studies have shown that nearly all individuals with type 1 diabetes [also known as insulin-dependent diabetes mellitus (IDDM)] and more than 60% of individuals with type 2 diabetes (non-insulin-dependent diabetes mellitus) have some degree of retinopathy after 20 years. Current population-

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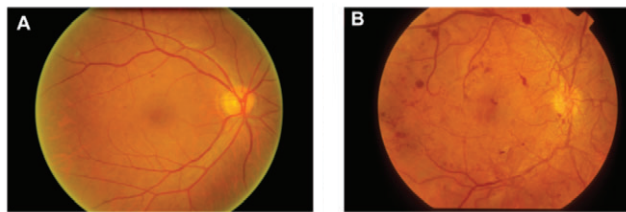


Fig. 1. Clinical features of DR. Fundus photographs of human patients showing (A) early non-proliferative diabetic retinopathy (NPDR) and (B) proliferative diabetic retinopathy (PDR).

based studies suggest that about one-third of the diabetic population have signs of DR and approximately one tenth have vision-threatening stages of retinopathy, including PDR and DME (Wong et al., 2006; Wong et al., 2008; Wang et al., 2009; Zhang et al., 2010).

People with diabetes are 25 times more likely to become blind than non-diabetics. In fact, reports have shown that 50% of diabetics will become blind within 5 years following diagnosis of PDR, if left untreated (Ciulla, 2004; Klein, 2008; Wong et al., 2009). The number of people with DR is rapidly increasing owing to a dramatic rise in the prevalence of type 2 diabetes, reflecting the increased prevalence of obesity and metabolic syndrome observed in recent years (Cheung et al., 2010; Raman et al., 2010).

The three major risk factors for DR are prolonged (1) diabetes, (2) hyperglycemia and (3) hypertension, which have been shown to be consistently associated with DR in epidemiological studies and clinical trials (Wong et al., 2006; Wong et al., 2008; Wang et al., 2009; Cheung et al., 2010; Grosso et al., 2011). Dyslipidemia and body mass index might also be risk factors for DR, but associations have not been as consistent (Lim and Wong, 2011; Benarous et al., 2011; Dirani et al., 2011; Sasongko et al., 2011). Emerging evidence supports a genetic component for DR, but specific genes associated with the disease have not been clearly identified despite large studies (Liew et al., 2006; Abhary et al., 2009; Sobrin et al., 2011). It remains difficult to predict which diabetic individuals will progress from NPDR to PDR.

Pathophysiology of DR

The pathogenesis of the development of DR is highly complex owing to the involvement of multiple interlinked mechanisms leading to cellular damage and adaptive changes in the retina (Frank, 2004). Hence, the fundamental cause(s) of DR has not been elucidated completely despite years of clinical and laboratory investigation. In the past, retinopathy has been characterized largely by its microvascular abnormalities, including endothelial cell dysfunction, vessel leakage, and vascular occlusion and degeneration (Curtis et al., 2009). Recent evidence, however, indicates that retinal complications of diabetes are a composite of functional and structural alterations in both the microvascular and neuroglial compartments (Antonetti et al., 2006; Curtis et al., 2009; Villarreal et al., 2010; Barber et al., 2011). The exact mechanisms by which hyperglycemia initiates the vascular or neuronal alterations in retinopathy have not been completely defined (Curtis et al., 2009; Villarreal et al., 2010). The cellular damage in the retina has been speculated to be caused by several mechanisms, including

increased flux through the polyol pathway, production of advanced glycation end products (AGEs), increased oxidative stress and activation of the protein kinase C (PKC) pathway, but many of these hypotheses have yet to be validated in human studies or clinical trials (Frank, 2004; Cheung et al., 2010).

DR also shares similarities with chronic inflammatory diseases: it causes increased vascular permeability, edema, inflammatory cell infiltration, tissue destruction, neovascularization, and the expression of pro-inflammatory cytokines and chemokines in the retina. Increased expression of vasoactive factors and cytokines probably plays an important role in mediating the structural and functional changes in the retina (Khan and Chakrabarti, 2007; Wirostko et al., 2008). Recent studies strongly suggest that inflammation is also important in the pathogenesis of early stages of experimental DR (Kern, 2007; Liou, 2010; Tang and Kern, 2011), although studies in humans have not found a consistent association between systemic markers of inflammation and retinopathy (Nguyen et al., 2009; Lim et al., 2010). Thus, it remains uncertain whether inflammation also plays a crucial role in the development and progression of DR in humans. Some of the major pathways and factors involved in the pathogenesis of DR are shown in Fig. 2.

Outstanding questions regarding DR etiology and treatment

The currently available treatments for advanced stages of DR, including PDR and DME, are laser photocoagulation, surgical vitrectomy or intraocular injections of steroids and vascular endothelial growth factor (VEGF) inhibitors. Although these treatments have had high success rates, they are not useful for early stages of DR, and do not completely eliminate the risk of blindness (Cheung et al., 2010). Laser therapy is inherently destructive, with unavoidable side effects, and is not effective in reversing vision loss. The new approaches involving anti-VEGF therapy also have potential ocular and systemic risks (Cheung et al., 2010; Truong et al., 2011). Thus, new treatment strategies that are preventative and/or can provide interventions earlier in diabetes to delay or prevent the progression of early NPDR are needed.

In this regard, it is crucial to more fully establish the underlying mechanisms and causes for DR. Studies suggest that multifactorial approaches intervening at the hemodynamic, metabolic and cytokine levels will delay the development of DR. However, the basic fundamental mechanism(s) of hyperglycemic influence or regulation of retinal vessels is not completely understood. Despite the large number of experimental studies being conducted on the etiology and treatment of DR in various laboratories, some of the important issues that remain unanswered are: (1) the mechanisms that link neural impairment in early diabetes to the development of retinal vascular abnormalities; (2) the potential role of inflammation in diabetes-induced retinal neurodegeneration; (3) the specific retinal layers that are vulnerable to metabolic imbalance in DR; and (4) the genetic basis of DR.

Further studies using appropriate animal models are required to provide answers to some of these questions, which will provide the basis for new treatment approaches that prevent or delay the onset of DR.

