

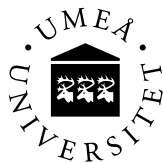
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# Superoxide Dismutase 1 and Cataract

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Cover: Mouse lens photographed in retro-illumination on a grid.  
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*"Pro captu lectoris habent sua fata libelli"*

*("According to the capabilities of the reader, books have their destiny")*

Terentianus Maurus, 2<sup>nd</sup> century roman grammarian

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# Abbreviations

AGE	Advanced glycation end-product
AR	Aldose reductase
DETA/NO	(Z)-1-[N-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (NO generator)
DHA	Dehydroascorbate (oxidation product of ascorbate)
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
LDCL	Lucigenin-derived chemiluminescence
LDH	Lactate dehydrogenase
L-NAME	N-nitro-L-arginine methyl ester (NOS inhibitor)
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide (coenzyme)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP <sup>+</sup>	Nicotinamide adenine dinucleotide phosphate (coenzyme)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NF-κB	Nuclear factor-kappa B (transcription factor)
NOS I	Neuronal nitric oxide synthase (nNOS)
NOS II	Inducible nitric oxide synthase (iNOS)
NOS III	Endothelial nitric oxide synthase (eNOS)
NO <sub>x</sub>	Nitric oxide end-products
PSSG	Protein-thiol mixed disulfides
PSSP	Protein-protein disulfides
RAGE	Receptor for advanced glycation end-products
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD1	Copper-zinc superoxide dismutase (CuZnSOD)
SOD2	Manganese superoxide dismutase (MnSOD)
SOD3	Extracellular superoxide dismutase (ECSOD)
STZ	Streptozotocin
TRx	Thioredoxin
TTase	Thioltransferase, (glutaredoxin)
UV	Ultraviolet (as in UV radiation)

# Chemical Formulas

$\text{Cu}^+$	Copper ion (oxidation number I)
$\text{Cu}^{2+}$	Copper ion (oxidation number II)
$\text{Fe}$	Iron (ground state)
$\text{Fe}^{2+}$	Ferrous ion (oxidation number II)
$\text{Fe}^{3+}$	Ferric ion (oxidation number III)
$\text{H}_2\text{O}_2$	Hydrogen peroxide
$\text{NaHCO}_3$	Sodium bicarbonate
$-\text{NH}_2$	Amino group
$\text{NO}\cdot$	Nitric oxide
$\text{NO}_2^-$	Nitrite
$\text{NO}_3^-$	Nitrate
$\text{O}_2$	Molecular oxygen
$\text{O}_2^{\cdot-}$	Superoxide radical
$\text{OH}\cdot$	Hydroxyl radical
$\text{ONOO}^-$	Peroxynitrite
$^{86}\text{Rb}$	Rubidium isotope
$-\text{SH}$	Sulfhydryl group (thiol group)

# Original Articles

This thesis is based on the following original articles which are referred to in the text by their roman numerals.

- I. Olofsson, E.M.; Marklund, S.L.; Karlsson, K.; Brännström, T.; Behndig, A. ***In vitro* glucose-induced cataract in copper-zinc superoxide dismutase null mice.** *Experimental Eye Research*. 81:639-646; 2005.
- II. Olofsson, E.M.; Marklund, S.L.; Behndig, A. **Glucose-induced cataract in CuZn-SOD null lenses: An effect of nitric oxide?** *Free Radical Biology & Medicine*. 42:1098-1105; 2007.
- III. Olofsson, E.M.; Marklund, S.L.; Behndig, A. **Enhanced diabetes-induced cataract in copper-zinc superoxide dismutase null mice.** In press, *Investigative Ophthalmology & Visual Science*, 2009.
- IV. Olofsson, E.M.; Marklund, S.L.; Behndig, A. **Enhanced age-related cataract in copper-zinc superoxide dismutase null mice.** Manuscript.

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# Abstract

Light and oxygen generate harmful reactive oxygen species (ROS) in the lens, causing biochemical changes that gradually disarrange the lens fibres resulting in light scattering and loss of transparency. In the healthy eye, this chronic exposure to oxidative stress may lead to age-related cataract. However, there are also some conditions that accelerate cataract formation, such as diabetes mellitus, in which increased glucose levels may contribute to increased generation of ROS.

The superoxide dismutases (SOD) participate in the defence against ROS by catalysing the dismutation of superoxide radicals. The main SOD isoenzyme in the lens is copper-zinc superoxide dismutase (SOD1). The aim of this thesis was to explore if this antioxidant enzyme is important for the protection against age-related and diabetes-induced cataract development.

Lenses from wild-type mice and mice lacking SOD1 were incubated in high levels of glucose *in vitro* and their transparency and damage evaluated daily. Also, the impact of nitric oxide was studied by adding a nitric oxide synthase inhibitor. Furthermore, *in vivo* cataract formation in relation to the oxidative status of the lens was evaluated in streptozotocin-induced diabetic mice as well as in non-diabetic mice of both genotypes. Finally, the spontaneous age-related cataract development was studied in both genotypes.

*In vitro*, the SOD1 null lenses showed increased levels of superoxide radicals and developed dense nuclear lens opacities upon exposure to high levels of glucose. They also showed increased lens leakage of lactate dehydrogenase, reduced transport function across cell membranes, and increased water contents. However, the lens damage and cataract formation were eliminated when the synthesis of nitric oxide was inhibited. This indicates that both superoxide and nitric oxide have important roles in glucose-induced cataract development possibly through their reaction with each other which generates the highly reactive peroxynitrite.

*In vivo*, both the SOD1 null and the wild-type mice showed cortical cataract changes after 8 weeks of diabetes, although the SOD1 null mice showed a more pronounced cataract formation than the wild-type mice in relation to the level of hyperglycaemia. As cataract formation was accentuated the lenses showed diminishing levels of glutathione but increasing amounts of protein carbonyls, suggesting a reduced lens antioxidant capacity as well as increased lens protein oxidation. Non-diabetic young (18 weeks of age) SOD1 null mice did not show any signs of cataract. At 1 year of age they had developed some cortical lens obscurity as compared to the wild-type mice which did not show equivalent changes until 2 years of age.

The results presented in this thesis show that SOD1 null mice are more prone to develop diabetes-induced and age-related cataract than wild-type mice. The findings thus further endorse the importance of oxidative stress as a contributor to cataract development and indicate that both superoxide and nitric oxide may be damaging to the lens. I therefore conclude that the antioxidant enzyme SOD1 is important for the protection against cataract.

Keywords: cataract, diabetes mellitus, nitric oxide, SOD1 null mice, superoxide, superoxide dismutase 1.

# Introduction

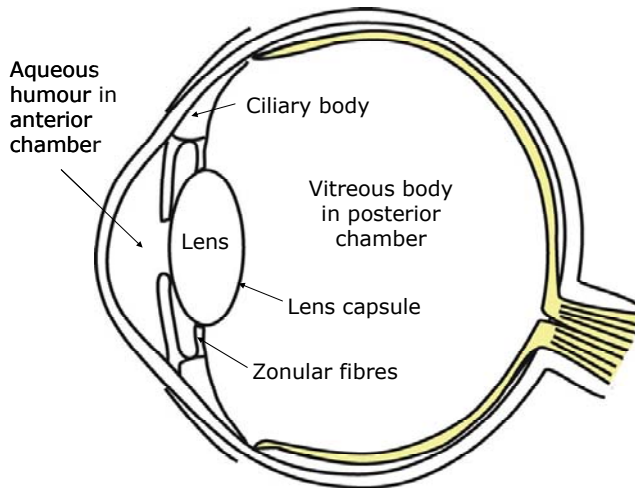
## **Cataract – the Leading Cause of Visual Disability**

Animals and humans are all dependent on the clarity of the eye's lens to maintain a good vision throughout life. The development of lens obscurity, known as cataract, thus leads to visual disability due to an increased scattering and a reduced transit of light, causing glare and a loss of visual acuity. The only treatment for cataract is surgical removal and replacement of the lens and even though the availability and quality of cataract surgery has improved vastly during the last decades, cataract is still the leading cause of visual disability, having rendered over 17 million people blind in the world today [1]. Unfortunately, cataract surgery is costly and not without risks as sight-threatening post-operative infections and other complications may occur. Also, as the artificial lens lacks some of the properties of the natural lens, such as accommodative power, visual correction (glasses or contact lenses) must be worn even after surgery. It is thus desirable to find alternative treatment strategies for cataract, preferably by delaying or even inhibiting the development of lens opacities as this would benefit both patients and health-economies the most. However, the mechanisms behind cataract formation must first be unravelled before a prophylactic or remedial agent can be achieved.

### ***Structure and Function of the Lens***

The lens provides  $1/3$  of the refractive power of the eye and its flexibility enables the eye to switch between sight at near and far distances [2]. The lens thus focuses the light on the retina allowing us to see sharp images of what we are looking at. The lens is surrounded by a capsule that is suspended from the ciliary body by the zonular fibres (Fig. 1). The anterior side of the lens is covered by a single layer of cuboidal epithelial cells which are metabolically highly active. At the lens equator these cells elongate, start to lose their organelles, and differentiate into lens fibres, pushing the older lens fibres towards the centre of the lens. The innermost part of the lens, the nucleus, is thus composed of the eldest fibres that have lost the ability to synthesize proteins or replace damaged ones, whereas the surrounding younger lens fibres constitute the lens cortex of which the most superficial fibres are still nucleated and metabolically active (Fig. 2). As fibre maturation continuous throughout life, the nucleus keeps expanding, displacing the oldest lens fibres further away from the protection provided

by the constituents of the epithelial cells, rendering the lens susceptible to damage [3].



**Figure 1.** The anatomy of the eye.

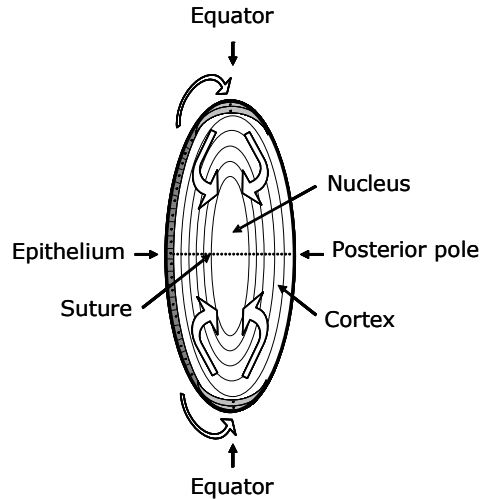
Drawing by Camilla Henriksson. Printed with permission from Berit Byström.

The lens fibres are mainly composed of proteins of which 90% are crystallins [4]. Actually, the protein concentration in the lens can be as high as 33-35% of its wet weight [4, 5] and lens transparency and refractive property are due to the high concentration and uniform arrangement of these crystallins forming stable and durable structures. The lack of protein turn-over may however lead to the accumulation of damaged components which gradually disarranges the lens fibres causing light scattering and loss of transparency. Lens crystallins are divided into two families:  $\alpha$ -crystallins and  $\beta\gamma$ -crystallins of which the  $\alpha$ -crystallins in addition to being important for lens structure, also act as protein chaperones, thus protecting  $\beta\gamma$ -crystallins from damage [6]. This is important since  $\beta\gamma$ -crystallins are susceptible to modifications due to their high contents of thiol groups. These thiols need to be in the reduced state to maintain lens clarity [5], which will be explained further on.

The lens has no blood supply and nutrients and oxygen must therefore be provided by diffusion from the surroundings. The anterior part of the lens where most of the metabolism takes place is bathed in the aqueous humour secreted by the ciliary body whereas the posterior part of the lens is adjacent to the vitreous body in the posterior chamber of the eye (Fig. 1). Glucose is

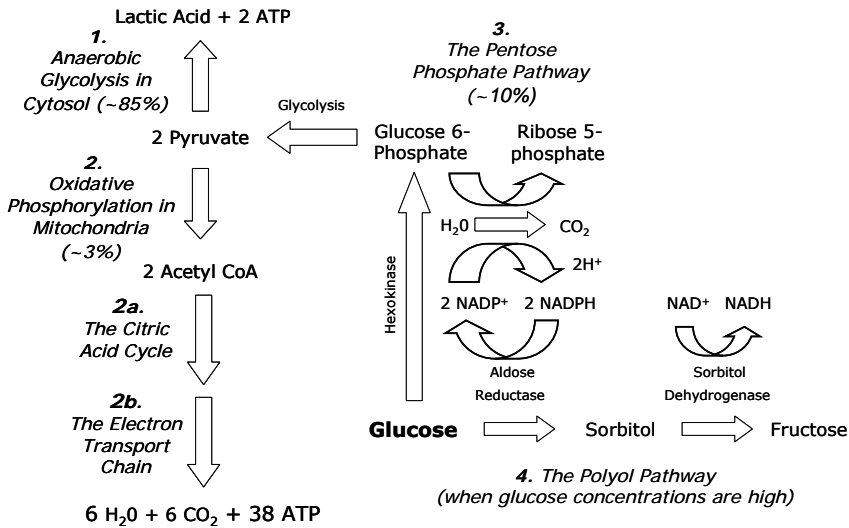
the principal fuel for lens metabolism and it is derived from the aqueous humour where its levels mirror those in the blood [7]. Glucose uptake into the lens is facilitated by transport proteins localized mainly in the lens epithelial cells but also in the cortical fibre cells [8]. It is however, only in the former, and possibly in the not yet fully differentiated fibre cells of the outermost cortex, that glucose is metabolised by oxidative phosphorylation [9]. The cells in the rest of the lens lack mitochondria and thus process glucose by anaerobic glycolysis. Due to the high concentration of fibre cells, this is the main energy source, supplying about 85% of the lens's total requirement of ATP [9]. Apart from energy production, glucose is also used by the pentose phosphate pathway (also known as the hexose monophosphate shunt) to provide nicotinamide-adenine dinucleotide phosphate (NADPH) [9], an electron carrier and an important cofactor in reactions involving lens protein protection. In addition, this cofactor is used by another glucose-processing pathway, the polyol pathway, in which glucose is converted to sorbitol and fructose in subsequent steps [10]. Hence, NADPH connects these two pathways which operate at an increased rate in high glucose concentrations (Fig. 3).

