Light scattering in the C57BL/6 mouse lens

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ABSTRACT.
Purpose: **To characterize inherent light scattering in the C57BL/6 mouse lens.**
Methods: **Lenses from 20 6-week-old female C57BL/6 mice were extracted from freshly enucleated globes and microsurgically cleaned of remnants of the ciliary body. Lens light scattering was measured quantitatively with a light dissemination meter (LDM). Morphological properties of the mouse lenses were documented using grid- and dark-field illumination photography. Analysis of variance was performed to establish variance for animals, variance between left and right eyes and variance for measurements.**
Results: **Average inherent light scattering in the C57BL/6 mouse lens is 0.16 ± 0.02 tEDC (transformed equivalent diazepam concentration). The mean size of a mouse lens at 6 weeks is 1.9 mm in diameter. Two lenses featured pre-existing cortical lens opacities. Variance for animals was assessed to be 7.9 10^{-4} $tEDC^2$, variance for measurements was 1.6 10^{-4} $tEDC^2$, and variance between left and right eyes was 8.8 10^{-4} $tEDC^2$. The tolerance limit for non-pathological light scattering was determined to 0.26 tEDC. No significant difference in light scattering between left and right mouse lenses was found. The minimum number of C57BL/6 mice required for detection of a 10% experimentally induced change in light scattering intensity was estimated to be 50 for independent group experiments and 25 for paired design experiments.**
Conclusions: **The C57BL/6 mouse is a suitable animal in which to conduct experiments on light scattering or cataractogenesis with high precision at reasonable sample sizes. Before including C57BL/6 mice into a study on cataractogenesis, pre-existing lens opacities such as congenital cataract must be excluded.**

Key words: lens – light scattering – C57BL/6 mouse – cataract – oxidative stress

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Introduction

The present study aims to characterize normal light scattering in the C57BL/6 mouse lens. This information is essential to establish the C57BL/6 mouse lens as an *in vivo* model for oxidative stress-induced cataract. Furthermore, it will serve as a reference for efficient study design when the C57BL/6 mouse is used as the experimental animal. It is anticipated that studies with C57BL/6 knockout mice will elucidate how the genome modulates lens susceptibility to oxidative stress.

Exposure of lens tissue to oxidative stress, from exposure to ultraviolet radiation type B (UVR-B), leads to a wide variety of alterations in lens micro architecture, resulting in increased lens light scattering as a measure of cataract formation. In lens crystallins, proteolysis, an increase in disulphide bridges, deamidation of asparagines and glutamine residues, racemization of aspartic acid residues, phosphorylation, non-enzymatic glycosylation, and carbamylation have been observed as a result of oxidative stress (Hejtmancik et al. 2001). These alterations lead to protein denaturation and thus protein insolubility with maintained protein folds (Hejtmancik & Kantorow 2004). Insoluble proteins cause refractive index shifts, giving rise to light scattering, which is perceived by an examiner as lens opacification. Increased lens light scattering causes visual impairment and is defined as 'cataract' (Söderberg et al. 2004).

Cataract is the leading cause of blindness (Leske & Sperduto 1983; Seidman-Ripley & Huang 1993; World Health Organization 2004). Cataract surgery is now the most commonly performed surgical intervention in industrialized countries (Hartmann 1997). No other surgical technique has been standardized and perfected in such a short period at any point in the history of medicine (Augustin 2003). Nonetheless, patient

distress and the financial burden on existing health care systems due to cataract are enormous. Recent studies demonstrate that a delay in the onset of age-related cataract by a mere 10 years would decrease the need for cataract surgery by approximately 45% (Kupfer 1985; Brian & Taylor 2001). Therefore, we urgently need to gather knowledge on genetic predispositions that alter lens sensitivity to UVR-B. Such knowledge could contribute towards the avoidance or delay of the onset of cataract due to UVR.