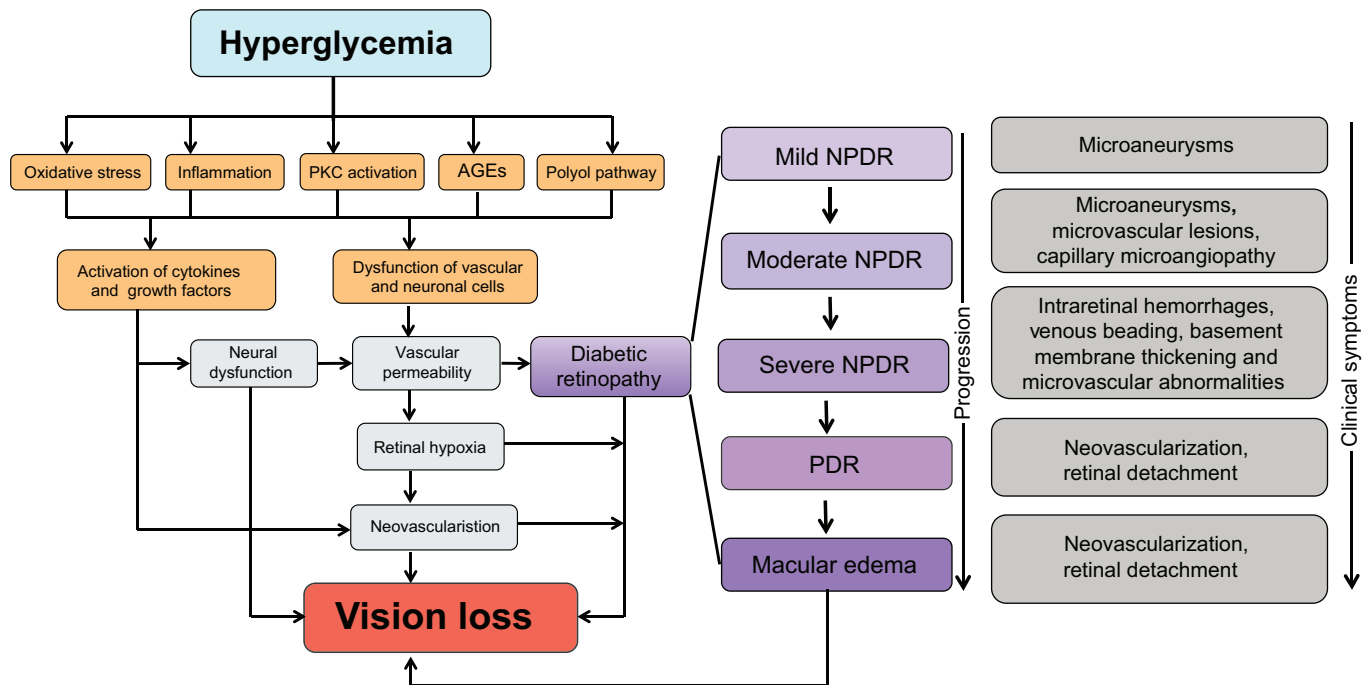


Fig. 2. Flow diagram showing the major key factors involved in the pathogenesis of DR and the clinical symptoms evident at different stages of DR. DR is a multifactorial disease involving several pathological mechanisms, including increased oxidative stress, inflammation, the polyol pathway leading to sorbitol accumulation, production of advanced glycation end products (AGEs) and activation of the protein kinase C (PKC) pathway. These pathways can in turn activate the production of cytokines and many vasoactive factors, such as vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF), which are vital in mediating the structural and functional changes of DR. Clinically significant diabetic macular edema (DME) can occur in the late stages of DR with non-proliferative or proliferative retinopathy and is the most common cause of vision loss.

Animal models of DR

During the last two decades, extensive research with animal models of diabetes has been carried out. To date, several species – including mice, rats, cats, dogs, pigs and non-human primates – have been used as models to provide valuable information on the cellular and molecular aspects of pathogenesis of DR. Diabetes in animals is usually induced with chemicals such as alloxan or streptozotocin (STZ), by surgical pancreatectomy, or spontaneously by selective breeding or genetic manipulation. Most experimental studies on DR to date have used animal models of type 1 diabetes (Cheta, 1998; Kern, 2009; Zheng and Kern, 2010). Recently, many transgenic and knockout mouse models have also been developed to study the molecular pathways involved in DR pathology. In the following sections, we review currently available animal models of DR, beginning with a description of the various techniques used for in vivo characterization. We then briefly discuss studies of DR performed using each model, comment on their contributions to investigating the pathogenesis of DR, and highlight their advantages and disadvantages. Retinal lesions of DR observed in small and higher animal models are summarized in Tables 1 and 2, respectively.

Characterization of DR in animal models

The use of appropriate imaging techniques to analyze animal models of human DR is essential to experimentally study and characterize the disease. Eyes collected at necropsy can be subjected to sensitive histological techniques (such as trypsin digest) to study the retinal vasculature in detail. In addition, various molecular and

biochemical techniques (such as quantitative PCR, microarray, western blotting, as well as protein, enzyme and cytokine assays) can be carried out to study the expression of various genes and proteins in the eyes. While the animals are alive, the vascular and non-vascular alterations of DR (Cunha-Vaz, 2007) can be monitored in the same animal over time using various techniques, including electrophysiological testing, fundus photography (FP), fundus fluorescein angiography (FFA) and optical coherence tomography (OCT); these techniques are briefly discussed below.

Electrophysiological tests

Electrophysiological tests of retinal function are the most commonly used non-invasive technique for studying visual function in animal models. These tests include electroretinography (ERG), pattern ERG (PERG), multi-focal ERG (mfERG) and visually evoked potential. Studies using ERG revealed functional abnormalities before the evidence of vascular changes in the eyes of diabetic rats (Kohzaki et al., 2008). Alterations in retinal electrical responses, which arise from the neural retina, are one of the earliest manifestations of DR. ERG studies on diabetic animals have shown reduced a- and b-wave amplitude (Li et al., 2002), and prolonged implicit time in the oscillatory potentials (OPs) (Hancock and Kraft, 2004).