The epithelial cells contain  $\text{Na}^+\text{-K}^+\text{-ATPases}$  that maintain osmotic balance through the regulation of intracellular water volume by transporting  $\text{Na}^+$  out of and  $\text{K}^+$  into the cell [4]. Hence, a loss of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  function will eventually result in swelling which is therefore often used as a parameter to quantify lens damage. Apart from uptake by epithelial cells, it appears as if water and small molecules circulate in the narrow extracellular spaces within the lens, causing a flow of solvents from the anterior and posterior poles to the equator while providing nutrients for the fibre cells. Ion channels and gap junctions permeable to ions and molecules of up to 1 kD enable this current to also cross cell membranes in order to pass through the lens [11]. Glucose may, for instance, be taken up by fibre cells from the extracellular



**Figure 2.** The structure of the lens in cross-section. Arrows show the migration of the epithelial cells to the equator where they differentiate into lens fibre cells while pushing the older fibres into the nucleus.

space within the lens cortex, in addition to uptake by epithelial cells directly from the aqueous humour [12]. It has actually been proposed that restricted diffusion in aged lenses contributes to a loss of lens clarity possibly through modifications of the gap junctions [11].



**Figure 3.** Four possible pathways of glucose metabolism in the lens. Glucose is converted by the enzyme Hexokinase to glucose 6-phosphate. Energy (ATP) is then produced either by anaerobic glycolysis in the cytosol (1) or by oxidative phosphorylation in the mitochondria (2). Glucose 6-phosphate may also be used for NADPH production (3). An excess of glucose activates the polyol pathway (4). The contribution of each pathway in the lens is indicated in brackets.

### *To summarize:*

The lens is a highly organized organ that is specialized in transmitting and focusing light on the retina, thereby providing us with a most valued sense. In addition to being part of a complex visual system, the lens is nevertheless an elegantly simple tissue, made of only two types of cells. However, damage to these cells give rise to changes that gradually may compromise lens function resulting in obscured vision. It is therefore important that harmful factors are identified so that their impact on the lens may be minimized.

### ***Risk Factors for Cataract Development***

The most important risk factor for cataract development is age [13] although many other factors, environmental as well as endogenous, seem to enhance the formation of lens opacities. Exposure to some known risk factors, such as smoking [14, 15], is preventable whereas others are more difficult to influence. For instance, in the daytime, the lens is constantly exposed to visible light and ultraviolet radiation, both of which are associated with cataract development [16-18]. Of course, risk factors may be interconnected. For instance, lens susceptibility to UV radiation is found to increase with age due to the decrease in free UV filters. These are tryptophan-derived molecules that protect the lens and the retina from photodamage by absorbing UV light [19]. Intriguingly, in aged lenses, these filters may actually have opposed effects as they may bond to lens proteins and act as photosensitizers, thereby exerting damage rather than protection [20].

In addition to light, the lens is constantly exposed to oxygen ( $O_2$ ) supplied by the aqueous humour. Paradoxically, as well as being essential for energy production in the mitochondria of cells,  $O_2$  is also highly toxic and known to induce cataract formation [21, 22]. Different studies have shown varying levels of oxygen tension in the aqueous humour ranging from 23 to 72 mmHg and an oxygen tension gradient that decreases from the corneal endothelium to the lens [23-27]. The oxygen tension levels in the lens itself range from 10 to 28 mmHg with the highest levels found directly behind the anterior capsule and the lowest in the nucleus and posterior pole where they are close to that of the adjacent part of the vitreous [24, 27]. Hence the oxygen tension in the lens is kept low as it is in other cells of the body to reduce the oxygen toxicity.

### ***Cataract in Diabetes Mellitus***

Cataract is also associated with many clinical disorders. For instance, patients suffering from diabetes mellitus endure a higher risk of visual loss due to lens opacities than others [28]. Apart from cataract, diabetic patients also develop other eye-related complications, such as retinopathy, and are subject to more complications at cataract surgery [29]. It is therefore even more important to find ways to retain lens transparency in these patients.

Diabetes mellitus is a chronic disease characterized by derangements in carbohydrate, fat, and protein metabolism derived from either a failed production of insulin in the pancreas (diabetes mellitus type 1) or an increased resistance to insulin in muscle and adipose tissue causing a

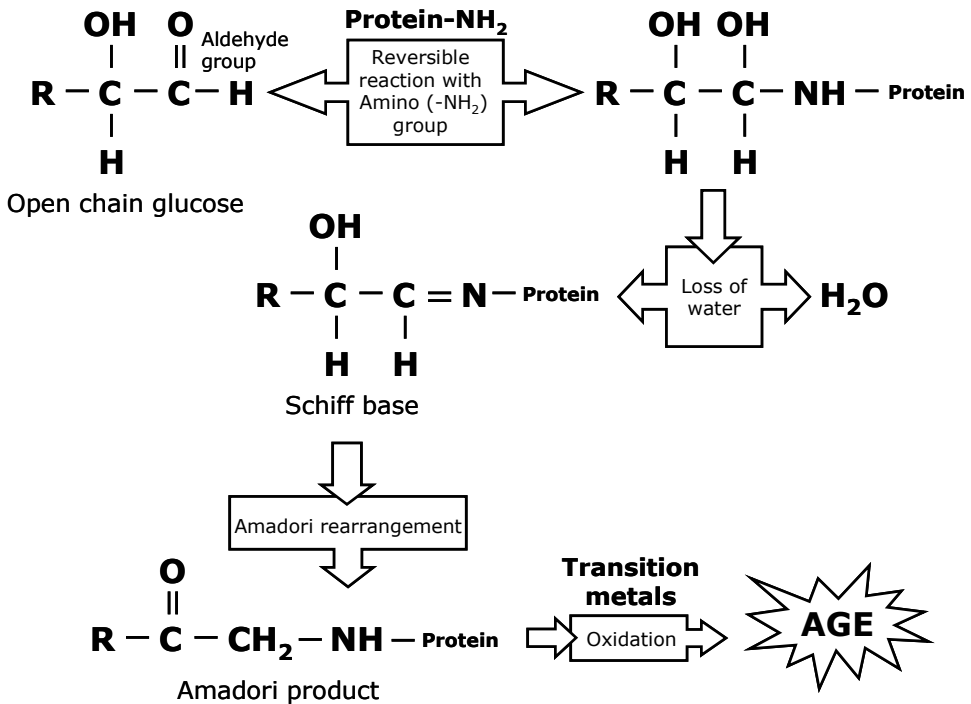
defective glucose uptake (diabetes mellitus type 2). Both variants cause elevated blood glucose levels, which is harmful to many tissues, including the eye.

Apart from hyperglycaemia, increased levels of glucose are also found in the aqueous humour which may affect the lens in many ways. For instance, diabetics have a thicker lens, are more myopic, and suffer episodes of transient refractive changes which correlate to changes in blood glucose levels. These changes may be due to direct osmotic effects caused by differences in glucose levels between the aqueous and the lens, resulting in dehydration or hydration of the lens tissue [4].

Lens swelling due to osmotic stress imposed by sorbitol accumulation within the lens was for a long time suggested to explain diabetes-induced cataract formation. It was proposed that hyperglycaemia would lead to a flux of sugars through the polyol pathway, in which the substrate would be converted into its sugar alcohol by the enzyme aldose reductase (AR) (Fig. 3). Glucose would thus be reduced to sorbitol, thereby inducing osmosis and lens swelling [4, 30]. This theory has however been questioned, and even contradicted by many, and it is now clear that osmotic stress is not the sole mediator of diabetes-induced cataract. AR may nonetheless participate in cataractogenesis in other ways [10, 31], as will be discussed further on.

The relevance of Maillard browning (after the French scientist Louis Camille Maillard who studied the reactions of carbohydrates and amino acids in the beginning of the 20<sup>th</sup> century) in cataract formation, especially in diabetes mellitus, is now becoming apparent. In these non-enzymatic reactions, sugars react with amino groups of proteins, yielding Schiff bases. These products are initially reversible but may slowly rearrange into more stable Amadori intermediates. Finally, oxidative reactions promoted by metal ions lead to irreversible protein-bound compounds collectively termed advanced glycation end-products (AGEs) (Fig. 4). The accumulation of AGEs causes fluorescence, browning, and protein cross-linking through the alterations of the lens fibre structure. In addition, the glycation of  $\alpha$ -crystallins and of other protective proteins and enzymes render the lens even more susceptible to further damage [32, 33]. An alternative and possibly even faster pathway for AGE formation is by primary oxidation of sugars followed by reactions with proteins. AGE formation is thus accelerated in the presence of oxygen and further enhanced by transition metals. These glycooxidation reactions generate highly reactive dicarbonyl compounds that rapidly may cause AGEs and subsequent protein cross-linking [34].

Although the development of diabetes-induced cataract is now considered to be of multifactorial origin, different contributors may be interconnected. For instance, in hyperglycaemic conditions, the sorbitol generated due to an excess of glucose, may be further turned into fructose, which is known to be a more potent glycation agent than glucose itself. The flux of glucose through the polyol pathway could thus increase AGE formation in the lens [31].



**Figure 4.** Non-enzymatic reactions of glucose with proteins which generate advanced glycation end-products (AGEs).

*To summarize:*

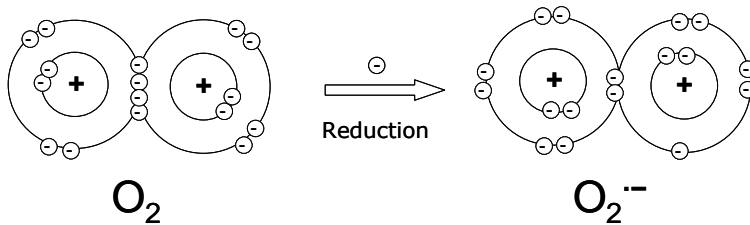
The mechanisms behind age-related and diabetes-induced cataract are not clear, but a complex model involving many contributing risk factors is beginning to evolve. UV radiation, smoking, diabetes, and of course oxygen are all connected as they all induce oxidative stress. The impact of oxygen-derived toxicity on the lens as a cause of damage and cataract formation has therefore been in focus for quite some time now and has grown into a



widespread research field [35-37]. I have in the following section aimed to cover the chemistry of some of these oxidative reactions that may occur in the lens with special attention to those that are most relevant for this thesis.

### **Oxidative stress – the Paradox of Life**

Fortunately, the oxygen in the air around us reacts sluggishly with most other compounds. However, the addition of an extra, unpaired electron (reduction) to the oxygen molecule generates the superoxide radical ( $O_2^{\cdot-}$ ) which weakens the oxygen-oxygen bond rendering it slightly more reactive (Fig. 5).



**Figure 5.** The reduction of ground-state oxygen ( $O_2$ ) generates the superoxide radical ( $O_2^{\cdot-}$ ).

Species that contain one or more unpaired electrons and are capable of independent existence are known as free radicals [34]. The reactivity of free radicals varies widely and some non-radical oxygen-derived compounds are in fact more reactive than the oxygen free radicals. The collective term, reactive oxygen species (ROS) is therefore usually instead used as it includes both the oxygen free radicals and some non-radical derivatives of  $O_2$ .

### ***Reactive Oxygen Species in the Lens***

ROS affecting the lens may arise both in the surroundings of the lens and inside its cellular compartments. In the lens epithelium and in the not yet fully differentiated fibre cells within the outermost cortex, where mitochondria are still present, ROS are mainly generated as by-products of energy production in the electron transport chain localized in the inner mitochondrial membrane. As electrons are passed through the chain, some

escape into the mitochondrial matrix and the intermembrane space where they react with  $O_2$ , producing  $O_2^{\cdot-}$  [3]. It has actually been proposed that ageing may be the result of progressive ROS-derived damage to mitochondria which has given rise to the free radical theory of ageing [38].

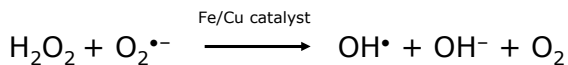
ROS also arise in the lens as a result of normal enzymatic function. For instance, low levels of  $O_2^{\cdot-}$  radicals are constitutively produced by the NADPH-oxidases and have been proposed to participate in the regulation of lens growth and function through redox-signalling [39]. The production of  $O_2^{\cdot-}$  by this specific enzyme can however be greatly enhanced in response to injuring external influences such as UV radiation [40]. Likewise, other lens enzymes such as xanthine oxidase [41] and inducible nitric oxide synthase (NOS II) [42], produce large amounts of  $O_2^{\cdot-}$  when activated by damaging insults [34, 43]. Enhanced xanthine oxidase activity has also been found to increase the generation of  $O_2^{\cdot-}$  in diabetic animal models [44].

Moreover, ROS can be generated in the lens by autoxidations which occur when  $O_2$  reacts with other compounds. These reactions which usually do not happen due to the low reactivity of  $O_2$  are, however, facilitated in the presence of transition metals. Since these metal ions are enabled to accept and donate electrons they are highly reactive and therefore involved in catalysing many reactions. The most well known is the Fenton reaction in which ferrous ions ( $Fe^{2+}$ ) and hydrogen peroxide ( $H_2O_2$ ) generate the highly reactive hydroxyl radical ( $OH^{\cdot}$ ). This reaction is also facilitated by other transition metals such as  $Cu^+$ :

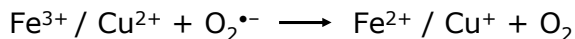


$H_2O_2$  has for long been known to participate in cataract development [35, 45, 46]. It is in itself generally poorly reactive, but since it is small and neutral it can cross membranes and therefore enter the lens from the aqueous humour. Once inside the lens, reactions with other compounds may be facilitated by the presence of transition metals [47]. The generation of external  $H_2O_2$  must therefore be considered to be important for cataract development, which also is supported by the finding that the aqueous humour surrounding opaque lenses contains about three times the normal concentrations of  $H_2O_2$  [46].

In the presence of both  $O_2^{\cdot-}$  and  $H_2O_2$ , the production of  $OH^{\cdot}$  may be accelerated through the superoxide-assisted Fenton reaction also known as the Haber-Weiss reaction [34]:



The role of  $\text{O}_2^{\bullet-}$  in this reaction is to recycle  $\text{Fe}^{3+}/\text{Cu}^{2+}$  into  $\text{Fe}^{2+}/\text{Cu}^+$  by donating an electron to the former:

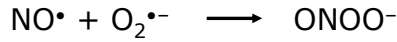


The reduced transition metal ion, which is then again available for the reaction with  $\text{H}_2\text{O}_2$ , keeps Fenton chemistry going, thus maintaining the generation of  $\text{OH}^\bullet$ . The relevance of the Haber-Weiss reaction may however be limited in normal physiological conditions due to its low rate constant in comparison to the fast dismutation of  $\text{O}_2^{\bullet-}$ . Instead, another ROS, singlet  $\text{O}_2$ , may participate in these reactions [48]. Singlet  $\text{O}_2$  is generated as excitation energy from an illuminated photosensitizer is transferred to  $\text{O}_2$ , converting it into a more oxidizing species. For instance, UV-A radiation (320-400 nm) is thought to induce singlet  $\text{O}_2$  formation in the lens through photosensitization reactions [11, 20, 49]. Other ROS, such as  $\text{O}_2^{\bullet-}$ , may also be produced by these photochemical reactions, especially in older lenses in which chromophores have been found to accumulate [49].

