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Title page

Full title: The effect of varying glucose levels on the ex vivo crystalline lens; implications for hyperglycaemia-induced refractive changes

Running head: The effect of hyperglycaemia on the ex vivo crystalline lens

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Abstract

Purpose: Refractive changes in diabetic eyes have long been reported but with equivocal results. The lens has been a more recent focus as the source of any change but it is possible that multiple sources of variation have made it difficult to demonstrate a systematic change clinically. The aim of this study was therefore to use a bovine lens model to investigate the optical changes in hyperglycaemia and when lenses are returned to normal glucose levels as would occur following commencement of treatment.

Method: Bovine eyes were obtained and their lenses excised under sterile conditions before placing them in culture medium within an incubator using standard tissue culture techniques. In the first experiment, lenses were transferred into culture medium containing 5mM (N=12), 15mM (N=12) and 30mM (N=12) glucose. Measurements were made of the change in back vertex focusing distance with equatorial lens diameter using the ScanTox™ measurement system. From these measurements, the back vertex focal length and primary longitudinal spherical aberration were derived. In a second experiment, lenses maintained at 30mM glucose (N=7) were stepped down to 5mM glucose to simulate starting diabetic therapy and measured in the same way. Changes over time were assessed with a linear regression model.

Results: A trend towards myopia was observed with increasing hyperglycaemia, this was not statistically significant. When lenses were stepped-down from hyperglycaemia to normal physiological levels of glucose, a hyperopic shift was observed in line with published clinical studies that again failed to reach statistical significance. High variability in the measurement on longitudinal spherical aberration prevented any significant trends being measured.

Conclusions: Our results suggest that there are no consistent crystalline lens-induced refractive changes following exposure to hyperglycaemia for time-periods up to five days used in the current study. It is possible that bovine lenses are able to offset the raised osmotic pressure from high glucose levels in the short-term by a process of osmoregulation and that repeated osmotic stress or longer term exposure may be required to induce the changes in refraction that are seen clinically.

Introduction

The occurrence of transient refractive changes in diabetic patients is well documented and blurred vision is a common presenting symptom of diabetes mellitus. The first systematic analysis of refractive changes in diabetes was conducted by Duke-Elder who presented a case series drawn from his own practice and from the published literature.[1] He concluded that the refractive power of the eye was directly correlated with glycaemic state with a tendency to myopia associated with increased blood glucose and hypermetropia with decreased blood glucose. Although several studies have subsequently supported Duke-Elder's conclusions linking acute hyperglycaemia to myopia,[2-4] others have reported hyperopic shifts.[5-7] It has been shown that the sudden reductions in blood glucose that occur with intensive diabetic therapy can induce significant hyperopic refractive shifts.[8-10] Hyperopia usually occurs within a few days following an abrupt decrease in plasma glucose, peaking at days 7-14, and regressing gradually over the subsequent four weeks.[9] In addition to studies reporting myopic and hyperopic shifts, there are two that have reported no significant change in refractive status with fluctuating glucose levels.[11,12] In summary, there is no clear evidence of a consistent refractive shift associated with glycaemic changes in diabetes.

There are several mechanisms that could potentially explain the observed refractive fluctuations in diabetes. For example, changes in axial length, corneal curvature or crystalline lens parameters. Most commentators have hypothesised that alterations in lens thickness, shape or refractive index represent the most likely aetiology. The diabetic lens is prone to osmotic fluxes arising from changes to the osmolarity of the surrounding media and the accumulation of intracellular osmolytes, which could in turn lead to alterations in lens hydration and volume.[13,14] However, attempts to measure geometrical changes in the lens during hyperglycaemia have produced mostly equivocal results.[9,10,15,16] Recently Charman has reviewed the optical parameters that potentially explain transient refractive changes in diabetes.[17] He concluded that parameter changes required for a clinically significant change in refraction were capable of being measured by modern instrumentation and implicated refractive index change as the mechanism to explain refraction shifts in diabetic eyes. In a subsequent study the changes required in the gradient refractive index profile were investigated using optical modelling.[18] Their results are in agreement with previous studies although this doesn't rule out small multiple parameter changes, as noted by Charman.[17]

The human eye has varying amounts of higher order aberrations, the most significant of which is spherical aberration. It has long been known that higher order aberrations can lead to blurred vision, a symptom often reported by diabetics. Wiemer and co-workers have published the only study to our best knowledge to have reported on higher-order aberrations as well as biometric changes and found an increase in root mean square higher-order aberrations of 0.07±0.02microns.[11] He considered that this was too small to affect visual acuity. (Average RMS higher-order aberration values in normal eyes are around 0.6micron for a 6mm pupil).[19] Less is known therefore about higher order aberrations in diabetics but it is possible that subtle optical changes could be seen in the higher-order aberrations particularly if changes in gradient refractive index profile are implicated as noted in the previous paragraph.