FP and FFA

Retinal vasculature photographs obtained with FP provide a relatively insensitive tool to monitor the progression of DR, but are adequate for providing important information about the severity

Table 1. Retinal lesions reported in small animal models of DR

Animal model	Type of diabetes	Age of onset of diabetes	Retinal lesions	References
Rats				
STZ	Type 1	3 days after STZ injection	<ul style="list-style-type: none"> • Pericyte loss • Vascular leakage • Blood retinal barrier breakdown • Ganglion cell loss • Endothelial cell damage • Vascular occlusion of retinal capillaries • Thicker capillary BM 	Robison et al., 1991; Anderson et al., 1995; Miyamoto et al., 1999; Xu et al., 2004; Gastinger et al., 2006; Zheng and Kern, 2010
Galactosemia	–	–	<ul style="list-style-type: none"> • Pericyte loss • Acellular capillaries • IRMAs • Thicker capillary BM 	Kern and Engerman, 1995
BB	Type 1	2-5 months	<ul style="list-style-type: none"> • Pericyte loss • Acellular capillaries • Blood retinal barrier breakdown • Thicker capillary BM 	Blair et al., 1984; Sima et al., 1985
WBN/Kob	Type 1	9-21 months	<ul style="list-style-type: none"> • Acellular capillaries • Thicker capillary BM 	Miyamura et al., 1998; Bhutto et al., 1999; Tsuji et al., 2009
SDT	Type 1	5-6 months	<ul style="list-style-type: none"> • Pericyte loss • Acellular capillaries • Vascular leakage • Retinal detachment with fibrous proliferation 	Yamada et al., 2005; Kakehashi et al., 2006; Sasase et al., 2010
ZDF	Type 2	1-2 months	<ul style="list-style-type: none"> • Pericyte loss • Acellular capillaries • Thicker capillary BM 	Danis et al., 1993; Yang et al., 2000; Behl et al., 2008
OLETF	Type 2	4-5 months	<ul style="list-style-type: none"> • Microaneurysms • Tortuosity • Loop formations of capillaries • Vessel caliber irregularity • Thicker capillary BM 	Bhutto et al., 2002; Lu et al., 2003
GK	Type 2	1-2 months	<ul style="list-style-type: none"> • Increased EC:pericyte ratios 	Agardh et al., 1997
Mice				
STZ	Type 1	3 days after STZ injection	<ul style="list-style-type: none"> • Pericyte loss • Acellular capillaries • Apoptosis of vascular cells • Ganglion cell loss • Thinning of retina 	Martin et al., 2004; Leichman et al., 2005
Galactosemia			<ul style="list-style-type: none"> • Pericyte loss • Acellular capillaries • Microaneurysms • Thicker capillary BM 	Kern and Engerman, 1996a
NOD	Type 1	8 months	<ul style="list-style-type: none"> • Loss of retinal microvessels • Disordered focal proliferation of new vessels 	Makino et al., 1980; Shaw et al., 2006; Lee et al., 2008
db/db	Type 2	1-2 months	<ul style="list-style-type: none"> • Pericyte loss • Acellular capillaries • Blood-retinal barrier breakdown • Thicker capillary BM 	Midena et al., 1989; Clements et al., 1998; Cheung et al., 2005
<i>Ins2^{Akita}</i>	Type 1	1 month	<ul style="list-style-type: none"> • Increased vascular permeability • Acellular capillaries • Thinning of retina • Ganglion cell loss 	Barber et al., 2005; Gastinger et al., 2008
Akimba	Type 1	1 month	<ul style="list-style-type: none"> • Microaneurysms • Vascular leakage • Venous beading • Tortuosity • Capillary dropout • Hemorrhage • Possible neovascularization • Retinal edema 	Rakoczy et al., 2010

Table 2. Retinal lesions of DR reported in higher animal models of DR

Animal models	Type of diabetes	Retinal lesions	References
Dog	Type 1	<ul style="list-style-type: none"> • Pericyte loss • Microaneurysms • Thicker capillary BM 	Gardiner et al., 1994; Kern and Engerman, 1996b
Galactosemic dogs	–	<ul style="list-style-type: none"> • Pericyte loss • Acellular capillaries • Microaneurysms • IRMAs • Retinal hemorrhages 	Takahashi et al., 1993; Kador et al., 1995; Kern and Engerman 1996b; Kobayashi et al., 1998
Cat	Type 1	<ul style="list-style-type: none"> • Acellular capillaries • Microaneurysms • Vascular leakage • Tortuosity • Thicker capillary BM 	Mansour et al., 1990; Hatchell et al., 1995; Linsenmeier et al., 1998; Budzynski et al., 2005
Pig	Type 1	<ul style="list-style-type: none"> • Retinal microvasculopathy • Thicker capillary BM 	Hainsworth et al., 2002; Lee et al., 2010
Non-human primate	Type 1	<ul style="list-style-type: none"> • Microaneurysms • IRMAs • Cotton wool spots • Macular edema 	Tso et al., 1988
	Type 2	<ul style="list-style-type: none"> • Acellular capillaries • Cotton wool spots • Intraretinal hemorrhages • Microaneurysms • IRMAs • Hard exudates in the macula • Decreased RGC layer 	Kim et al., 2004; Johnson et al., 2005

of retinopathy in patients, including vessel caliber changes, swelling of vessels, abnormal new growth of vessels on retinal surface and tortuosity. FFA, which monitors the flow of a fluorescent dye through the retinal vasculature, is useful for demonstrating vascular abnormalities, non-perfusion and vascular leakage. Neither FP nor FFA has been extensively used in animal studies, owing to the rapid development of cataracts in many species, and the fact that small animals' eyes are small, highly curved globes, which prevent light rays from focusing on the retina. Moreover, FFA cannot be used easily in albino animals or in the non-pigmented portions of retina in animals having a tapetum (dogs, cats) (Hawes et al., 1999). Fig. 3 shows a comparison of FFA performed in both B6 wild-type (pigmented) and Balb/c albino (non-pigmented) mice.