Reactions generating  $\text{OH}^\bullet$  may be facilitated in diabetic lenses in which  $\text{Cu}^+$  has been found to be accumulated [50].  $\text{Cu}^+$  may thus contribute to accelerated cataract formation in diabetic patients by inducing a Fenton's-type reaction resulting in harmful ROS generation. The surplus of glucose and the presence of  $\text{Cu}^+$  in diabetes may also facilitate autoxidation reactions between glucose and  $\text{O}_2$  leading to  $\text{O}_2^{\bullet-}$  production [51]. In addition,  $\text{O}_2^{\bullet-}$  is produced by metal catalysed oxidative reactions with glycated proteins [52] while simultaneously accelerating the formation of stable AGEs [33] (Fig. 4). Oxidative stress is also induced as AGEs interact with their specific cell surface receptors (RAGEs) [53]. As many receptor-mediated signalling pathways are suggested to be initiated by the binding of AGEs to RAGEs, these interactions may be responsible for many complications seen in diabetic patients and have also been implicated in cataract formation [54].

Reactions involving  $\text{O}_2^{\bullet-}$  must occur at its site of origin due to its negative charge which renders it unwilling to cross membranes. Once generated,  $\text{O}_2^{\bullet-}$  reacts readily with certain iron-sulphur clusters as well as with radicals but less readily with other compounds. The combination with the radical nitric

oxide (NO<sup>•</sup>) is especially favoured [55] and gives rise to the highly toxic peroxynitrite (ONOO<sup>-</sup>).



NO<sup>•</sup> can cross membranes and diffuse between and within cells freely. It has multiple important roles as a signalling molecule, for example in neurotransmission [56], vasodilatation [57], and host defence [58]. NO<sup>•</sup> is synthesized from L-arginine by three different isoforms of NOS. The constitutively NOS I and NOS III produce short-lasting and usually small amounts of NO<sup>•</sup> whereas the inducible form NOS II produces large amounts of NO<sup>•</sup>, as well as O<sub>2</sub><sup>•-</sup> which was mentioned earlier, when activated by endotoxins or cytokines [59]. An excessive production of NO<sup>•</sup>, which is known to be cytotoxic, has been implicated in cataract formation [60-64], especially in patients showing accelerated development of lens opacities induced by cigarette smoking [65], lens trauma [66], and diabetes mellitus [67]. There are though some contradicting assumptions regarding the role of NO<sup>•</sup> in diabetic complications. In the eye, NO<sup>•</sup> levels and NOS expression have for instance been found to be increased in diabetic retinopathy by some [68-70], whereas others propose that NO<sup>•</sup> levels and NOS expression decrease in response to hyperglycaemia [71, 72]. However, NO<sup>•</sup> is a very complex molecule due to its dual nature: cytoprotection versus cytotoxicity [73], and its production is tightly regulated. Yet, if synthesis of both NO<sup>•</sup> and O<sub>2</sub><sup>•-</sup> are enhanced, as suggested to be in diabetes [74], NO<sup>•</sup> may be consumed and ONOO<sup>-</sup> instead formed [75]. Furthermore, NOS expression and NO<sup>•</sup> production may depend on the degree of lens opacification [64], as well as on the severity of the diabetes-induced damage [68]. These mechanisms may thus partly explain why NO<sup>•</sup> findings differ between studies.

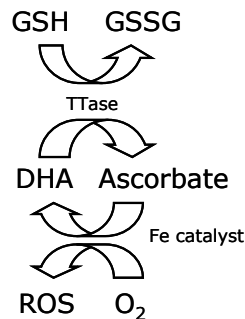
*To summarize:*

Many of the deleterious effects of O<sub>2</sub><sup>•-</sup> may be due to secondary reactions leading to more damaging compounds such as OH<sup>•</sup> and ONOO<sup>-</sup>. As soon as these are formed, they react quickly with molecules in their immediate vicinity, causing damage through peroxidation of lipids in cell membranes, DNA-strand breaks, and oxidation and nitration of proteins [34]. These deleterious effects may be augmented in diabetes mellitus due to increased levels of both O<sub>2</sub><sup>•-</sup> and NO<sup>•</sup>. It is thus important that the lens is protected by efficient antioxidative systems.

### ***Antioxidative Defence in the Lens***

Any substance that delays, prevents or removes oxidative damage to a target molecule is known as an antioxidant [34]. The lens contains many different antioxidative systems which consist of both first line defence and second line repair mechanisms. Two protective systems have already been mentioned: the  $\alpha$ -crystallins which are stress-induced chaperones that aid the refolding or removal of denatured proteins thus preventing their aggregation in the lens, and the free UV filters which absorb damaging UV radiation. However, the latter may in aged lenses become protein-bound and paradoxically initiate oxidative damage through ROS generated by photosensitization reactions [11, 20, 49].

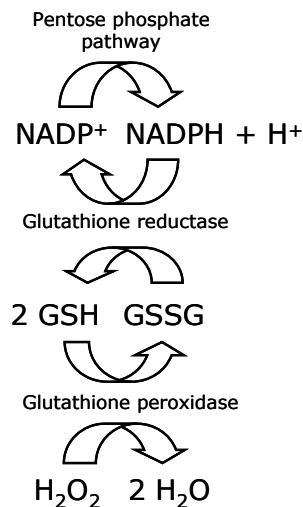
Ascorbate (vitamin C) is another compound with contradicting properties as it may exert both antioxidative as well as pro-oxidative effects [76]. As it is a powerful reducing agent it scavenges many ROS [77], after which the resulting oxidation end-product, dehydroascorbate (DHA), is recycled back to ascorbate. However, as ascorbate also reduces  $\text{Fe}^{3+}/\text{Cu}^{2+}$  to  $\text{Fe}^{2+}/\text{Cu}^{+}$ , it may in the presence of transition metals facilitate Fenton reactions and auto-oxidations, which, in turn, may result in ROS production (Fig. 6). Furthermore, DHA may cause glycation of proteins [78], leading to AGE formation which also contributes to increased oxidative stress. This may be especially prominent in diabetic and aged lenses, in which the impaired antioxidant capacity hinders the recycling of DHA to ascorbate [79]. The role of ascorbate may thus depend on the oxidative status and the presence of free transition metal ions [77]. Nevertheless, ascorbate has mainly been considered to be an antioxidant in the lens [80-82]. This is supported by the fact that the eye has an ability to concentrate ascorbate, resulting in more than 10 times higher levels in the lens and aqueous humour than in plasma [83]. These high levels are found especially in the aqueous of diurnal animals [84, 85] suggesting that ascorbate exerts an important protection against lens damage caused by UV radiation from the sunlight [86]. However, the ascorbate levels decrease during normal ageing [83], and are also compromised by cigarette smoking and diabetes [34], resulting in increased susceptibility to oxidative damage which may lead to the development of cataract.



**Figure 6.** Recycling of ascorbate. See text or page 6 for abbreviations.

Ascorbate is also involved in the recycling of  $\alpha$ -tocopherol (vitamin E), an antioxidant that is especially important for the prevention of lipid peroxidation in biological membranes [34]. This lipid-soluble antioxidant scavenges singlet  $O_2$  and  $OH\cdot$  radicals, but probably more importantly, also removes lipid peroxy radicals that are derived from peroxidation of polyunsaturated fatty acids [77]. Once generated, these radicals react with other polyunsaturated fatty acids, creating new lipid radicals, thereby propagating chain reactions. By inhibiting this process,  $\alpha$ -tocopherol appears to stabilize lens cell membranes and may thus protect against lens damage and cataract development [87-89]. However,  $\alpha$ -tocopherol is, like ascorbate, consumed by its antioxidant activity and an  $\alpha$ -tocopheryl radical arises in its place. This new radical may in itself impose pro-oxidant effects unless it is quickly recycled. Another similarity to ascorbate is that  $\alpha$ -tocopherol may reduce  $Fe^{3+}/Cu^{2+}$  into  $Fe^{2+}/Cu^+$ , thereby promoting transition metal catalysed oxidative reactions [34].

Glutathione (GSH) is another antioxidant that is abundant in the lens. Actually, the lens and the liver show the highest tissue concentrations of GSH, with varying levels in different species [5]. GSH is a low molecular mass tripeptide synthesized in the cytoplasm of lens epithelial cells from L-glutamate, L-cysteine and glycine [34], which results in a concentration gradient with decreasing levels towards the lens centre [5]. GSH contributes to the redox state of the lens in different ways. It participates in the recycling of ascorbate from DHA (Fig. 6), scavenges ROS and reactive nitrogen species (RNS), and prevents protein-SH oxidation and cross-linking [90]. Reduced GSH is also a cofactor for the glutathione peroxidases (GPx) which catalyse the degradation of  $H_2O_2$  into water. This reaction generates oxidized GSH (GSSG) which may then be recycled back to reduced GSH by glutathione reductase (GR), thereby keeping the lens bulk of GSH in the reduced state (Fig. 7). There is an age-dependent decrease in lens GSH contents, which may be due to slowed synthesis and inefficient recycling [5], rendering the lens susceptible to oxidative damage. GSSG that is not recycled may instead form protein-thiol mixed disulfides (PSSG) with lens proteins [91]. Contrary to GSH,



**Figure 7.** Recycling of glutathione.

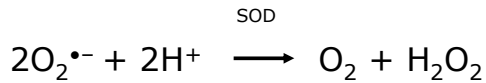
PSSG accumulate in the lens with age [92], especially in cataractous lenses and after exposure to different oxidants causing oxidative stress [5], thus suggesting oxidation as a unique process causing protein thiolation. Interestingly, lens PSSG levels have been shown to decrease in diabetic conditions which may be attributed to an increased lens leakage of thiolated proteins [93]. Thiolation changes protein conformation which can lead to further modifications resulting in protein-protein disulfide cross-linking, followed by protein aggregation, light scattering, and finally lens opacity. The lens has however a repair system, the GSH-dependent enzyme thioltransferase (TTase), also called glutaredoxin [94] which can dethiolate these proteins before they are further modified. In addition to keeping proteins in the reduced state, thioltransferase also catalyses the reaction between GSH and DHA, which enhances ascorbate recycling [5] thereby replenishing the antioxidative capacity of the lens (Fig. 6). If further modifications of proteins still happen despite the presence of thioltransferase, the NADPH-dependent thioredoxin/thioredoxin reductase system may dissociate disulfide bridges and hinder cross-linking [95, 96].

*To summarize:*

The lens contains several antioxidants that participate in both the first line of defence against oxidative stress but also in the reparation of modified proteins. The different antioxidative systems in the lens also seem to interact. For instance, GSH participates in the recycling of both  $\alpha$ -tocopherol and ascorbate. The latter may also, in itself, enhance  $\alpha$ -tocopherol regeneration [89]. Furthermore, ascorbate regeneration by GSH is accelerated by thioltransferase. Likewise, the enzymatic degradation of  $H_2O_2$  is a complex interplay between different systems. It may be degraded in the lens by the glutathione peroxidases, catalase, and the peroxiredoxins [97-100]. In addition to all of the here mentioned antioxidants, several other compounds serve as scavengers of ROS in the lens. In this thesis, I have focused on the antioxidant enzymes mainly responsible for the removal of  $O_2^{\cdot-}$  radicals, namely the superoxide dismutases.

***The Superoxide Dismutases***

The  $O_2^{\cdot-}$  theory of  $O_2$  toxicity proposes that  $O_2^{\cdot-}$  is a major factor in  $O_2$  toxicity. Hence, it is of great importance that generated  $O_2^{\cdot-}$  radicals can be detoxified by the superoxide dismutases (SODs) [101]. These antioxidant enzymes catalyse the dismutation of  $O_2^{\cdot-}$  radicals by oxidizing one molecule of  $O_2^{\cdot-}$  to  $O_2$  while reducing another to  $H_2O_2$ .



$\text{O}_2^{\bullet-}$  do not cross membranes and must therefore be scavenged by the SOD isoenzyme that is present in the cellular compartment in which the radicals originate. Presently three different human isoenzymes expressed in different locations are known. Copper-zinc SOD (CuZnSOD or SOD1) was the one first discovered and it was originally named haemocuprein as it was isolated as a copper containing protein in bovine blood [34]. As it later also was isolated from other tissues and discovered to contain zinc as well as copper, its function was suggested to be as a metal storage. However, it was renamed when its catalytic activity was found which also opened the door to the  $\text{O}_2^{\bullet-}$  theory of  $\text{O}_2$  toxicity [102].

SOD1 is a homodimer of about 32 kD in which the two subunits each contain an active site consisting of copper and zinc ions. The copper ions participate directly in the dismutation reactions by alternatively oxidizing and reducing  $\text{O}_2^{\bullet-}$  molecules. The zinc ions and the presence of disulfide bonds contribute to the stability and render the enzyme unusually resistant to inactivation. There are, however, some known inhibitors of enzyme activity, such as  $\text{H}_2\text{O}_2$  and cyanide [34].

Although the dismutation of  $\text{O}_2^{\bullet-}$  can occur spontaneously, the reaction is greatly accelerated in the presence of a SOD. The activity of SOD1 is enhanced by an electrostatic field gradient caused by negatively charged residues on the surface and positively charged pathways leading to the active sites in the enzyme. This drives the negatively charged  $\text{O}_2^{\bullet-}$  molecules to the catalytic binding sites at the bottom of the channels [103]. Apart from  $\text{O}_2^{\bullet-}$  dismutation, SOD1 is recently shown to also catalyse the nitration of tyrosine residues by ONOO<sup>-</sup> [104].

SOD1 is mainly located in the cytosol, but some is present also in the nucleus, mitochondrial intermembrane space, and the peroxisomes [105, 106]. Another isoenzyme, manganese SOD (MnSOD or SOD2) is found largely in the mitochondrial matrix [107]. SOD2 is thus highly important in the defence against endogenously produced  $\text{O}_2^{\bullet-}$  derived from the electron transport chain in the mitochondrial inner membrane. Apart from the function to dismutate  $\text{O}_2^{\bullet-}$ , SOD1 and SOD2 are completely different. SOD2 has for instance another ion, manganese, at its active site. It also consists of



four subunits and is generally unstable, but is, unlike SOD<sub>1</sub>, not inactivated by cyanide.