Materials and Methods

Experimental animals

Six-week-old female C57BL/6 mice were studied in this experiment. Ethical approval was obtained from the Stockholm Norra Djurförsöksnämnd, protocol number N227/03. All mice were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental devices

The intensity of forward light scattering in the mouse lens was measured with a light dissemination meter (Söderberg et al. 1990). This instrument uses the principal of dark-field illumination. The illuminating light transilluminates a transparent object (e.g. a mouse lens) at 45 degrees against the horizontal plane. At this angle, the light cannot enter the objective aperture. If the object scatters light in the forward direction, a defined fraction of light reaches the objective and is measured by a photodiode.

The scattering standard was a lipid emulsion of diazepam (Stesolid Novum; Dumex-Alphapharma, Copenhagen, Denmark). Light scattering was therefore expressed as transformed equivalent diazepam concentration (tEDC) (Söderberg et al. 1990).

Experimental procedure

The animals were killed, the eyes enucleated and the lenses extracted microsurgically. Remnants of the ciliary body were removed from the lens equator under a microscope, keeping the lens in balanced salt solution (BSS). The intensity of forward light scattering was then quantitatively measured with the light dissemination meter. Thereafter, the macroscopic appearance of the lens was documented in incident illumination against a grid and in light- and dark-field illumination photography.

Experimental design

A total of 20 C57BL/6 mice were studied. Two lenses together with their contralateral lenses had to be excluded from the data analysis because of the presence of pre-existing cataract. One lens was damaged during dissection and, together with its contralateral lens, was excluded from the statistical analysis. Intensity of light scattering was measured in both lenses from each animal. Each lens was measured three times.

Statistical parameters

Given the sample size, the significance limit and the confidence coefficient were set to 0.05 and 0.95, respectively.

Estimation of the tolerance limit for non-pathological light scattering and baseline

The tolerance limit was established according to a condition allowing light scattering in 5% of the examined lenses to be classified as pathological, although it was non-pathological. The baseline for native light scattering in the mouse lens was defined as the 95% confidence interval (95% CI) for the mean light scattering in all lenses.

Results

Morphology

The mean diameter of the C57BL/6 mice lenses at 6 weeks of age was estimated to 1.9 mm (Fig. 1).

The majority of the lenses were clear when judged under the microscope (Fig. 1). In dark-field illumination, a diffuse increased scattering was observed close to the equator.

In some lenses, the nucleus contained multiple, incipient, round opacifications (Fig. 2).

Two lenses out of 40 showed pre-existing cortical opacities (Fig. 2).

Tolerance limit for non-pathological light scattering and baseline

Adopting the estimated mean and standard deviation assuming that one measurement of a lens is the average of three measurements, the 95% tolerance limit for non-pathological light scattering was estimated to be 0.26 tEDC. The baseline for non-pathological light scattering was defined as the 95% CI for the mean light scattering in all lenses and was assessed to be 0.16 ± 0.02 tEDC.

Frequency distribution of light scattering in C57BL/6 mice lenses

The frequency distribution for light scattering in C57BL/6 lenses was determined by randomly selecting the right or left lens from each animal. The light scattering values of the selected lenses were plotted as a frequency distribution (Fig. 3).