OCT

Measurement of retinal thickness has been used as an indicator of progressive neural retinal pathology in animal models of retinal degeneration, but the degree of degeneration that occurs in diabetic animals is considerably less than in retinal degeneration models and is more difficult to measure reproducibly. High-resolution OCT measures retinal thickness non-invasively in rodents (as in humans), and recent machines can produce images with some features of retinal histology. Studies have also shown that ERG amplitude loss is highly correlated with retinal thinning as measured by OCT (Li et al., 2001; Fischer et al., 2009). Similarly, reduced retinal thickness has been demonstrated in diabetic mice using OCT (Rakoczy et al., 2010). Retinal thickening has been detected with OCT in

monkeys (Sakurai et al., 2009) and dogs (Panzan et al., 2004), and, using magnetic resonance imaging, also in diabetic rats (Berkowitz et al., 2007). Detailed validation of structural and functional lesions of DR in mice is available in a review by Kern et al. (Kern et al., 2010a).

Emerging techniques to measure retinal vascular caliber

Human epidemiological studies have shown that measurement of retinal vascular caliber from photographs might provide clues to early pathological processes in pre-diabetes, diabetes and DR (Nguyen et al., 2007; Nguyen et al., 2008; Rogers et al., 2008; Wong, 2011). An insight into the retinal vessel caliber changes in animal models of DR could thus potentially help to characterize the structure and pathology of the microcirculation, and to examine its relationship to systemic vascular diseases in diabetes. Currently, there are no well-established methods to study the retinal vessel caliber changes in animal models. Recently, a semi-automated computer-based quantitative program to measure retinal vascular caliber from retinal photographs of rodents has become available, which is identical to the program used in large epidemiological studies of both diabetic and non-diabetic human populations (Fig. 4A,B). This novel imaging software measures subtle retinal vessel caliber changes in rodents (Fig. 4C,D), which might be markers of early microvascular dysfunction in diabetes. Such developments will open the door for advanced quantitative assessments in animal models, which could substantially contribute to a better understanding of the pathogenesis and prediction of DR.

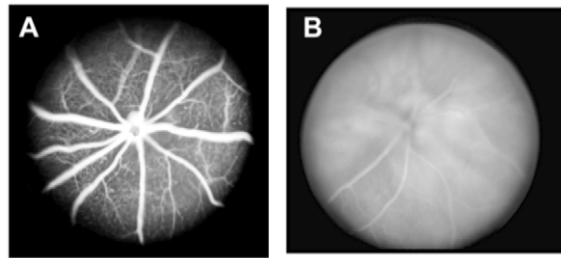


Fig. 3. Fundus fluorescein angiography (FFA) images show the comparison between normal B6 (pigmented) and Swiss albino (non-pigmented) mice. Normal (A) B6 (pigmented) and (B) Swiss albino (non-pigmented) mice. FFA cannot be used in albino mice owing to the absence of pigment, which produces severe glare.

Rodent models of DR

Various rodent models have been used for studying the molecular mechanisms underlying the pathogenesis of DR. These models are easy to handle, relatively inexpensive, have short reproductive cycles and have a similar genetic background to humans, hopefully allowing experimental results to be extrapolated. Rodent models of DR vary with respect to species (predominantly rat or mouse), strain, method of diabetic induction and duration of diabetes. Advanced techniques of genetic manipulation, such as tissue-specific transgenic expression and targeted gene knockout, have increased the relative importance of mouse models for experiments that specifically require genetically engineered models. Thus, these animals provide a remarkable platform to investigate the pathogenesis of at least the early stages of the retinopathy, because genetic alterations of selected metabolic and pathophysiological mechanisms are now possible.

However, a major criticism of using rodents to model DR is that they might not exactly mirror the human condition, especially with regard to the extent of pathology. Rodent models reproduce most

aspects of the early stages of DR, but have not been found to reproducibly develop the late, neovascular stage of the disease, probably owing to the short lifespan of the animals and thus the shorter duration of diabetes.

Chemically induced diabetic models

Diabetes can be induced in animals using STZ or alloxan, which destroy pancreatic β -cells and thereby induce type 1 diabetes. Rodents that have been made diabetic in this manner have been studied for up to 24 months in a hyperglycemic yet healthy condition, by providing them with small amounts of insulin every few days. This approach reproduces early symptoms of DR, such as loss of retinal pericytes and capillaries, thickening of the vascular basement membrane (BM), vascular occlusion and increased vascular permeability (Kern and Mohr, 2007; Kern, 2009; Zheng and Kern, 2010). Physiological and biochemical changes in the retina begin to appear between 1-2 months after the onset of hyperglycemia in STZ-induced diabetic rats. Diabetes-induced non-vascular changes (neuronal and glial) are seen before the development of changes in vascular cells and might contribute to the pathology of the vascular disease in this model (Barber et al., 1998; Zeng et al., 2001; Kohzaki et al., 2008). However, there are variations in the reported retinal biochemistry and histopathological response to diabetes between species and also within the same species. In fact, a recent report studied the differences in the rate at which early stages of DR develop in three different rat strains (Sprague Dawley, Lewis and Wistar) with diabetes induced by STZ. After 8 months of diabetes, Lewis rats showed the most accelerated loss of retinal capillaries and retinal ganglion cells (RGCs), whereas Wistar rats showed degeneration of the capillaries without significant neurodegeneration and Sprague Dawley rats showed no lesions at this time point (Kern et al., 2010b).

STZ-induced mouse models were not frequently used for studies on DR in the past because it was more difficult to induce diabetes in mice than in rats and was difficult to keep the tiny animals alive once diabetic. These problems have been overcome more recently. STZ-induced diabetic B6 mice demonstrated acellular capillaries, apoptosis of vascular cells and pericyte ghosts in the retina, the hallmark of early characteristics of DR (Feit-Leichman et al., 2005), at ~6 months after the onset of diabetes. Advanced proliferative retinal changes did not develop in these mice within the study duration (18 months of diabetes). Whether or not mice develop loss of RGCs is controversial. At 10-14 weeks after STZ treatment, these mice demonstrated loss of RGCs, and significant thinning of the inner and outer layers of the retina (Martin et al., 2004; Barber et al., 2005). Other studies, however, found no evidence of RGC loss in diabetic mice (Asnaghi et al., 2003; Feit-Leichman et al., 2005; Gastinger et al., 2006). These diabetic models are mostly used to demonstrate early changes of DR. Studies of advanced proliferative retinal changes cannot be carried out in these models because they die before PDR could be detected.