The third human SOD is extracellular SOD (ECSOD or SOD<sub>3</sub>) which, as its name implies, catalyses the dismutation of O<sub>2</sub><sup>-</sup> in the extracellular fluids [108] where levels of other antioxidative enzymes usually are low. This isoenzyme is a tetrameric glycoprotein with a molecular mass of about 135 kD [109]. After secretion to the extracellular space, it attaches to heparan sulphate proteoglycans on cell surfaces [110, 111]. Its affinity to these glycosaminoglycans is due to a carboxy-terminally located heparin-binding domain. The middle portion of the SOD<sub>3</sub> sequence shows, however, a strong homology to the part of the SOD<sub>1</sub> sequence that defines the active site [112]. SOD<sub>3</sub> thus also contains copper and zinc ions and is inhibited by the same substances as SOD<sub>1</sub>.

The relative importance of the different SOD isoenzymes can be explored by studying animal models in which the genes responsible for SOD expression are either overexpressed or knocked out. For instance, mice totally lacking SOD<sub>2</sub> die shortly after birth [113], whereas heterozygous mice in which some SOD<sub>2</sub> activity remain, survive but show increased mitochondrial oxidative damage as they age [114]. Contrary, SOD<sub>3</sub> knockout mice appear healthy but are more susceptible to oxidative stress and die prematurely if exposed to high oxygen tension [115]. Regarding the eye, these mice show decreased corneal endothelial cell viability, indicating that SOD<sub>3</sub> may participate in protecting the cornea against oxidative stress [116, 117]. Although SOD<sub>1</sub> knockout mice also appear healthy, they have reduced body weights and shortened lifespans [118], the latter being mainly due to their increased risk of developing hepatocellular carcinomas [119]. SOD<sub>1</sub> null mice also show signs of premature ageing such as neurological degenerations [120], accelerated skeletal muscle atrophy [121], hearing loss [122], age-related macular degeneration [123, 124], and age-related cataract formation [125], as well as reduced female fertility [126].

The activities of the three SOD isoenzymes have previously been studied in our laboratory and found to differ in various organs as well as in the eye itself. Regarding SOD<sub>1</sub>, activity has been found to be highest in the retina (11,000 ± 1,200 U/g) with similar levels to that found in many other human tissues (14,500 ± 7,000 U/g) [127] apart from the liver, which shows the highest, and the kidney, which shows the second highest activities in the body [128, 129]. The cornea, sclera, and especially the lens showed less SOD<sub>1</sub>, whereas activities were almost negligible in the aqueous humour and vitreous body [127]. Although the lens had a low SOD<sub>1</sub> activity (970 ± 310 U/g) compared to the other tissues examined, this isoenzyme accounted for

virtually all of the total SOD activity in the lens, which is in accordance with the almost entirely cellular and mitochondrial free character of this tissue [127]. Interestingly, the accumulation of inactivated SOD1 protein was higher in the lens than in any other part of the human eye [127], suggesting that SOD1 specifically participates in processes occurring in the lens. Supporting this is the reported loss of human lens SOD1 activity during ageing [130-133] and cataract development [130, 134-136]. In fact, in our laboratory, younger lenses were found to have higher SOD1 activities (2,800 U/g) [137]. Alas, since young human lenses are difficult to obtain, these results came from analyses performed on murine lenses.

*To Summarize:*

Although the SODs have for a long time been known to provide a first line of defence against oxidative stress in the lens [138, 139], they have been given more attention in recent years [125, 132, 135, 137, 140]. There are however some contradicting results regarding the protective effects of SOD1 [141], especially in diabetic patients in which both decreased and increased activities of SOD1 have been reported in blood samples [134, 142] and lenses [135, 136]. Apparently, the role of SOD1 in the lens needs to be clarified, especially in diabetic lenses in which the mechanisms behind the loss of transparency seem to be more complex.

This thesis appraises the results of four studies on cataract development in lenses from mice lacking SOD1. Previously, one study, conducted in our laboratory, found accelerated photochemical-induced cataract in lenses from these knockouts *in vitro* [137]. To further evaluate the role of SOD1 in other cataract models, I have, in two of the studies presented in this thesis, instead explored glucose-induced cataract development in lenses from SOD1 null mice. In the following paper, I delved into this further by assessing diabetes-induced cataract development *in vivo* and searched for oxidative mechanisms behind the lens changes found in this genotype. Finally, as another research group has shown enhanced age-related cataract development in SOD1 null mice [125], I supplemented their study by comparing the cataract development in different age-groups of SOD1 null mice and investigated if these lenses showed signs of oxidative stress.

# Aims

The aims of this thesis are outlined below.

- To investigate the role of SOD1 in glucose-induced cataract *in vitro*.
- To investigate if  $O_2^{\cdot-}$  and  $NO^{\cdot}$  may participate in the formation of *in vitro* glucose-induced cataract.
- To evaluate the effect of diabetes mellitus on cataract formation in SOD1 null mice *in vivo*.
- To evaluate age-related cataract formation in different age-groups of SOD1 null mice *in vivo*.
- To assess the oxidative states of lenses from SOD1 null and wild-type mice showing varying levels of cataract formation.

# Methods

This summary will describe the methods used in this thesis. However, specific details regarding materials and equipment are given in the respective paper.

## **SOD1 Knockout Mice**

### ***Ethical Considerations and Animal Care***

The use of laboratory animals in all four studies was approved by the regional animal ethical committee (Umeå djurförsöksetiska nämnd). The mice were handled according to the animal welfare legislation of Sweden and the ARVO *Statement for the Use of Animals in Ophthalmic and Vision Research* was followed.

The mice were kept in ventilated plastic (Makrolon) cages and had free access to water and standard mouse chow (SDS standard CRM(E) diet). The animal facility provided a controlled thermo-neutral environment (20 – 21°C) with a light intensity of around 50 lux for 12 hours, followed by complete darkness for 12 hours. The mice were group-housed and thereby enabled to socialise with mice of the same gender. They were provided with paper rolls for enrichment and handling was kept to a minimum to reduce stress. Each animal was identified by an ear clipping performed after weaning. At the same time a blood sample was drawn from the tip of the tail for SOD1 analysis. All animals were killed by cervical dislocation at the appropriate time according to the protocols of the studies.

### ***Generation and Breeding of SOD1 Null Mice***

Mice lacking SOD1 originated from another laboratory [120]. To develop these mice, the entire coding sequence of the mouse SOD1 gene had been deleted by homologous recombination in a 129/Sv mouse embryonic stem cell line. The mutant embryonic stem cells were injected into embryos from CD-1 donor mice. The resulting chimeras were then bred to CD-1 females, resulting in heterozygotes that were crossed to obtain SOD1 null mice.

In our laboratory the original 129/CD1 mice were backcrossed 10-20 times into C57BL/6J mice to get congenicity. Since female SOD1 null mice show reduced fertility [126], breeding was accomplished with heterozygous mice

which generated homozygous SOD1 null (SOD1<sup>-/-</sup>), heterozygous SOD1 null (SOD1<sup>+/-</sup>), and wild-type littermates (SOD1<sup>+/+</sup>). SOD1 null and wild-type control mice derived from the same breeding were thus used in the following studies.

### ***Mouse Genotyping by SOD1 Activity Analysis***

The genotype of each mouse was determined by analysing its SOD enzyme activity in the blood [143]. To prepare the blood samples, they were first haemolysed and centrifuged, after which the supernatants were collected and assayed at pH 9.5 to reduce the risk of non-enzymatic O<sub>2</sub><sup>-</sup> dismutation. Thereafter, KO<sub>2</sub> was added to generate O<sub>2</sub><sup>-</sup> radicals and the resulting loss of absorbance, due to the disproportionation of KO<sub>2</sub> by SOD, was observed in a spectrophotometer at 250 nm. Since the content of SOD3 in plasma is low, and erythrocytes lack SOD2, the resulting activity in the blood samples represented that of SOD1. Catalase was also added to reduce the risk of SOD1 inactivation by H<sub>2</sub>O<sub>2</sub>. Finally, the activity calculated was related to the haemoglobin concentration in the sample.

## **Study Designs and Preparation of Lenses**

### ***Papers I and II***

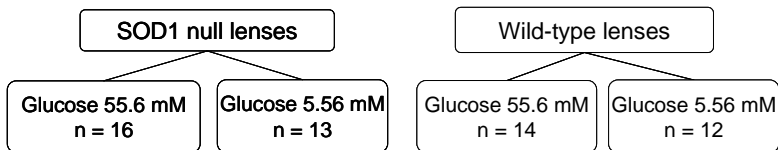
A summary is given in Table 1 regarding the number and the male/female distribution of the animals used in studies I and II.

**TABLE 1.** Number and Mean Ages  $\pm$  Standard Deviations in Weeks, of Animals Used in Papers I and II.

		SOD1 null mice		Wild-type mice	
Paper I	Males	11		15	
	Females	10		6	
	Total	21		21	
	Age	19 $\pm$ 8		20 $\pm$ 10	
Paper II		Part 1	Part 2	Part 1	Part 2
	Males	12	11	11	15
	Females	11	9	25	5
	Total	23	20	36	20
	Age	30 $\pm$ 5	27 $\pm$ 7	25 $\pm$ 5	26 $\pm$ 7

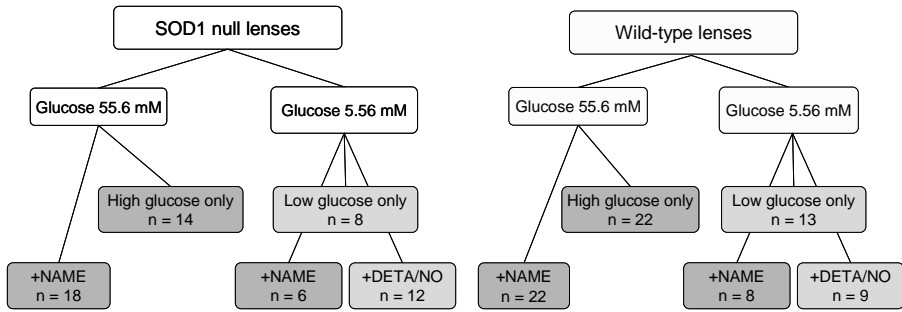
In papers I and II, the lenses were removed from the eye globes and immediately placed in Medium 199 supplemented with  $\text{NaHCO}_3$ , HEPES (pH 7.2) and benzylpenicillin. All lenses were incubated at 37 °C in a humidified atmosphere of 95% air/5%  $\text{CO}_2$  in 24-well culture plates. After 24 hours (day 1), the clarity of the lenses and their membrane leakage were evaluated. Cloudy lenses as well as lenses with a high permeability during the first 24 hours were presumed to have been damaged during dissection and therefore discarded. The different treatments were started on day 1 and the culture medium was changed daily throughout the whole incubation time of six days.

In paper I, one lens out of a pair from one eye, was kept in medium with 55.6 mM glucose [144] (10 times the normal concentration in the medium), while the other lens was kept in medium with a normal (5.56 mM) glucose concentration (Fig. 8). The osmolalities of the high and normal glucose media were 348 and 306 mOsm/kg, respectively.



**Figure 8.** Study design and number of lenses (n) in paper I.

In paper II, the study was performed in two parts. In the first part, the lenses from the SOD1 null and the wild-type mice were randomized into six different treatment groups and incubated either in medium with normal (5.56 mM) or high (55.6 mM) levels of glucose. The arginine analogue, N-nitro-L-arginine methyl ester (L-NAME), was added to some of the lenses to inhibit NO formation by blocking the active sites of all three NOSs (Fig. 9). In the second part of study II, four different treatment groups were used. One lens, out of a pair from one eye, was incubated in medium with normal glucose levels (5.56 mM) with the addition of the NO-donor (Z)-1-[N-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA/NO), whereas the other lens was kept in medium with normal levels of glucose only (Fig. 9).



**Figure 9.** Study design and number of lenses (n) in paper II. Dark grey = lenses in part 1 of study II. Light grey = lenses in part 2 of study II.

### ***Papers III and IV***

A summary is given in Table 2 regarding the number and the male/female distribution of the animals used in studies III and IV.

**TABLE 2.** Number and Mean Ages  $\pm$  Standard Deviation in Weeks, of Animals Used in Papers III and IV.

		SOD1 null mice			Wild-type mice		
Treatment group		STZ	Control*		STZ	Control*	
Paper III	Males	6	3		11	7	
	Females	7	4		9	7	
	Total	13	7		20	14	
	Age	15 $\pm$ 5	18 $\pm$ 3		17 $\pm$ 5	19 $\pm$ 4	
Age group (years)		0	1	2	0	1	2
Paper IV	Males	3	7	7	6	10	3
	Females	4	5	2	5	5	8
	Total	7	12	9	11	15	11
	Age	18 $\pm$ 3	54 $\pm$ 12	98 $\pm$ 6	18 $\pm$ 4	52 $\pm$ 11	106 $\pm$ 5

\* Age-matched mice from these control groups were used as young adult (0-year old) control mice in paper IV.

In paper III, SOD1 null and wild-type mice were given either intraperitoneal injections of streptozotocin (STZ) (60 mg/kg or 52 mg/kg respectively) or the STZ vehicle only (100 mM sterile citrate buffer pH 4.2) for 5 consecutive days (Table 2). Mean plasma glucose for each mouse was calculated from blood samples drawn from the tail and immediately analysed, at 2 and 8 weeks after the initial injection. Mouse weight was recorded before the first injection, after 2, and after 8 weeks. Urine samples were collected only at 8 weeks and used for U-glucose analysis on a 6-level scale; 0 mmol/l; 1 – 5.5 mmol/l; 6 – 14 mmol/l; 15 – 28 mmol/l; 29 – 55 mmol/l; and > 55 mmol/l. The mice were non-fasting and unanaesthetized at the time of plasma and urine sampling. The animals were killed and the lenses dissected after 8 weeks of diabetes. In paper IV, lenses from healthy SOD1 null and wild-type mice of different age-groups (Table 2) were dissected. In both studies III and IV, the lenses were immediately weighed, photographed in retroillumination, and thereafter homogenized and lysed in 500 µl of ice-cold phosphate-buffered saline by Ultraturrax, followed by sonication. The samples were then centrifuged for 10 minutes at 20,000 x g after which the lens supernatants were stored at -80 °C until further analysed.

## **Cataract Quantification**

Cataract development was followed by comparing photographs of the lenses taken daily with a digital camera in a dissection microscope at 16x magnification. The lenses were photographed on top of a steel grid in retroillumination from a light table, and the degree of lens opacification was quantified from the photographs both by a digital image analysing program and by subjective evaluation.