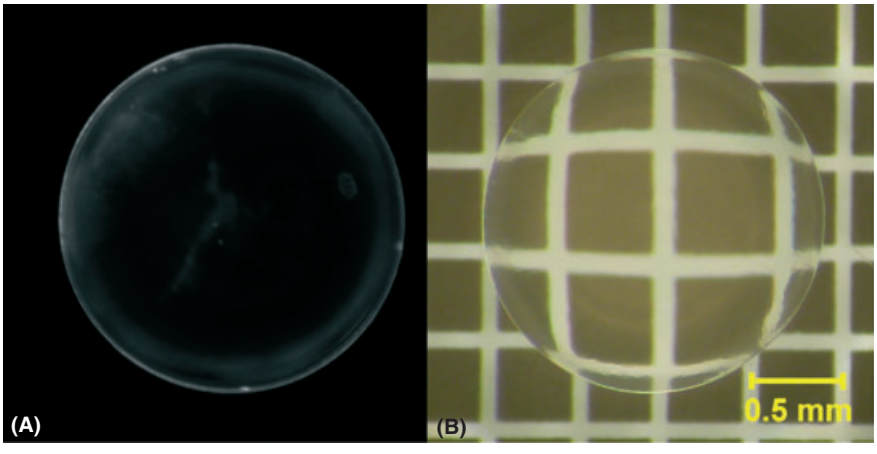

Fig. 1. C57BL/6 mouse lens under dark-field illumination (A) and incident illumination on top of a white grid (B). Grid marks are 0.5 mm apart.

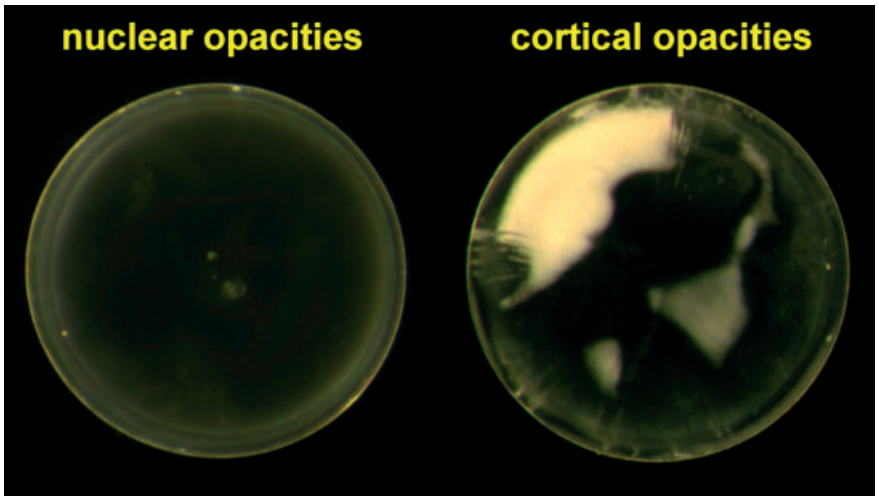

Fig. 2. Pre-existing opacities in C57BL/6 mouse lenses.

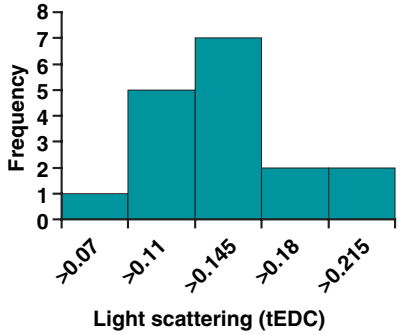

Fig. 3. Frequency distribution for forward light scattering in C57BL/6 lenses.

Testing for deviation of the estimated frequency distribution from a normal distribution with chi-square analysis revealed that light scattering in C57BL/6 mouse lenses is distributed according to a normal distribution ($\chi^2 = 1.938$; ($\chi^2_{0.05;4} = 9.488$) (Snedecor & Cochran 1980).

Systematic difference in light scattering intensity between left and right eyes in C57BL/6 mice

There is no systematic difference in light scattering between the left and right eyes in C57BL/6 mice because the 95% CI for the mean difference, 0.00 ± 0.03 tEDC, includes zero.

Sources of variation

Measurements were analysed using analysis of variance according to the model given in the Appendix (Equation 1).

Table 1 presents the results of the analysis of variance.

The variance for animals (Table 1) was estimated to be 7.9 10^{-4} tEDC^2. The variance for sides (Table 1) was estimated to be 8.8 10^{-4} tEDC^2. The variance for measurements (Table 1) was estimated to be 1.4 10^{-4} tEDC^2 based on the estimated mean square.