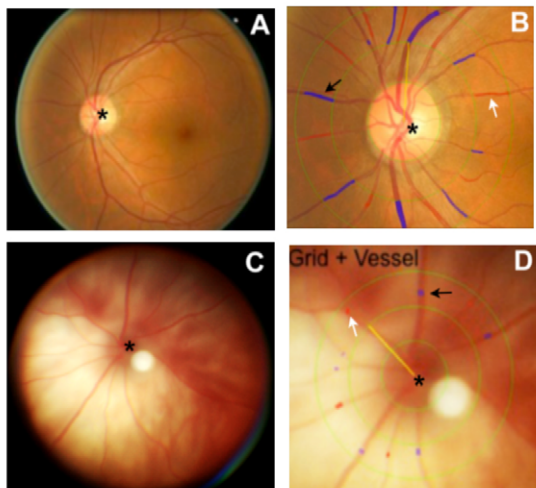


Fig. 4. Retinal vascular caliber measurement. The measurement of retinal vessel caliber in human (A,B) and mouse (C,D) with fundus imaging, using semi-automated computer-based quantitative program (SIVA). The white arrow indicates the artery, the black arrow indicates the vein and an asterisk (*) shows the optic nerve head.

eNOS^{-/-} mice

Recently, the effects of single genes on the development of DR have been assessed by inducing diabetes with STZ in transgenic or gene knockout mice. For example, Li et al. investigated the pathogenic role of endothelial nitric oxide synthase (eNOS) dysfunction in the

development of DR by inducing diabetes using STZ in eNOS knockout (*eNOS*^{-/-}) mice (Li et al., 2010). The retinal vasculature of *eNOS*^{-/-} mice develops normally and is associated with increased vascular-associated neuronal NOS activity that compensates for the eNOS deficiency in the retina. STZ-induced diabetes in these mice showed accelerated retinal complications of DR when compared with age-matched STZ-induced diabetic B6 wild-type mice. The retinal complications included increased vessel leakage, gliosis, acellular retinal capillaries and retinal capillary BM thickening. Further studies using this model will be useful for investigating the cellular and molecular mechanisms of DR, including gliotic responses in retinal Muller cells.

Spontaneously diabetic rat models

Zucker diabetic fatty rats

Zucker diabetic fatty (ZDF) rats are genetic models of type 2 (non-insulin-dependent) diabetes and become hyperglycemic at 6-7 weeks of age. These rats usually die at ~1 year of age, but can be maintained without treatment if supplemented with glucose of more than 500 mg/dl. Studies demonstrated pericyte loss, a thicker retinal capillary BM, and an increased number of endothelial intercellular junctions and focal nodules in ZDF rats. This model is thought to be useful for pharmacological intervention studies because it is naturally and severely type 2 diabetic, showing quantifiable retinal vascular changes. In addition, same-sex littermates can be used as controls (Danis and Yang, 1993; Ottlecz et al., 1993; Yang et al., 2000).

WBN/Kob rats

The WBN/Kob rat is also a spontaneously type 2 diabetic strain, in which hyperglycemia occurs at 9 months of age. Thickened capillary BM and acellular capillaries in the retina have been reported in this model (Miyamura and Amemiya, 1998; Matsuura et al., 1999). Proliferative changes were reported in the pre-retinal vitreous of these rats, showing intra-retinal angiopathy accompanied by newly formed vessels and significant hyalinization of intra-retinal vessels (Tsuji et al., 2009). Hence, this might be useful as an animal model for progressive DR, but the neovascularization has not been confirmed. Microaneurysms, the early clinical sign of human NPDR, and arterio-venous shunts, which are associated with severe stages of human NPDR, were not observed in this model (Bhutto et al., 1999).

Otsuka Long-Evans Tokushima fatty rats

Otsuka Long-Evans Tokushima fatty (OLETF) rats spontaneously develop type 2 diabetes with severe obesity. The retinal ultrastructural changes observed in OLETF rats are similar to those seen in diabetic individuals (Miyamura et al., 1999; Lu et al., 2003), but do not include hemorrhages or exudates. There is significant thinning of the inner nuclear and photoreceptor layers of the retina, a decrease in the height of the retinal pigment epithelial (RPE) cells, thickened capillary BM and poorly developed basal infoldings in these rats. Abnormal ERG was also reported in sucrose-fed OLETF rats (Hotta et al., 1997). However, it has been suggested that OLETF rats are not suitable for studying DR because the formation of acellular capillaries and pericyte ghosts typical of human DR are not accelerated in these rats (Matsuura et al., 2005).

Goto-Kakizaki rats

The Goto-Kakizaki (GK) rat is a spontaneous model of non-obese type 2 diabetes and develops chronic hyperglycemia at 4-6 weeks of age (Goto et al., 1988). Diabetic GK rats demonstrated an increased ratio of retinal endothelial cells to pericytes (Agardh et al., 1997), and reduced retinal blood flow without changes in major retinal vessel diameters, at an early stage of diabetes (Miyamoto et al., 1996). Because of the moderate and stable diabetic state, this rat model is useful for investigating the retinal microcirculatory changes caused by type 2 diabetes over an extended period of time (Miyamoto et al., 1996).

Spontaneously diabetic Torii rats

The spontaneously diabetic Torii (SDT) rat is a non-obese type 2 diabetes model that develops hyperglycemia at 20 weeks of age and can survive for long periods without insulin treatment. SDT rats exhibit tractional retinal detachment with fibrous proliferation, and possibly neovascularization, without retinal ischemia. The development of neovascularization in the absence of ischemia makes this model considerably different from the neovascularization that has been observed in diabetic individuals (Yamada et al., 2005). Thus, the model needs additional study before it can be considered as a model of DR. Studies showed a reduction in the amplitude of ERG b-waves and OPs. Because OPs arising from amacrine cells are a sensitive measure of retinal ischemia, these studies indicate the development of inner retinal ischemia in these rats (Sasase, 2010).