### ***Digital Image Analysis (Papers I, II, III and IV)***

Using a digital image analysing program (the public domain NIH Image program for papers I and II, whereas Image J, a public domain Java image processing program for papers III and IV, since the latter was more compatible with the operating system of the computer), the central circular 1 mm<sup>2</sup> area of the lens on a 256-level grey scale photograph was marked. The standard deviation (SD) and the mean density (MD) in NIH Image, or mean grey value (MG) in Image J, were obtained for each pixel within the marked area. In a clear lens, most marked pixels are black or white due to the pattern of the steel grid underneath the transparent lens, rendering the SD correspondingly high. The maximal SD was established by photographing



the grid through the medium but without a lens, which yielded a SD of 53.89. Development of cortical cataract will render more grey (and less black/white) pixels and thereby lower the SD. As a darker central area develops in a nuclear cataract, the SD will again increase due to an increasing difference between dark and light pixels. The MD or MG is therefore used to compensate for this effect. The MD and MG, which are little affected by cortical cataract, will increase as a nuclear cataract develops making the pixels generally darker. The highest theoretical MD or MG (255) and the highest SD obtained for a clear lens in the material (48.48) were used to construct the two formulas presented below. The results obtained from the two formulas are comparable and presented in arbitrary units (au).

*For NIH Image;*

$$\text{Lens opacity in arbitrary units (au)} = \left(1 - \frac{SD}{48.5}\right) \times \left(\frac{MD}{255}\right) \times 100$$

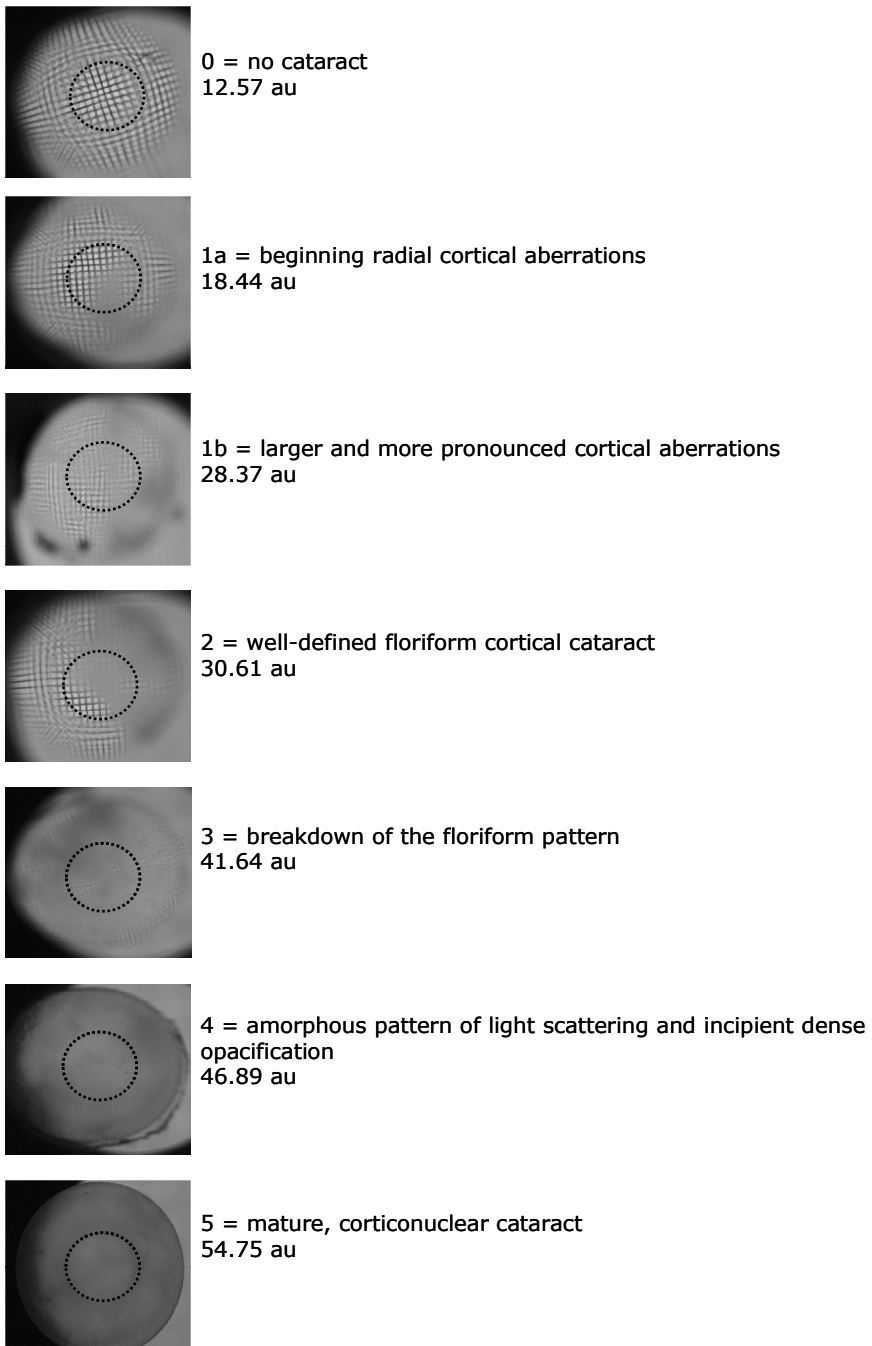
*For Image J;*

$$\text{Lens opacity in arbitrary units (au)} = \left(1 - \frac{SD}{48.5}\right) \times \left(\frac{255 - MG}{255}\right) \times 100$$

Lenses with more than 25 au on day 1 were excluded in papers I and II since the possibility of dissection damage could not be ruled out in these lenses.

### ***Subjective Staging (Papers I and II)***

The degree of cataract was subjectively assessed from the photographs by three independent evaluators and staged on a scale from 0 to 5 [145] (Fig. 10). The medians of the three evaluators' assessments were used for the staging of each lens. Lenses with a median of 1a or more on day 1 were excluded as they were presumed to have been damaged during dissection.



**Figure 10.** Subjective cataract quantification on a scale from 0 to 5 of lenses photographed on a grid in retroillumination. The cataract was also assessed by digital evaluation in arbitrary units (au) of the opacity in the central 1 mm<sup>2</sup> of each lens (area marked on each picture).

## **Parameters of Lens Damage**

### ***Lens Water Contents (Papers I and II)***

The water contents of the lenses were assessed as a marker of lens damage and swelling. The lenses were weighed before and after drying at 60 °C for 24 hours and the differences used to calculate the percentages of lens water.

### ***<sup>86</sup>Rubidium Uptake (Paper I) and [<sup>14</sup>C]Choline Uptake (Paper II)***

The transport function across cell membranes was examined by assessing the lens uptake of radiolabeled <sup>86</sup>Rb (paper I) or [<sup>14</sup>C]Choline (paper II). Whereas [<sup>14</sup>C]Choline transport is carrier mediated [146, 147], <sup>86</sup>Rb enters the lens through the Na<sup>+</sup>-K<sup>+</sup>-ATPase in which K<sup>+</sup> is replaced by Rb<sup>+</sup>. A reduced influx of these isotopes thus reflects damaged membrane functions which may lead to lens swelling. <sup>86</sup>Rb was primarily used because it had been the method of choice in our laboratory [137, 148]. However, the isotope was changed because choline transport in the lens appears to be more sensitive to oxidative stress than Na<sup>+</sup>-K<sup>+</sup>-ATPase [149].

After 6 days of incubation in the medium described above (see study design and preparation of lenses), the lenses were transferred into medium containing either 75 ng/ml <sup>86</sup>Rb for 10 minutes, or 5 μM [<sup>14</sup>C]Choline for 60 min. The initial specific activity of the <sup>86</sup>Rb was 62 MBq/mg, and of the [<sup>14</sup>C]Choline 14.7 MBq/mg. The lenses were then rinsed, dried at 60 °C overnight, and thereafter lysed in 10% trichloroacetic acid. After centrifugation, the supernatants were mixed with scintillation liquid and the radioactivity of both the samples and the incubation medium with respective isotope were determined in a scintillation counter. The ratio of the lens/medium radioactivity was finally calculated.

### ***Lactate Dehydrogenase Leakage (Papers I and II)***

The leakage of lactate dehydrogenase (LDH) out of the lens was determined to assess the lens damage [150]. As the culture medium was changed daily, the LDH activity in the medium reflected the lens leakage during the past 24 hours. For each day, the previous days' leakages were thus added to get the accumulated activity.

The LDH activity was analysed using a kit from Roche/Hitachi in which the LDH present in the medium catalysed the conversion of pyruvate to lactate

while also oxidizing NADH to NAD<sup>+</sup>. The rate of NADH decrease reflected the LDH activity in the sample and was determined spectrophotometrically at 340 nm. Fresh culture medium served as blank. If the LDH activity on day 1 exceeded 1.25 U/g of lens dry weight, the lens was presumed damaged during dissection and therefore excluded.

## **Analysis of Superoxide Radicals**

### ***Lucigenin-Derived Chemiluminescence (Papers I and II)***

Lucigenin-derived chemiluminescence (LDCL) was used to analyse the amount of superoxide anion radicals generated in the lenses. After 5 days of cultivation as described above, the lenses were transferred to vials containing medium with a low concentration of lucigenin and the resulting luminescence was measured in a luminometer during 60 seconds.

To validate the specificity of the LDCL assay for the superoxide radical, the ability of the SOD mimic, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) to quench the LDCL generated by xanthine oxidase in the presence of xanthine, was first shown. Secondly, as the low molecular weight MnTMPyP may penetrate into the lens and quench the internally formed superoxide, the LDCL generated by both wild-type and SOD1 null lenses before and after the addition of MnTMPyP was measured.

Although lucigenin at a low concentration is considered to be specific for O<sub>2</sub><sup>•-</sup> [151], it is a controversial method. For instance, it does not react directly with O<sub>2</sub><sup>•-</sup> and must first be reduced to the lucigenin cation radical. This radical will react with O<sub>2</sub><sup>•-</sup> to yield an unstable dioxetane which decomposes to the detected light-emitting species. However, LDCL may arise also in the presence of other reducing agents than O<sub>2</sub><sup>•-</sup> [152, 153], which renders the assay problematic. In addition, the radical intermediate may interfere with the assay by contributing to increased O<sub>2</sub><sup>•-</sup> generation by itself [154]. This confounding effect may though be less important in systems that produce significant amounts of O<sub>2</sub><sup>•-</sup> [151].

### **Analysis of Nitric Oxide (not included in papers)**

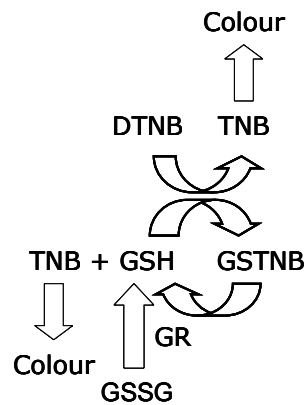
The amount of NO<sup>•</sup> in the lens medium was determined indirectly by measuring the amounts of stable NO<sup>•</sup> oxidation end-products (nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>)) generally named NO<sub>x</sub>, using an assay from R&D Systems Europe (19 Barton Lane, Abingdon Science Park, Abingdon, Oxon OX14

3NB, UK). After conversion of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  by nitrate reductase, the further reaction of  $\text{NO}_2^-$  with sulphanilamide in an acidic solution of N-(1-naphthyl)ethylenediamine (the Griess reaction) was measured spectrophotometrically at 540 nm. The amount of  $\text{NO}_2^-$  in each sample was then calculated from a standard curve.

## Parameters of Oxidative Stress

### *GSH Analysis (Papers III and IV)*

The contents of reduced (GSH) and oxidized (GSSG) glutathione were determined to assess the redox states of the lenses. The aqueous lens homogenate was deproteinated by adding 10% metaphosphoric acid and the clear supernatants collected after centrifugation. Then, to increase the pH in the samples, a freshly prepared solution of triethanolamine was added. After the above preparations, the samples were split onto two 96-well plates. The samples on the first plate were prepared further for GSSG analysis by blocking available sulfhydryl groups on GSH using 2-vinylpyridine. The remaining GSSG was then reduced to GSH by added glutathione reductase (GR) (Fig. 11). The second plate was left as it was for total GSH analysis. An assay kit from Cayman Chemical Company was thereafter used for the quantification of GSH. The sulfhydryl groups of the GSH in the samples on both plates were first allowed to react with DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) to produce the yellow coloured 5-thio-2-nitrobenzoic acid (TNB). The concomitantly produced mixed disulfide, GSTNB, was then recycled by added glutathione reductase (GR), producing more GSH and thus further colour change measured in a standard 96-plate reader at 405 nm (Fig. 11). The rate of colour produced was directly proportional to the concentration of GSH in the samples which was calculated from a standard curve. The GSSG concentrations derived from the first plate were finally subtracted from the total GSH concentrations measured on the second plate, to obtain the amounts of reduced GSH.



**Figure 11.** GSH recycling in the GSH and GSSG assay.

### ***Protein Oxidation (Papers III and IV)***

To further assess the redox states of the lenses, their contents of carbonyl groups were determined using an assay for immunoblotting (OxyBlot™ Protein Oxidation Detection Kit, Chemicon International).

The oxidative modification of protein side chains by ROS results in the formation of carbonyl groups (aldehydes and ketones). In the lens homogenates, these carbonyl groups were derivatized by 2,4-dinitrophenylhydrazon (DNPH) and then separated by 15% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and electroblotted onto polyvinylidene difluoride membranes (GE Healthcare). Non-specific sites on the blots were blocked with milk before the addition of antibodies. The primary antibody was a rabbit antibody specific to the DNP moiety of the proteins and the secondary a horseradish peroxidase-labelled goat anti-rabbit IgG antibody. After treating the blots with chemiluminescent reagent (ECL-Advance), the light was detected in a Chemidoc XRS imager and the resulting bands visualized using Quantity One software (Bio-Rad). The carbonyl content of each lens was quantified by comparing the signal intensities of its bands to the intensities derived from a standard curve, attained by running a series of dilutions of a protein mixture (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase and trypsin inhibitor) with known amounts of carbonylated proteins, on each blot. The specificity of the primary antibody for the DNP residue was also tested by substituting the DNPH solution for a negative-control solution. This prevented the reaction between the antigen and the primary antibody and subsequently rendered the blots blank.