Estimation of sample sizes for different experimental designs

When we know the variation for measurements, animals, and left and right sides, it is possible to derive the minimal required sample size that is needed to detect a 10% difference in level between two samples $\leq 0.1\ \mu$. This information can be applied to the experimental design for independent groups experiments and dependent paired groups experiments, respectively (Appendix, Equations 2 and 4).

Adopting the variance estimate for measures, σ_x^2 (Appendix, Equation 3), where each measure is the mean of the two lenses from the same animal and the value for each lens is the mean of three measurements, the minimal number of animals in an independent group design, considering 5% and 10% changes in light scattering level as statistically significant, was estimated to be 200 and 50 mice, respectively.

Similarly, the minimum number of animals in each group, using an independent group design, but using the difference between left and right eyes for each animal, considering three measurements in each lens, can also be estimated from Equation 3 (Appendix), using the variance estimate, σ_d^2, from Equation 5 (Appendix).

For a paired design, the minimum sample size can be estimated with Equation 4 (Appendix), adopting σ_d^2 from Equation 5 (Appendix) as the variance estimate. Thus, the minimal number of mice in a paired group design, considering 5% and 10% changes in light scattering level as statistically significant, was estimated to be 100 and 25 animals, respectively.

Table 1. Analysis of variance of light scattering in normal C57BL/6 mice lenses.

Source of variation	df	Estimated mean square ($_t$EDC 100)^2	Expected mean square
Animals	16	0.007542	$\sigma_\varepsilon^2 + n\,\sigma_B^2 + b\,n\,\sigma_A^2$
Sides	17	0.002794	$\sigma_\varepsilon^2 + n\,\sigma_B^2$
Measurement error	68	0.000156	σ_ε^2

df = degrees of freedom; σ^2 = the expected variance for the indexed source; A = animals (n = 17), B = number of sides (n = 2), ϵ = number of measurements (n = 3).

Discussion

The current study was designed to elucidate light scattering in non-pathological C57BL/6 mouse lenses. Our results provide valuable knowledge for the design of future experiments on light scattering and cataractogenesis in the mouse lens.

Macroscopic appearance

Of interest is the finding that the healthy, untreated C57BL/6 mouse lens occasionally features incipient nuclear opacities (Fig. 2). However these slight opacifications generate baseline scattering in normal lenses. To our knowledge this finding has not been previously described.

Two animals featured pre-existing cortical opacities (Fig. 2), underlining the necessity to examine the animals carefully in mydriasis prior to including them in a study involving cataractogenesis. Without this examination, it would have been difficult to

attribute increased lens light scattering to an experimental cataractogenic factor, such as oxidative stress from exposure to UVR-B.

Tolerance limit for non-pathological light scattering and baseline

The assessed baseline for normal light scattering and the presently estimated tolerance limit for non-pathological light scattering in the mouse lens, 0.26 tEDC, may be applied as a threshold for pathology or as a reference for inherent light scattering in the C57BL/6 mouse lens in a future dose–response experiment. The finding that light scattering in the mouse lens does not deviate from a normal distribution implies that collected data on light scattering in these animals can be treated with normal distribution statistics.

Estimated sample sizes for different experimental designs

The finding that detection of a 10% experimental change in the level of light scattering requires only 50 animals in an independent group design and 25 in a paired group design demonstrates that the current measurement technology used in this study provides reasonable precision for future experiments using genetically engineered mice with altered antioxidant genes. This is reflected by the very low measurement error with three light scattering measurements taken for each lens. Thus, experimental designs including the C57BL/6 mouse are both efficient and cost-effective, and therefore fulfil ethical demands for experimental studies.

In the late 1950s, Russell and Burch elaborated the 'Three R concept' (*R*eplacement, *R*eduction, *R*efinement) for the responsible use of animals in experiments (Van Zupthen 2003). In this concept, 'reduction' refers to a decrease in the number of animals required for a given experiment. This can be achieved by choosing suitable experimental procedures, by controlling environmental factors and by standardizing the animal population, resulting in a decrease in variation of the results. Our data allow for the highest possible reduction of animal numbers with no cutbacks in the desired precision and significance of the results.