Biobreeding rats

The biobreeding (BB) rat is a spontaneous model of type 1 diabetes that develops diabetes between the age of 40 and 140 days. These rats exhibit retinal lesions, including pericyte loss, BM thickening, capillary degeneration and an absence of microaneurysms after 8-11 months of diabetes (Sima et al., 1985). Pancreas transplantation has been shown to inhibit the development of retinal microvascular lesions in this model (Chakrabarti et al., 1987). However, very few studies of DR have been reported using this model, so its advantages and disadvantages cannot be judged.

Spontaneously diabetic mouse models

Non-obese diabetic mice

Non-obese diabetic (NOD) mice spontaneously develop type 1 diabetes owing to autoimmune destruction of insulin-producing pancreatic β -cells by CD4+ and CD8+ T cells (Makino et al., 1980). Studies have shown the loss of retinal microvessels, reduced perfusion of the retina and disordered focal proliferation of vessels in NOD mice (Shaw et al., 2006; Lee and Harris, 2008). It is reported that angiotensin II and thromboxane mediates the venule-dependent arteriolar vasoconstriction. Only a few reports on DR have been published using this model.

db/db mice

The db/db (*Leprd*) mouse is deficient for the leptin receptor and spontaneously develops type 2 diabetes associated with obesity at 4-8 weeks of age. Six-month-old db/db mice have been shown to exhibit early features of DR, such as pericyte and endothelial cell loss (Midena et al., 1989), BM thickening (Clements et al., 1998) and increased blood flow (Tadayoni et al., 2003) in the retina. By

15 months, these mice demonstrated distinct DR symptoms, including blood-retinal barrier (BRB) breakdown, pericyte loss, neuroretinal apoptosis, glial reactivation, possible neovascularization and acellular capillaries in the retina (Cheung et al., 2005). Reports show that db/db mice have some specific advantages for the study of the retinal microcirculation. These mice are darkly pigmented and hence the fluorescence of labeled elements circulating in the choroid can be masked by their pigment epithelium.

Ins2^{Akita} mice

The *Ins2^{Akita}* (Akita) mouse contains a dominant point mutation in the gene encoding insulin-2 that induces spontaneous type 1 diabetes in the B6 mouse strain. Heterozygous male Akita mice develop hyperglycemia as early as 4 weeks of age. After 12 weeks of hyperglycemia, the retinas were found to have increased vascular permeability, degenerate capillaries and alterations in the morphology of astrocytes and microglia with increasing duration of diabetes. Furthermore, increased retinal cell apoptosis was identified, accompanied by a distinct reduction in the thickness of the inner plexiform layer and inner nuclear layer (Barber et al., 2005). These mice showed loss of RGCs from the peripheral retina within the first 3 months of diabetes, as well as marked alterations to the morphology of surviving cells (Gastinger et al., 2008). Another study demonstrated a significant reduction in the total number of cholinergic and dopaminergic amacrine cells in Akita mice (Gastinger et al., 2006). This model has been studied up to 15-18 months of age, but mortality increased significantly towards the end of this duration (T. S. K., unpublished data).

This model could be useful for exploring the molecular mechanisms involved in the initiation and early progression of DR. In addition, it is an ideal model for evaluating the neuroprotective effects of drugs because of the quantifiable loss of RGCs in a relatively short time (4-5 months). Use of this model is increasing among researchers interested in DR.

Mouse models of proliferative retinopathy

Despite many attempts to establish suitable models that accurately reflect the features of late human DR, very few animal models develop severe DR with large areas of retinal non-perfusion and neovascularization. Hence, researchers have turned to non-diabetic animals to study proliferative retinopathy. The widely used rodent models of proliferative retinopathy to study neovascularization are those in which VEGF is overexpressed in photoreceptors (such as Kimba mice and mice in which VEGF expression is driven by the rhodopsin promoter) (Okamoto et al., 1997; Tee et al., 2008), mice overexpressing insulin-like growth factor-1 (IGF1) in the retina (Ruberte et al., 2004), oxygen-induced retinopathy (Gole et al., 1990; Holmes and Duffner, 1996) and branch retinal vein occlusion (Zhang et al., 2007). These models induce possible neovascularization and retinal detachment in the absence of diabetes. For example, transgenic mice with increased expression of IGF1 in the retina exhibit signs of diabetes-like eye conditions, including pericyte loss, retinal capillary BM thickening, venule dilatation, IRMAs and possibly retinal neovascularization (Ruberte et al., 2004). Kimba mice, generated through photoreceptor-specific overexpression of human VEGF165 protein, demonstrate retinal neovascular changes, increased permeability, pericyte and

endothelial cell loss, vessel tortuosity, leukostasis, and capillary blockage, dropout and hemorrhage (Tee et al., 2008).

Akimba mice

A potential transgenic mouse model of DR named 'Akimba' has been developed recently by crossing Kimba mice with Akita mice (Rakoczy et al., 2010). These mice showed key features exhibited by the parent strains: overexpression of VEGF (as in Kimba mice) and spontaneous type 1 diabetes (as in Akita mice). In this model, advanced retinal lesions resembling human PDR were caused in diabetic mice by 'on top' alternative approaches (e.g. neovascularization due to transgenic expression of human VEGF165 in photoreceptors). Interestingly, vascular changes in this model include microaneurysms, increased prevalence of leaky capillaries, venous beading, tortuous vessels, capillary dropout and attenuation of vessels. Fig. 5 shows a comparison of retinal fundus, FFA and histology of Kimba, Akita and Akimba mice.

The neovascular changes observed in the Akimba mouse are not due to long-term hyperglycemia, as in human DR, but are due to the presence of the human *VEGF165* transgene in the photoreceptors. Hence, this model might not be suitable for studying the etiology of DR or the factors associated with the development of pre-retinal neovascularization. The mechanisms of enhanced vascular and neuronal retinal changes, neuroprotection and the role of inflammation in Akimba mice have not yet been studied. This newly developed model is an important tool to improve our understanding of the complex processes underlying the progression of DR.