### **Statistical Calculations**

SPSS statistical software was used for data analysis. Transformations were performed on data that violated the assumptions of parametric testing or the assumptions of the statistical model.

In papers I, II and IV, factorial analyses of variance with two or three independent factors (paper I: genotype and glucose, paper II: genotype, glucose and NO<sup>•</sup>, paper IV: genotype and age-group) were used to find their effects upon cataract formation and lens damage/oxidation, and how they may have interacted with one another. In paper III, linear regression models and analyses of covariance were instead performed to explore the effects of

hyperglycaemia and lens oxidation status on cataract formation in the two genotypes. Pearson's correlation coefficient was also given as a measure of the strength of the relationship between two variables.

Student's T-test or one-way analysis of variance followed by appropriate *post hoc* analysis was applied when testing the differences between means of two or more groups. Non-parametric testing using Mann-Whitney test or Kruskal-Wallis test followed by appropriate *post hoc* analysis was applied to data of two or more groups that could not be transformed to fulfil the assumptions mentioned above.

The level of significance for rejecting the null hypothesis of no treatment effect, was decided to be  $p \leq 0.05$ .

## Main Findings and Discussion

The main findings will here be presented and their contributions to the research field discussed. Parts of the results are summarized in tables and figures for easier comparisons between the studies. The rest of the results are presented in graphs and tables in respective paper. Some tables present data that were not included in the papers, but are here shown to emphasize or amplify the results and conclusions made in the four studies.

### Enhanced Glucose-Induced Cataract in SOD1 Null Lenses (Papers I and II)

#### *In Vitro* Cataract Development and Lens Damage

Upon exposure of the lenses to high levels of glucose (55.6 mM) *in vitro*, the lenses from the SOD1 null mice became more opaque and developed more signs of lens damage than the lenses from the wild-type mice (Table 3). This accelerated cataract development was evident in the SOD1 null lenses already on the second day upon subjective staging and on days 2-3 (see Fig. 2 in papers I and II) on digital quantification when the cataract seen was mainly cortical. By this time, these lenses also showed increased leakage of LDH (see Fig. 3 in papers I and II), indicating lens damage. Cataract formation kept progressing and reached the maximal subjective stage, corresponding to mature corticonuclear cataracts, on days 3-4 (papers I-II). Contrary, the digital cataract quantification system showed increasing values of lens opacification throughout the whole incubation period of 6 days, although the highest rate of cataract progression occurred during the first 2-3 days which corresponded to the peak of LDH leakage.

**TABLE 3.** Digital Cataract Quantification in Arbitrary Units (au) (Means  $\pm$  SD) and Subjective Staging (Medians and 1<sup>st</sup> to 3<sup>rd</sup> Interquartile Ranges) in Lenses Incubated *in Vitro* for 6 Days.

Paper	Treatment	Wild-type lenses		SOD1 null lenses	
		Au	Stage	au	Stage
I	Low glucose	29 $\pm$ 11	1b (1b-2)	27 $\pm$ 9	1b (1b-b)
	High glucose	34 $\pm$ 8	3 (1b-2)	46 $\pm$ 9	5 (5-5)
II	Low glucose	32 $\pm$ 5	3 (2-4)	30 $\pm$ 4	3 (3-4)
	High glucose	24 $\pm$ 12	2 (2-3)	46 $\pm$ 3	5 (5-5)
	Low glucose + L-NAME	18 $\pm$ 10	1b (1-2)	25 $\pm$ 14	1b (0-4)
	High glucose + L-NAME	21 $\pm$ 10	2 (1-2)	24 $\pm$ 7	1b (1-3)
	Low glucose + DETA/NO	34 $\pm$ 4	4 (4-5)	45 $\pm$ 7	5 (4-5)



The addition of the NOS inhibitor, L-NAME, attenuated the cataract formation and the damage in the glucose-exposed SOD1 null lenses (see Fig. 2 and 3 in paper II). Contrary, the addition of the NO generator, DETA/NO, enhanced cataract development and lens damage, especially in the SOD1 null lenses (see Fig. 4 and 5 in paper II). These findings imply that NO· may have contributed to the development of cataract.

### ***Superoxide and Nitric Oxide in Cataract Development***

In paper I, the lenses from the SOD1 null mice showed increased levels of O<sub>2</sub><sup>-</sup>, detected by LDCL, compared to the lenses from the wild-type mice. This was in agreement with a previous study from our laboratory [137] and also confirmed in paper II when comparing all of the SOD1 null lenses to all of the wild-type lenses regardless of treatment (Table 4).

**TABLE 4.** Interquartile Ranges and Maximal Values of LDCL Detected in all of the SOD1 null and Wild-Type Lenses Included in Paper II Regardless of Treatment (These calculations are not Shown in Paper II).

	1 <sup>st</sup> Quartile	Median	3 <sup>rd</sup> Quartile	Maximum
SOD1 null lenses* (n = 58)	0	1.4	2.3	4.5
Wild-type lenses (n = 73)	0	0	1.9	2.8

\*Significantly higher LDCL in the SOD1 null compared to the wild-type lenses (U = 1614, p = 0.01).

The O<sub>2</sub><sup>-</sup> levels were however not affected by the presence of glucose in the incubation media as neither the wild-type nor the SOD1 null lenses exposed to high levels of glucose for 5 days showed higher LDCL than their corresponding low-glucose controls (papers I and II). This was somewhat surprising as we had anticipated increased levels of O<sub>2</sub><sup>-</sup> in the opaque glucose-exposed SOD1 null lenses. Possibly, the O<sub>2</sub><sup>-</sup> radicals may have been consumed by the reactions with other ROS, keeping the LDCL unaffected. For instance, since O<sub>2</sub><sup>-</sup> and NO· are known to readily react with one another, their reaction may have been facilitated in the SOD1 null lenses exposed to high levels of glucose, and in that way caused cataract through the generation of ONOO<sup>-</sup>. Since this highly reactive compound does not readily oxidize lucigenin [155], the LDCL in the lenses would remain unaffected. However, by reducing the amount of available NO·, through the addition of L-NAME, as in paper II, the formation of ONOO<sup>-</sup> would be prevented and the LDCL instead increased. Alas, even though the LDCL seemed to be

elevated in the SOD1 null lenses exposed to both glucose and L-NAME, it was not statistically significant (see Table 1 in paper II).

Some difficulties were encountered when detecting the LDCL, especially in paper II, in which the  $O_2^{\cdot-}$  levels in the SOD1 null lenses were generally lower, and in the wild-type lenses generally higher than in the previous paper, leaving less differences between the treatments. Also, the LDCL showed large variances within the groups and for many lenses the LDCL was below the detection limit (Table 4). However, the short half-life of  $O_2^{\cdot-}$  renders its assessment difficult, which naturally may have contributed to the encountered problems. Another concern is that lucigenin in itself may generate  $O_2^{\cdot-}$  through autoxidation [154], although this interference is minimized by the use of a low concentration of lucigenin [151], as in our studies. Nevertheless, despite being debated, this method appears to be rather specific for  $O_2^{\cdot-}$  [155], also in our lens system. This was concluded since the addition of bovine SOD1, which is not taken up by cells during a short incubation time [152], did not quench the detectable LDCL in intact SOD1 null lenses [137], whereas the low molecular weight SOD mimic, MnTMPyP, which is more likely to penetrate into the lens, reduced the LDCL by 75% (paper I). These two findings indicate that the LDCL detected by our system was indeed generated by elevated  $O_2^{\cdot-}$  levels within the lens.

In papers I and II, the LDCL was only assessed at day 5. To investigate if the  $O_2^{\cdot-}$  generated was affected by the length of incubation, the LDCL was also measured in SOD1 null and wild-type lenses straight after, as well as after 2 and 5 days following dissection. Interestingly, the LDCL was reduced during the incubation time (Table 5). Possibly, the metabolism in the lens epithelium may be decreased due to damage caused by the *in vitro* environment, rendering a slowed endogenous mitochondrial production of  $O_2^{\cdot-}$  and thus lowered LDCL in both genotypes over time. In addition, gradual cataract development may obliterate photons generated by LDCL in the deeper cortical layers, and mask some of the luminescence. However, most importantly, this finding may indicate that other oxidants than  $O_2^{\cdot-}$  may be the cause of the lens damage seen during *in vitro* cultivation. I therefore again propose that the  $O_2^{\cdot-}$  radicals may be consumed by other ROS, yielding more toxic compounds which are not detected by LDCL. This may lead to a gradual lens damage and cataract formation, especially in lenses subjected to increased oxidative stress as demonstrated by the accelerated cataract development in the SOD1 null lenses exposed to high levels of glucose (papers I and II).

**TABLE 5.** Repeated Measurements of LDCL (Relative Light Units/ $\mu\text{g}$  Lens Wet Weight) in Lenses During *In Vitro* Cultivation (not Included in the Papers). Medians with 1<sup>st</sup> to 3<sup>rd</sup> Interquartile Ranges are Shown.

	Day 0	Day 2	Day 5
SOD1 null lenses (n = 11)	2.2 (2.1 – 2.7)	2.4 (2.1 – 2.6)	1.9 (1.8 – 2.1)* <sup>1</sup>
Wild-type lenses (n = 15)	1.2 (0 – 1.9)	1.9 (1.7 – 2.2)	0 (0 – 1.7)* <sup>2</sup>

\*<sup>1</sup>Lower LDCL than days 0 and 2 ( $H_2 = 10.2$ ,  $p = 0.006$ , *post hoc tests*  $p \leq 0.02$ ).

\*<sup>2</sup>Lower LDCL than day 2 ( $H_2 = 6.9$ ,  $p = 0.03$ , *post hoc test*  $p = 0.03$ ).

Besides the  $\text{O}_2^{\cdot-}$  levels, end-products of  $\text{NO}^{\cdot}$  ( $\text{NOx}$ ) were also examined to assess the effects of L-NAME and DETA/ $\text{NO}$  (data not included in the papers). As expected, the medium from the lenses exposed to normal glucose levels (5.56 mM) showed low concentrations of  $\text{NOx}$ . Interestingly, the high glucose medium (55.6 mM) from both the SOD1 null and the wild-type lenses showed increased levels, indicating elevated  $\text{NO}^{\cdot}$  generation. As suspected, the addition of L-NAME reduced the amount of detectable  $\text{NOx}$  to the basal levels, whereas the DETA/ $\text{NO}$  increased the levels greatly (Table 6).

**TABLE 6.** End-Products of  $\text{NO}$  ( $\text{NOx}$ ) Detected in Medium From Wild-Type and SOD1 Null Lenses, Exposed to High (55.6 mM) or Normal Levels (5.56 mM) of Glucose with and Without L-NAME or DETA/ $\text{NO}$  (Means  $\pm$  SD). Analysed on Lenses Included in Paper II but Data Previously not Presented.

Treatment	N	$\text{NOx}$ ( $\mu\text{mol/l}$ )	
		* <sup>1</sup>	* <sup>2</sup>
SOD1 null	8	9 $\pm$ 1	
Wild-Type	13	10 $\pm$ 2	
SOD1 null + Glucose + L-NAME	18	11 $\pm$ 4	
Wild-Type + Glucose + L-NAME	15	12 $\pm$ 9	
SOD1 null + Glucose	14	26 $\pm$ 11	
Wild-type + Glucose	14	27 $\pm$ 16	
SOD1 null + DETA/ $\text{NO}$	12		185 $\pm$ 23
Wild-Type + DETA/ $\text{NO}$	9		198 $\pm$ 10
Comparisons within columns		p = ns	P = ns

There was a significant overall effect of  $\text{NOx}$  ( $F_{7,38} = 503$ ,  $p \leq 0.001$ ). Planned contrasts revealed that lenses with high glucose showed significantly higher  $\text{NOx}$  than lenses with or without L-NAME \*<sup>1</sup>( $t_{26} = -5.9$ ,  $p \leq 0.001$ ) and that lenses with DETA/ $\text{NO}$  showed significantly higher  $\text{NOx}$  than all other lenses \*<sup>2</sup>( $t_{18} = -45$ ,  $p \leq 0.001$ ).

Possibly, the glucose added to the lenses may have formed AGEs which through the binding of RAGEs in the lens epithelium [54] may have triggered the expression of the transcription factor nuclear factor-kappa B (NF- $\kappa$ B) [53]. This would lead to activation of NOS II within the lens [60, 75] and subsequently increased production of NO $\cdot$ . Apart from increased NO $\cdot$  formed intracellularly in hyperglycemic conditions as here proposed, an elevation of lens NO $\cdot$  in diabetic patients [67], may be derived from enhanced diffusion into the lens from increased levels found in the aqueous humour surrounding the lens [68].

NO $\cdot$  may contribute to lens damage and cataract formation even at normal glucose concentrations, especially in situations when there are elevated levels of this radical, induced for instance by smoking or trauma [66]. However, generally the evidence for NO $\cdot$  involvement has been provided indirectly, either by the inhibition of NOS [61] or by the addition of a NO $\cdot$  donor [156], after which cataract progression and different parameters of lens damage have been followed. These models are similar to that used in paper II and support the findings presented in this thesis.

### ***The Reliability of the In Vitro Model and the Methods Used***

*In vitro* cataract models are often used to study basic lens mechanisms. Likewise, the addition of a high glucose concentration (10 x the normal) to the lens culture medium, as in papers I and II, has been used in the past [144, 157] to provoke susceptible lenses to develop cataractous changes before the controls. High levels of glucose may influence the lens in many ways, for instance by activating the enzyme aldose reductase (AR) which converts glucose to sorbitol in the polyol pathway (Fig. 3). The accumulation of sorbitol used to be considered the sole mediator of glucose-induced cataract by causing osmotic stress [30]. More recently, the polyol pathway has been shown to be deleterious by other means, for example by contributing to oxidative stress. Firstly, AR may deplete its cofactor NADPH which is also needed for GSH regeneration. Secondly, the next step in the pathway involves the oxidation of sorbitol to fructose by sorbitol dehydrogenase. Simultaneously, NAD $^+$  is reduced to NADH, resulting in less available NAD $^+$  for glycolysis and energy production. This in turn activates protein kinase C which triggers the O $_2^{\cdot-}$  generating enzyme NADPH-oxidase. Thirdly, fructose as a potent glycating agent may form AGEs which may lead to further oxidative stress through interactions with RAGEs. Finally, AR activity may somehow impair the functions of many antioxidants, including SOD1 [10, 31]. Apart from inducing oxidative stress by the mechanisms mentioned above, AR may actually protect against the same by catalysing the

reduction of other aldehydes than glucose, such as those generated by lipid peroxidation [158]. If not detoxified, these lipid peroxidation products may accumulate and cause damage [158]. Nevertheless, despite these controversies, it is evident that AR plays a complex but vital role in cellular metabolism and it is therefore important to further explore the mechanisms by which AR may regulate the development of diabetic complications. However, the contributions of the polyol pathway in the model presented in papers I and II is likely minor due to lower levels of both AR and sorbitol dehydrogenase in the mouse lens compared to that found in many other animals [159]. Moreover, since NO<sup>•</sup> donors are shown to down-regulate the activity of AR in lenses incubated *in vitro* [72] and thus prevent osmotic and oxidative stress generated by the polyol pathway, whereas our results in paper II instead showed increased lens damage when adding NO<sup>•</sup>, the relative importance of AR as a cataract contributor in our model must be considered minimal.