The C57BL/6 mouse as a cataract model

Limitations of animal models in studies of damage caused by optical radiation originate mostly in anatomical differences in the eyeball between species. For example, mouse and rat corneas are thinner than the human cornea (Dillon et al. 1999). Thus, higher sensitivity of the lens is expected in these animals due to higher levels of UVR-B transmittance through the cornea. Despite these differences, the mouse is a suitable model for the study of genetic modulation of lens susceptibility to oxidative stress. Families that are available for detailed genetic investigations are usually too small in size. Therefore, it is necessary to establish appropriate animal models in order to identify the genes responsible for cataract formation and prevention and to analyse the mechanisms leading to lens opacification. The mouse is one of the best model systems because it is very well characterized among mammals genetically and because observable pathological alterations are comparable with those in humans (Graw & Loster 2003).

The basic information on inherent light scattering in the C57BL/6 mouse lens presented here is expected to serve as a reference. This reference can be used to design experiments on genetically altered susceptibility of the lens to oxidative stress.

Baseline and tolerance limits for non-pathological light scattering in the C57BL/6 mouse lens were defined. Light scattering variability was established. Additionally, the data provided will enable optimized sample size estimation in future experimental designs involving the C57BL/6 mouse as the experimental animal.

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Appendix

Analysis of the sources of variation

In order to define the components of variation in the measurements of the entire sample of lenses, the experimental data were analysed according to the following model:

$$x_{ijk} = \mu + A_i + B_{j(i)} + \varepsilon_{k(ij)} \qquad (1)$$

Light dissemination in an individual lens, x_{ijk}, equals the sum of the expected total mean, μ, a term for the variation among mice, A_i ($i = 1, \ldots 17$), a term for the random variation among eyes within mouse, $B_{j(i)}$ ($j = 1,2$), and a term for the measurement error, $\varepsilon_{k(ij)}$ ($k = 1,2,3$).

Estimation of sample sizes, independent group design

The minimum number of animals in each group, a, using an independent group design and averaging between sides can be estimated from Equation 2, considering a minimum difference, d, to be significant (Zar 1999):

$$a = \frac{2\sigma^2\left(Z_{1-\alpha/2} + Z_{1-\beta}\right)^2}{d^2} \qquad (2)$$

Here, σ^2 is the variance for animals. The parameters, $Z_{1\text{-}\alpha/2}$ and $Z_{1\text{-}\beta}$ are the standardized normal distribution values for the indexed probabilities. The variance for animals σ^2 depends on: the variation among animals, ; σ_A^2 the variation among lenses within animals, σ_B^2 and the variation among measurements within lenses, σ_ε^2 , if lenses are averaged with animal and the number of measurements, n, per lens (Equation 3):

$$\sigma_x^2 = \sigma_A^2 + \frac{\sigma_B^2}{2} + \frac{\sigma_\varepsilon^2}{2n} \qquad (3)$$

Estimation of sample sizes, paired group design

The minimum number of animals, a, using a paired group design and averaging between sides can be estimated from Equation 4 considering a minimum difference, d, to be significant (Zar 1999):

$$a = \sigma_d^2 \frac{\left(Z_{1-\alpha/2} + Z_{1-\beta}\right)^2}{d^2} \qquad (4)$$

Here, σ_d^2 is the variance for differences between lenses from the same animal. The variance for difference between lenses from the same animal depends on the variation among animals, σ_B^2 , and the variation among measurements within the same lens, σ_ε^2 , and the number of measurements, n (Equation 5):

$$\sigma_d^2 = 2(\sigma_B^2 + \frac{\sigma_\varepsilon^2}{n}) \qquad (5)$$