Galactosemia models

Galactosemia animal models of DR are induced in rodents by supplementing with a diet containing 30-50% galactose. Galactose-fed rats and mice develop retinal microangiopathy that resembles the early stages of DR (Kern and Engerman, 1995), including pericyte loss, acellular capillaries, thickened retinal capillary BM, IRMAs and, in dogs, also microaneurysms and intra-retinal hemorrhages. Galactosemic mice showed increased retinal capillary width and microaneurysms at 8 months of age. Retinal microaneurysms, acellular capillaries, pericyte ghosts and capillary BM thickening became increasingly prevalent in mice on the 30% galactose diet for longer durations. The development of cataracts has been extensively probed using galactosemic rats and mice (Kern and Engerman, 1996a). Galactosemic rodents lack many of the metabolic abnormalities that are characteristic of diabetes, but develop many of the retinal complications of diabetes and thus can be considered a valuable tool for the study of the pathogenesis of diabetic complications.

Large animal models of DR

Diabetic dogs

Retinopathy that develops in spontaneously or experimentally induced diabetic dogs is morphologically similar to human DR (Kern and Mohr, 2007; Kern, 2009; Zheng and Kern, 2010). Studies of retinopathy using dog models have used mostly type 1 diabetes induced by alloxan, STZ, growth hormone or pancreatectomy. Studies have also been carried out on long-term galactose-fed dogs (Engerman and Kern, 1984). Retinal lesions reported in diabetic dogs include microaneurysms, degenerate (acellular and non-

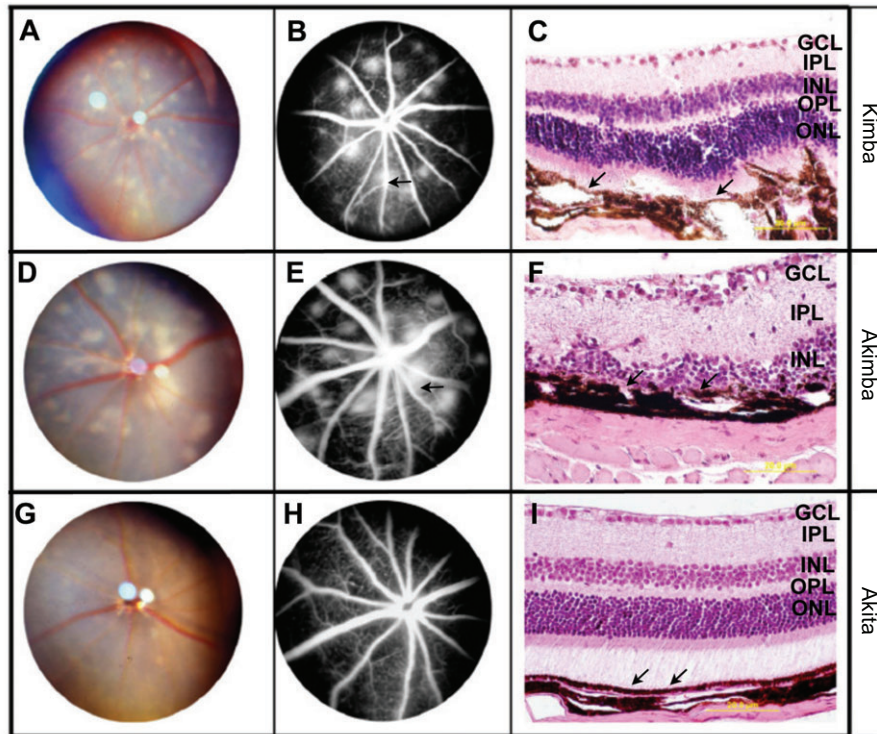


Fig. 5. Comparison of retinal fundus, FFA and histology of Kimba, Akimba and Akita mice. Fundus, FFA and retinal histology of Kimba (A-C), Akimba (D-F) and Akita (G-I) mice (courtesy of Lions Eye Institute, Perth, Australia). FFA shows the differences in retinal vasculature between Kimba (B), Akimba (E) and Akita (H) mice. Kimba (B) and Akimba (E) mice have foci of fluorescein leakages (arrows). Akita (H) mice have sharply defined retinal vessels and retinal capillary network with vessels radiating from the optic nerve head. Light micrographs of paraffin-embedded eyes of Kimba (C), Akimba (F) and Akita (I) mouse eyes show retinal histology. The sections were stained with H&E. Arrows in C and F point to breaks in the RPE cells of Kimba and Akimba mouse eyes, whereas arrows in (I) point to intact RPE cells in Akita eyes. Ganglion cell layer (GCL); inner nuclear layer (INL); inner plexiform layer (IPL); outer plexiform layer (OPL); and outer nuclear layer (ONL).

perfused) capillaries, pericyte loss, IRMAs, thicker capillary BM, and dot and blot hemorrhages (Gardiner et al., 1994; Kern and Engerman, 1996b). Similar to diabetic dogs, long-term galactose-fed galactosemic dogs have retinal lesions that resemble those seen in human DR (Takahashi et al., 1993; Kern and Engerman, 1996b; Kobayashi et al., 1998). Galactose-fed dogs have been shown to develop diabetes-like retinal vessel changes associated with both the early and moderately advanced stages of retinopathy. However, it is 3-5 years before severe retinopathy develops, accompanied by occasional intra-retinal neovascularization, in dog models (Engerman and Kramer, 1982; Engerman and Kern, 1984; Takahashi et al., 1992; Wallow and Engerman, 1997). In addition, the cost, lack of specific antibodies or molecular reagents, heightened ethical concerns and difficulty in maintenance mean that dog models are less useful than small animal models for the study of DR (Kobayashi et al., 1998).