The methods used in this thesis for the assessment of cataract formation and lens damage have previously been extensively employed by others [137, 149, 150, 160]. Nonetheless, to confirm the results of the digital image analysis of the cataract formation in the lenses, subjective staging was also performed. Despite generally a good correlation between the two methods (Table 7), there were some discrepancies. More specifically, the digital quantification seemed to be less sensitive than subjective staging for the earliest forms of lens changes but more sensitive for advanced forms. In the former case, the incipient cortical aberrations may be small and localized thus generating a sharp contrast which does not differ from that of the grid. Whereas these opacities may be more readily detected by the human eye on subjective staging, they render a high SD and a low cataract value on digital quantification. Also, a small peripheral opacity may be missed by the digital quantification system as it may lie outside the measured area. However, a perfect focus of the underlying grid is only obtained through a relatively small central part of the murine lens due to its pronounced biconvexity which renders the periphery fuzzy. It was therefore essential to only measure this sharply focused central lens area. However, any lens that was evaluated to a higher stage than 0, or showed a value above 25 au on day 1, was discarded. In this way, the lenses that were damaged during dissection or came from animals with congenital lens opacifications [161], as well as those that showed discrepancies between the two methods, were excluded which served as a standardization of the baseline in the studies. Contrary to incipient cataract, the more advanced stages of lens obscurity seemed to be well detected by the digital analysis system. Probably, the continuous scale of the latter was better at detecting subtle gradual changes than the ordinal scale of the subjective staging. This is exemplified by the fact that the values

for digital cataract quantification kept increasing even after the maximal subjective stage had been reached (papers I and II).

**TABLE 7.** Pearson's Correlation Coefficients Between the Different Methods of Quantifying Opacification (Digital Analysis and Cataract Staging) and Cell Damage (Lens Leakage of LDH, Lens Water Contents, and Lens Uptake of  $^{86}\text{Rb}$  or Choline) in Lenses from SOD1 null and Wild-Type Mice (Combined Data From Papers I and II).

	Digital Analysis	Cataract Staging	Lens Leakage of LDH	Lens Water content	$^{86}\text{Rb}$ Uptake (paper I)	Choline Uptake (paper II)
Digital Analysis	1	0.86†	0.55†	0.74†	-0.24	-0.76†
Cataract Staging		1	0.50†	0.72†	-0.30*	-0.78†
Lens Leakage of LDH			1	0.57†	-0.21	-0.69†
Lens Water content				1	-0.54†	-0.72†
$^{86}\text{Rb}$ Uptake (paper I)					1	-
Choline Uptake (paper II)						1

\*Significant at  $p \leq 0.05$ . †Significant at  $p \leq 0.005$ .

The different methods used for lens damage assessment were also well correlated with one another (Table 7). However, lens uptake of  $^{86}\text{Rb}$  showed low correlations with most of the other parameters and this method was therefore replaced in paper II by the uptake of [ $^{14}\text{C}$ ]Choline which, as well as being more sensitive to oxidative stress [149], proved to be better correlated to the other parameters.

*To summarize:*

The results presented in papers I and II, showed that merely the absence of the antioxidant enzyme SOD1 was not enough to induce an immediate lens opacity and damage *in vitro*. However, the combination with high glucose levels gave a rapid and progressive cytotoxic and cataractogenic effect which seemed to be more than an additive effect of the two separate stresses. Since the toxicity of  $\text{O}_2^-$  is mainly due to its reactivity with other reactive species, which may produce more damaging compounds, I suggest, that the

increased generation of  $O_2^{\cdot-}$ , found in the SOD1 null lenses, may have promoted reactions with  $NO^{\cdot}$ ; elevated in the lens by the high levels of glucose. These reactions yield  $ONOO^-$ , which, in turn, may have damaged the lens, thereby justifying the development of lens opacity. This proposed mechanism is supported by the finding of increased  $ONOO^-$  production during diabetic cataract formation [75]. Furthermore, lens GSH contents have been found to be depleted by  $ONOO^-$  [62], implicating the specific contribution of this compound to oxidative reactions occurring in the lens.

## Enhanced Diabetes-Induced (Paper III) and Age-Related Cataract (Paper IV) in SOD1 Null Mice

### *In Vivo Cataract Development*

The SOD1 null genotype showed an enhanced diabetes-induced (paper III) as well as a somewhat earlier onset of age-related (paper IV) cataract development compared to the wild-type mice, when cataract was assessed by the digital quantification system (Table 8).

**TABLE 8.** Digital Cataract Quantification in Arbitrary Units (au) (Means  $\pm$  SD) and Subjective Staging (Medians and 1<sup>st</sup> to 3<sup>rd</sup> Interquartile Ranges) in Lenses From Mice Rendered Diabetic by Streptozotocin, From Mice of Different Age-Groups, and From Non-Diabetic 18-week old Control Mice (Means  $\pm$  SD).

Paper	Treatment	Wild-type lenses		SOD1 null lenses	
		Au	Stage	au	stage
III and IV	Control	15 $\pm$ 5	1a (0-1a)	14 $\pm$ 6	0 (0-1a)
III	Diabetic	22 $\pm$ 7	1a (1a-b)	29 $\pm$ 6	1b (1b-b)
IV	1-year old	15 $\pm$ 7	0 (0-0)	22 $\pm$ 9	1a (0-1a)
IV	2-year old	27 $\pm$ 11	1a (0-1a)	21 $\pm$ 8	0 (0-1a)

Regarding the diabetes-induced cataract, the degree of opacification increased linearly with the level of plasma glucose in both the SOD1 null and wild-type mice, although the rate of cataract formation was accelerated in the former (see Fig. 1 in paper III). The relationship between cataract development and age was though somewhat different (see Fig. 1 in paper IV). Despite showing an earlier commencement of cataract, evident at 1 year of age, as also previously noted [125], no additional lens obscurity was observed in the 2-year old SOD1 null mice, which at that age showed equivalent lens changes as the wild-type controls. It must, however, be emphasized that SOD1 null mice show reduced survival, with maximum lifespans of 108-130

weeks compared to wild-type controls that show maximum lifespans of 150-180 weeks [118, 119]. This is exemplified by the fact that only about 15% of the SOD1 null mice generated in our breeding facility survive at 2 years of age whereas the majority of the wild-type mice are still alive at that age [118]. Consequently, only the most vigorous SOD1 null mice may have survived to 2 years of age in study IV and thereby influenced the results as their good health also may have protected them against cataract formation. Hence, this may have contributed to the absence of cataract progression from 1 years of age in the SOD1 null genotype. Contrary to the SOD1 null mice, the wild-type mice did not develop cataract until 2 years of age which is in accordance with previous findings in the C57BL/6 strain [162]. The discrepancy in lifespan between the two genotypes also caused a difficulty in getting age-matched groups which resulted in slightly younger SOD1 null mice ( $98 \pm 6$  weeks) in the 2-year old age-group compared to the wild-types ( $106 \pm 5$  weeks) ( $t_{18} = -3.3$ ,  $p = 0.004$ ). The fact that the wild-type mice were almost 2 months older than the SOD1 null mice in this age-group should of course also be considered when interpreting the resulting lack of difference in cataract development at this time-point.

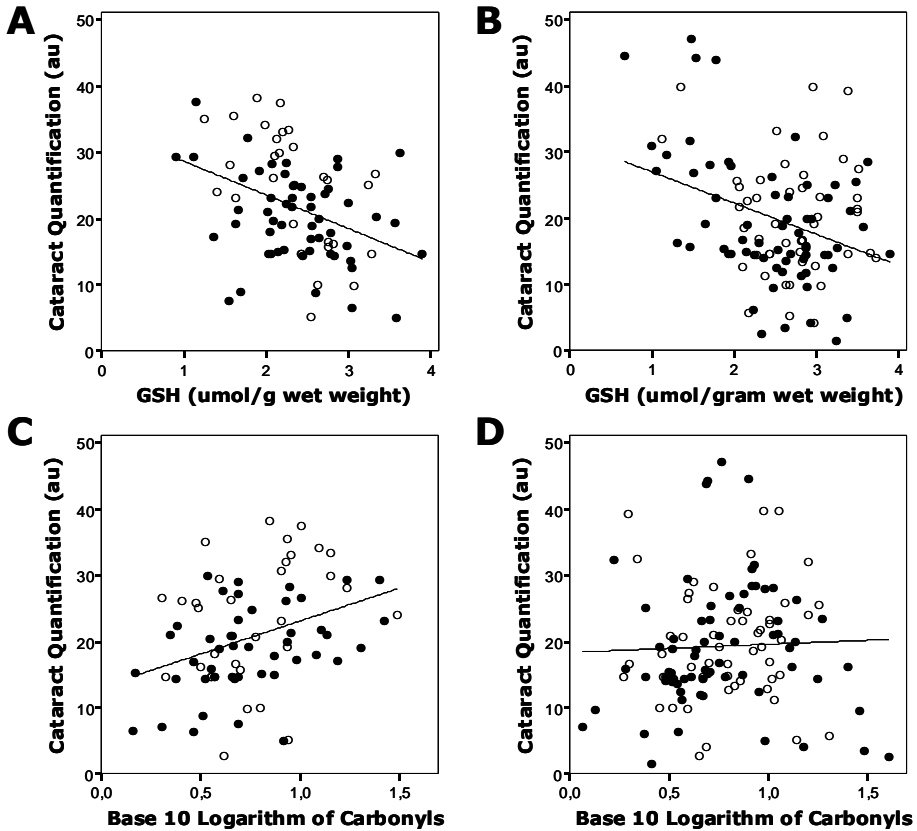
Generally, the lens opacities formed *in vivo* were less pronounced than those previously seen during *in vitro* lens cultivation (compare Fig. 1 in papers I and II to Fig. 2 in papers III and IV), and were mainly classified as cortical cataracts (Table 8). The diabetic SOD1 null mice showed, nevertheless, slightly more cataracts than the subtle senescent cataractous changes that developed over time in the non-diabetic lenses. Actually, the fine distinction between the cataractous lenses and the controls rendered the ordinal scale of the subjective staging too crude to detect differences, whereas the continuous scale of the digital quantification system proved to be more sensitive.

### ***GSH and Protein Oxidation in Age-Related and Diabetes-Induced Cataract Development***

GSH which is especially abundant in the lens where it plays a vital role as a first line of defence against ROS [90], was shown to decrease with progressive cataract formation (paper III and IV) (Fig. 12). Accordingly, lower levels of GSH were found in the SOD1 null lenses from the diabetic mice that also showed more cataract than the non-diabetic controls (see Table 1 in paper III). Surprisingly though, there was no decrease in GSH levels in the diabetic wild-type lenses which is contrary to another study [163]. However, the unaffected GSH levels in the diabetic wild-type mice in



our study accord well with the very limited cataract development seen in these lenses.



**Figure 12.** Digital cataract quantification as a function of oxidative stress in SOD1 null (empty circles) and wild-type (black circles) lenses. **A+B.** Lenses with cataract show reduced levels of GSH. **A.** Lenses from paper III ( $y = 34 - 5x$ ,  $r^2 = 0.15$ ,  $p < 0.001$ ). **B.** Lenses from paper IV ( $y = 32 - 5x$ ,  $r^2 = 0.11$ ,  $p < 0.001$ ). **C.** Lenses with cataract show increased levels of carbonylated proteins. Lenses from paper III (nmol/g lens wet weight) ( $y = 13 + 10x$ ,  $r^2 = 0.13$ ,  $p = 0.001$ ). **D.** No association between cataract development and levels of carbonyls. Lenses from paper IV ( $y = 18 + 1x$ ,  $r^2 = 0.00$ ,  $p = 0.67$ ).

In paper IV, we found an age-related decline in GSH levels which was independent of the genotype (see Fig. 4 in paper IV). The lack of difference in GSH levels between the SOD1 null and the wild-type lenses despite a difference in cataract development at 1 year of age was somewhat surprising. However, since the SOD1 null genotype shows lowered activities of glutathione peroxidase in many other tissues [118, 119], it may have a slowed

consumption of GSH also in the lens. This would thus mean that GSH levels could, at least initially, remain unaffected by oxidative stress in SOD1 null lenses. However, in advanced stages of oxidative damage, GSH levels are reduced, not only by a direct oxidation of GSH partly assisted by glutathione peroxidase, but also through a decreased synthesis [164], and through a loss of GSH regeneration due to a leakage of GSSG and other protein-thiol mixed disulfides out of the lens [5, 90]. These two latter mechanisms would probably affect the SOD1 null and the wild-type lenses equally, rendering a loss of GSH, which at this stage would be independent of the glutathione peroxidase activity. It would probably therefore be more accurate to compare GSH levels in more damaged lenses from these two genotypes than in the only slightly affected lenses in paper IV.

Contrary to GSH, the lens contents of carbonyls increased with progressive diabetic cataract formation (paper III) (Fig. 12), as previously shown [165]. However, despite finding more cataracts in the diabetic SOD1 null mice (paper III), as well as in the non-diabetic 1-year old SOD1 null mice (paper IV) compared to their respective wild-type controls, no differences in carbonyls were detected between the two genotypes. Large variances within the carbonyl data may though have contributed to this discrepancy. Nevertheless, we could only conclude that the contents of carbonyls were increased by diabetes as well as by age, but not by the absence of SOD1. When interpreting the carbonyl data, it should, however, be born in mind that carbonyl levels have not previously been studied in SOD1 null lenses. For comparison, it has been studied, by the same method as used in papers III and IV, in liver tissue from SOD1 null mice [119]. Interestingly and surprisingly, it was then found that only highly damaged cells in terminal SOD1 null mice suffering from hepatocellularcarcinoma, contained increased protein carbonyl contents. Since other indices of oxidative damage appeared much earlier in the diseased livers, it was proposed that undefined mechanisms may have removed oxidized proteins, thereby preventing their accumulation in early disease in this genotype [119]. According to this, it may be somewhat misleading to determine oxidative stress by assessing the carbonyl levels in only slightly damaged SOD1 null lenses.

### ***The Specificity of the Diabetic Cataract Model***

It is well known that blood glucose levels are affected differently by STZ depending on the strain of mice rendered diabetic [166]. Generally though, multiple intraperitoneal injections with a low dose of STZ have been shown to induce a gradual elevation in plasma glucose due to beta-cell toxicity resulting in insulin deficiency [167]. However, SOD1 null mice have shown a

slightly different response to this method. For instance, one laboratory found that blood glucose levels started to decline in the SOD1 null mice one month after the last injection of STZ, rendering a lower mean blood glucose level measured over a five month period in this genotype than in diabetic wild-type mice [168].

Besides the previously reported problem regarding the plasma glucose levels over time, we encountered a resistance to the hyperglycaemic effect of STZ in the SOD1 null mice during diabetes induction. More specifically, our SOD1 null mice showed lower levels of plasma glucose than the wild-type mice even after having increased the dosages given to the former (see Table 1 in paper III). Unfortunately, this dose adjustment may of course have increased the risk of non-specific cytotoxic effects caused by STZ, such as acute kidney damage [169]. Since the SOD1 null mice also showed increased morbidity, it is tempting to attribute the cataract development in these mice, at least to some extent, to the toxic effects of this broad-spectrum antibiotic and anti-neoplastic agent. However the use of STZ in different animal models of diabetic cataract is well established and seems not to be considered to negatively influence the lens [163, 170, 171]. Nevertheless, the effect of STZ on the lens needs to be clarified by future studies.

We do not know why the diabetic SOD1 null mice showed lower levels of plasma glucose than the diabetic wild-type mice. Possibly, this is related somehow to the fact that the SOD1 null genotype also shows lower body weights throughout life [118]. In paper III, we suggested that the absence of SOD1 might cause an increased metabolic rate which subsequently would increase the glucose consumption as well as lower the body weights.

The diagnosis of diabetes mellitus is usually based on two fasting blood glucose samples. After diagnosis, the progression of the disease is monitored by following the levels of glycated haemoglobin (HbA1c) which gives an indication of the glycaemic state during the last few weeks. In our study in paper III, we instead based our diagnosis on two non-fasting glucose levels which of course is somewhat inadequate. However due to ethical reasons, we did not want to use metabolic cages, nor expose the animals to repeated blood samplings. To complicate this further, the SOD1 null genotype has shown an accelerated turnover rate of erythrocytes, thus making HbA1c unsuitable to measure in this mouse strain [172]. We did however also analyse urine levels of glucose which were quite well correlated to the mean levels of plasma glucose (see Table 1 in paper III).

### ***Oxidative Stress in SOD1 Null Mice***

The dismutation of  $O_2^{\cdot-}$ , resulting in the formation  $H_2O_2$ , is greatly accelerated by the SOD isoenzymes, but may also, in the absence of the catalysing enzymes, proceed spontaneously although at a slower and pH dependent rate. However,  $O_2^{\cdot-}$  radicals are also reduced by ascorbate, which actually results in the production of twice as much  $H_2O_2$  compared to reactions with SOD [34]. In the human lens, SOD1 and ascorbate are considered to be equally important for  $O_2^{\cdot-}$  scavenging [127], whereas SOD1 is more important in the mouse lens, in which SOD1 accounts for about  $\frac{3}{4}$ , and ascorbate for  $\frac{1}{4}$ , of the total  $O_2^{\cdot-}$  scavenging capacity [137].

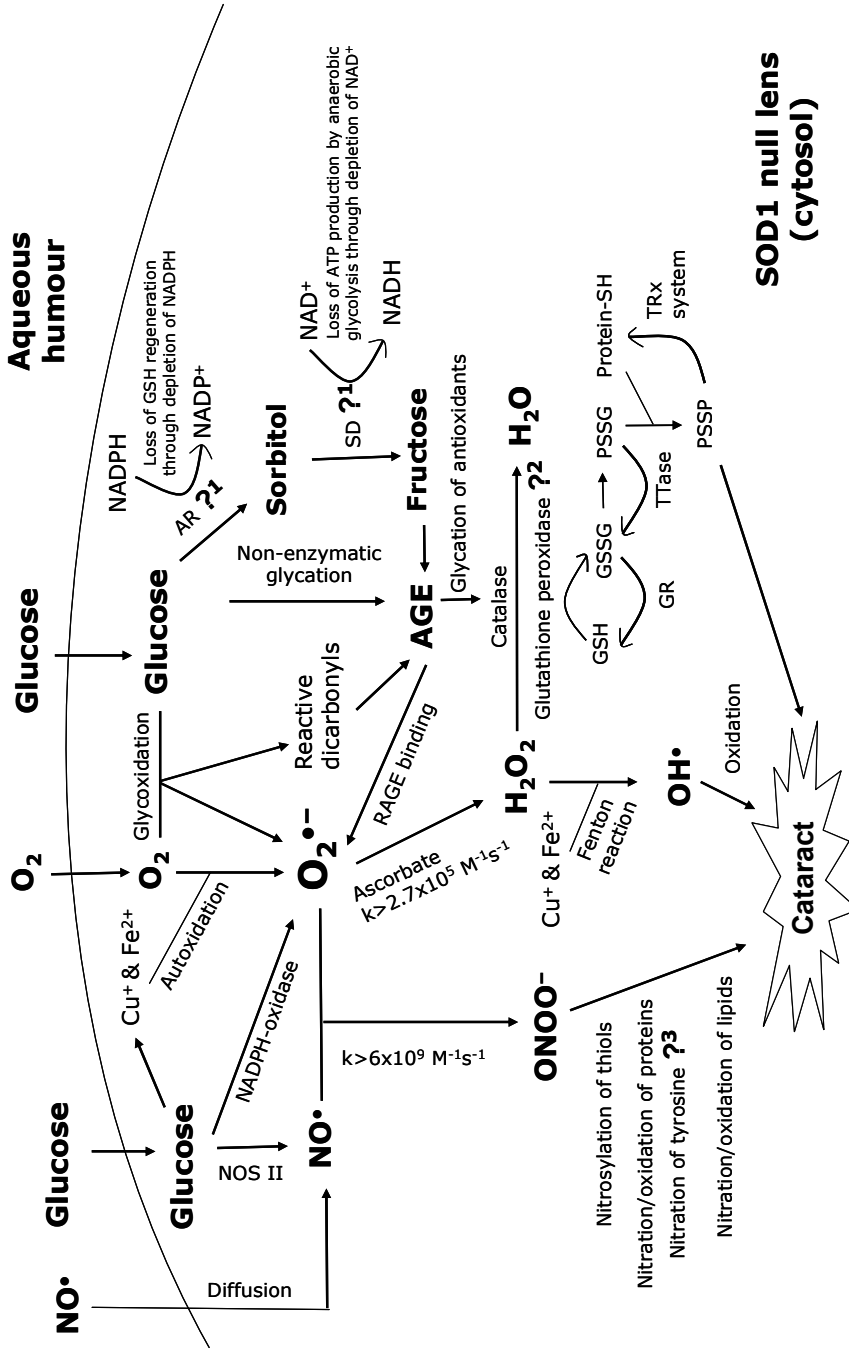
In lenses from SOD1 null mice, the relative importance of ascorbate as a  $O_2^{\cdot-}$  scavenger is probably enhanced by the absence of SOD1 [173], especially since mice, contrary to humans, may uphold ascorbate levels by *de novo* synthesis. Hence, in SOD1 null lenses,  $O_2^{\cdot-}$  may be reduced by ascorbate and give rise to increased levels of  $H_2O_2$ . Assuming that the lens has a reduced activity of glutathione peroxidase like other tissues in this genotype [118], the elimination of  $H_2O_2$  would be limited and its levels thus maintained high. This would promote further reactions, for instance with  $O_2^{\cdot-}$ , which would result in toxic  $OH^{\cdot}$  radical generation. These Haber-Weiss reactions may actually be facilitated in SOD1 null mice due to increased levels of iron [118], and even more so in diabetic conditions due to increased levels of copper [50].

In addition to reduction by ascorbate and participation in Haber-Weiss reactions,  $O_2^{\cdot-}$  may react with other reactive species such as  $NO^{\cdot}$  which yields the highly damaging  $ONOO^-$ . This pathway may be enhanced in diabetes due to activation of  $NF\kappa\beta$  which results in NOS II activation and thus increased  $NO^{\cdot}$  production [174]. Paradoxically though, SOD1 null mice have shown a resistance to presumed peroxynitrite-induced damage in liver tissue which has cast doubt upon the toxic role of  $ONOO^-$  in this genotype [141]. This controversy is boosted by the fact that SOD1 is known to catalyse the reaction between  $ONOO^-$  and tyrosine residues within proteins [104, 175]. The absence of this enzyme may thus protect against protein nitration. To complicate matters, the product of the reaction, nitrotyrosine, is a much used marker for peroxynitrate-induced damage [34], but hence, probably not a reliable parameter in SOD1 null mice.

It is still unclear how the different ROS that arise in the SOD1 null lenses interact with the remaining antioxidative systems and how these defence mechanisms are in themselves affected by the absence of SOD1. Although knockout models are very helpful when studying the effects of specific

proteins, interpretation of the results must be done with caution since the manipulations may lead to compensatory changes. In the SOD1 knockout model, some controversies do exist regarding compensatory antioxidative mechanisms in various tissues. For instance, GSH has been found to be up-regulated in the liver of healthy young adult (13 - 18 week old) SOD1 null mice, but not in other organs such as lung, kidney [118], or brain [120]. Likewise, we found no increase in lens GSH levels in the SOD1 null genotype in the corresponding age-group in papers III and IV. Surprisingly, the activity of glutathione peroxidase has been found to be much reduced in the liver and lung [118, 119], whereas no effect was detected in the same tissues in another study [126]. Catalase, also responsible for  $H_2O_2$  removal, has however not been found to be affected by the lack of SOD1 [118, 126].

In the absence of SOD1, the expressions of the other two isoenzymes have been studied in some organs, but, alas, not in the eye. One study found that SOD2 activity was increased by about 30% in the lung and liver of SOD1 null mice [118], whereas another study found no compensatory changes in these tissues [126] nor in the brain [120, 126]. An eventual compensatory increase in SOD2 in the lens would however only protect against raised  $O_2^{\cdot-}$  levels in the mitochondria localized mostly in the epithelium. Despite exerting an important protection against oxidation-induced injury in this part of the lens [125], SOD2 only accounts for about 10% of total lenticular SOD activity and is thus considered less important than SOD1 [137]. In addition, since the activity of the third isoenzyme, SOD3, is negligible [137] due to the very limited extracellular space between the lens fibres, SOD1 is clearly the main isoenzyme. In its absence, the lens thus shows a much reduced capacity to remove  $O_2^{\cdot-}$ , rendering the SOD1 null lenses suitable for studying the effects imposed by this specific radical. Due to the high specificity of this knockout model, I suggest that superoxide radical-induced oxidative stress most likely contributed to the enhanced cataract development in the SOD1 null mice.



**Figure 13.** Schematic illustration of possible reactions leading to  $O_2^{\bullet-}$  formation in diabetic SOD1 null lenses. The further generation of  $ONOO^-$  is favoured in the presence of  $NO$  due to the higher rate constant (k) for reactions between  $O_2^{\bullet-}$  and  $NO$  compared to that between  $O_2^{\bullet-}$  and ascorbate.<sup>1</sup>The roles of aldose reductase (AR) and sorbitol dehydrogenase (SD) are uncertain due to low levels in the mouse lens.<sup>2</sup>Glutathione peroxidase activity may be down-regulated in this genotype.<sup>3</sup>The nitration of tyrosine residues is less likely to occur in this genotype due to the lack of SOD1. See text or page 6 for abbreviations.

*To summarize:*

The participation of oxidative stress in both age-related and diabetes-induced cataract initiation and progression is today well established and many different ROS probably contribute to this process. However, although  $O_2^{\cdot-}$  radicals may well be the initiators of further reactions leading to more damaging species and subsequent cataract formation, its exact fate in the lens is not known. I therefore believe that the findings presented in papers III and IV of this thesis significantly contribute to the present knowledge about superoxide-derived lens damage. Most importantly, the findings prove that SOD1 exerts protection against age-related and possibly more so against diabetes-induced cataract development *in vivo*.

I now conclude this discussion by giving a simplified summary (Fig. 13) of the mechanisms that I propose may occur in the cytosol of the SOD1 null lenses in response to diabetes.

## Concluding Remarks and Future Perspectives

The development of cataract is usually a slow process that takes years before affecting vision to the degree that surgery is considered. However, some patients, such as those suffering from diabetes mellitus, show accelerated cataract development [28]. It is today well established that oxidative stress participates in both age-related and diabetes-induced cataract formation, although other factors probably also contribute, especially to the latter. Oxidative damage to the lens most likely arises as a consequence of an impaired defence system due to inactivation of antioxidative enzymes, both by age [130-133, 176] and diabetes [134, 135], in combination with an increased generation of ROS. In the future we may thus be able to control the development of cataract if we learn how to manipulate these two factors. As cataract surgery is costly and not without risks, alternative treatment strategies are highly desirable, and would especially benefit diabetic patients that also suffer from higher complication rates during surgery [29].

This thesis has focused on the antioxidative enzyme SOD1 in the lens and its participation as a first line of defence against cataract development both *in vitro* and *in vivo*. The role of SOD1 in the lens has been studied previously, although mainly by exploring the effects of its overexpression [125, 140]. The four studies presented in this thesis are instead conducted on lenses lacking SOD1. By using this knockout model, we have thus complemented and added to the present knowledge regarding the function of SOD1 in the lens. I therefore suggest that this antioxidative enzyme is important for the protection against cataract development, probably through cytosolic O<sub>2</sub><sup>-</sup>-scavenging during life-long exposure to light and oxygen. However, the importance of this specific enzyme is enhanced in situations when the lens is exposed to additional stresses, such as that imposed by high levels of glucose, which here has been shown for the first time.

I believe that it may be possible to delay the development of cataract, especially in high risk patients, in the future. This may be achieved by either additional intake of diet-derived antioxidants, or by the adjustment of the endogenous antioxidative defences. Supported by the findings presented in this thesis, I suggest that SOD1, as an important antioxidant in the lens, may be a potential target for future cataract intervention. Further studies are however needed to explore the regulation and expression of SOD1 in the lens before precise therapeutic strategies can be considered.



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