Diabetic cats

Most studies of diabetic cats involve type 1 diabetes induced by STZ and pancreatectomy, with or without alloxan. Retinal lesions include capillary BM thickening, increased vessel tortuosity, capillary non-perfusion, microaneurysms, fluorescein leakage and possibly neovascularization (Mansour et al., 1990; Hatchell et al., 1995; Linsenmeier et al., 1998; Budzynski et al., 2005). Diabetic cats

develop only mild cataract, which allows the use of FFA and other *in vivo* measurements for years after the induction of diabetes (Salgado et al., 2000; Richter et al., 2002). Cats also exhibit retinal hypoxia in diabetes, which might result from capillary plugging or altered flow through microaneurysms (Linsenmeier et al., 1998). However, the cost, lack of specific antibodies or molecular biology reagents, and slow development of lesions has made cat models less suitable than rodent models for studies of DR.

Diabetic pigs

The structure of the retinal vascular system in pigs is very similar to that of humans, which makes them very useful for research on eye diseases. Pigs developed retinal capillary BM thickening with several ultrastructural features, such as lamellation and rarefaction within BM, as early as 18 weeks after STZ treatment (Lee et al., 2010). Another study of type 1 diabetic pigs reported the fairly rapid development of features characteristic of early retinal microvasculature changes (Hainsworth et al., 2002). Further studies using this model might improve our understanding of DR progression, and might provide an important platform for investigating new treatments that prove promising in small animal studies, before progressing to clinical trials in humans. The disadvantages of pig models include high cost, lack of specific molecular reagents and antibodies, difficulty in maintenance, and

the fact that the techniques for genetic characterization and manipulation are currently less precise than in small animal models.

Diabetic non-human primates

The structural similarity of primate eyes to human eyes makes them potential models for research on eye diseases. The main advantage of primate models over the other models discussed above is the presence of the macula, an important site of damage in DR. The most common primate models used for studies of DR include rhesus monkeys with diabetes induced by alloxan, STZ or total pancreatectomy. Cynomolgus monkeys (*Macaca fascicularis*) and obese rhesus monkeys (*Macaca mulatta*) that develop diabetes spontaneously have also been used. Evidence shows that retinopathy develops very slowly in diabetic non-human primates (as in humans). STZ- or pancreatectomy-induced diabetic monkeys studied for up to 15 years showed retinal ischemia and defects in the BRB and macula. However, these models lack vascular lesions observed in human DR (Tso et al., 1988). Studies of aged monkeys with spontaneous diabetes revealed IRMAs and macular edema. Microaneurysms were also associated with the areas of non-perfusion (Johnson et al., 2005). Investigations on type 2 diabetic primate models revealed hemorrhages, large areas of retinal capillary non-perfusion, microaneurysms, cotton wool spots, intra-retinal hemorrhages and hard exudates in the macula (Kim et al., 2004). To date, retinal neovascularization or neuronal degeneration has not been reported in diabetic non-human primates.

Despite the structural similarity of the eye in humans and non-human primates, these models are not always preferred over other animal models owing to several limitations. Non-human primates are difficult to manipulate genetically, and molecular reagents for experimental studies are lacking. They also have a longer gestational period and lower birth rates. Other disadvantages include slow progression of DR, increased maintenance costs and the ethical issues of using non-human primates as animal models.

Future directions

As outlined in this Perspective, animal models of DR are important tools that will continue to enhance our understanding of the pathogenesis of DR, as well as the development of novel therapeutic approaches. However, it is clear that there is still no perfect animal model that recapitulates all aspects of human DR. Although most of the models discussed have demonstrated the basic features of NPDR, the key feature of human PDR – pre-retinal neovascularization secondary to diabetes per se – is not recapitulated in diabetic animal models. Nevertheless, current clinical tools to treat existing PDR are relatively successful, and thus the focus of future research should be to inhibit development of the early stages of the retinopathy, thereby preventing the subsequent progression to advanced retinopathy. Notably, retinal thickening (consistent with DME) has been detected in a small number of available models such as monkeys. Vision loss or impairment is also now being studied in mice and rats using the optokinetic response (Thomas et al., 2010); investigating the cause of a diabetes-induced reduction in visual function might provide new insight into the causes of vision loss in diabetic humans.

Some investigators feel that a remaining challenge is to develop animal models that mimic the progression of DR from the non-

proliferative to proliferative stage, as in human DR (Rakoczy et al., 2010; Li et al., 2010). This might be accomplished by accelerating the retinopathy in models through superimposing a particular genetic or metabolic abnormality on top of diabetes (e.g. as in *eNOS^{-/-}* or Akimba mice). Nevertheless, the extent to which the underlying pathogenesis of retinopathy in such models mimics that of human DR will be an important issue to address.

The application of appropriate techniques for the characterization of DR is an equally important challenge for the future. The current methods for in vivo characterization of DR, including ERG, FP, FFA and OCT, can monitor progressive pathological changes (vascular and non-vascular) in the same animal over time, but new methods that provide additional information (e.g. retinal vascular caliber measurements) are needed.

In terms of clinical issues, many areas of uncertainty remain. It is well known that, despite controlling systemic risk factors of hyperglycemia and hypertension, many patients still progress to develop the vision-threatening stages of DR (either PDR or DME). The current standard of care is laser therapy, which is inherently destructive, associated with side effects and is ineffective in reversing visual loss. New approaches, including intraocular administration of anti-VEGF agents, are promising but have potential risks (Cheung et al., 2010). Thus, preventing DR and targeting early stages of DR is desirable. For example, the ability to provide effective topical therapies that target multiple pathways underlying retinal neovascularization and edema could further improve the current management strategies for DR. Development of such therapies requires substantial basic and experimental studies, for which appropriate animal models of DR are essential.

Conclusions

New and cost-effective therapies for treating DR, particularly the early stages of DR, are urgently needed. Animal models that develop lesions that are characteristic of human DR will continue to play a crucial role in understanding pathogenesis and for testing new therapies before clinical trials. The success of each study depends largely on the choice of the appropriate animal model, which, in turn, is driven by experimental design and focus. Key features to consider when choosing an animal model of DR include: the structural and biochemical features of the visual system compared with humans; the ability to perform genetic manipulations; the availability and cost of the model; methods available for disease characterization and validation; the time course of pathological changes; and ethical, moral and legal issues. Overcoming current challenges in DR research requires more extensive experiments with the most promising models, incorporating advanced techniques for more accurate phenotyping. This approach will ultimately help to identify the best systems to better understand, prevent and treat the human disease.

COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

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