Several inter-subject variables could influence the direction and magnitude of any refractive change in diabetics presented with the same glucose challenge e.g. type and duration of the disease and degree of glucose tolerance. In an attempt to eliminate these variables the effect of hyperglycaemia has been investigated in normal healthy volunteers.[11,20] Furishima and co-workers measured refractive and ocular biometric measurements in seven subjects in which hyperglycaemia had been induced by the administration of a standardised oral glucose load and an injection of Somatostatin to suppress insulin secretion.[20] A consistent myopic shift in refraction was measured (mean -1.93D) together with an increase of lens thickness of 1mm within 150 minutes of the onset of hyperglycaemia. However, these results could not be replicated by Weimer and colleagues using the same methodology.[11] In this study, four out of five subjects showed no change in refraction (one showed a small hyperopic shift) and no change in ocular geometry could be measured.

An alternative approach to investigate the effect of hyperglycaemia on refractive change is to use an ex-vivo lens model. Ex-vivo models allow careful control of potentially confounding variables and could potentially clarify the role of the lens in determining the optical properties of the diabetic eye. The mammalian crystalline lens is ideal for long-term organ culture. The lens lacks a blood and nerve supply and can be maintained in standard cell culture conditions for several weeks. Bovine lenses are particularly suitable for lens research as they are readily available with minimal post-mortem delay [21] Although the bovine lens is significantly larger than its primate counterpart and has a limited accommodative range[22], its refractive properties have been extensively investigated.[23] In order to measure the optical properties of the lens we used a commercially available instrument, the Scan Tox™ laser scanning system.[24] This system, which was initially developed for toxicological applications, is ideal for both maintaining the lens in culture, while assessing the refractive properties of

the lens by measuring the variation in back vertex focal length of an incident laser beam at several points across the diameter of the lens.

The aim of this study is to investigate the effect of hyperglycaemia on the back vertex focal length and spherical aberration of the lens using an ex-vivo bovine lens model. Two specific experiments were performed: first an investigation of the optical properties of lenses maintained at medium and high physiological levels of glucose compared to controls and second, changes in the optical properties when lenses were rapidly returned to normal glucose levels simulating normalisation of aqueous glucose levels following the initiation of treatment.

Materials and Methods

Dissection and Bovine Lens Culture

Following local ethical committee approval, bovine eyes from young adult animals two to three years old were obtained from a local abattoir within three hours post euthanasia and transported to the laboratory on ice. After immersion in iodine solution, the eyes were bisected at the equator and lenses were carefully excised under sterile conditions. The lenses were then placed in specially designed holders made up of an aluminium base fitted with a silicone rubber insert and an upper chamber of optical quality glass.

The glass chamber was filled with fresh culture medium, which consisted of Medium 199, supplemented with Earle's salts, 0.1 mg/L L-Glutamine, 2.2 gm/L Sodium Bicarbonate, 5.96 gm/L HEPES, 8% FBS, 10mg/L Phenol Red and antibiotics (penicillin/streptomycin) (www.sigmaaldrich.com), which bathed both anterior and posterior lens surfaces. Lens holders were covered with a small sterile petri dish and then transferred to an incubator at 37℃, 5% CO_2 for 24 hours. At this stage, any lenses showing visible signs of mechanical damage or opacification were discarded and not used in subsequent experiments The culture medium and lens holders were prepared and maintained at all times under sterile conditions.

Optical measurements; repeatability

Optical measurements were made with a Scan Tox™ scanning laser system, which has been fully described by Dvorat and Sivak.[24] A brief description is given here for completeness. The scanner is designed to measure the back vertex distance at points across the diameter of the lens. A low power laser source (670 nm 4 mW diode) projects onto a plane mirror assembly inclined at 45 degrees which reflects the laser beam onto the

cultured lens. The mirror is mounted on a motorised carriage assembly which moves the mirror in 0.5mm steps under computer control allowing the laser to scan across the lens. A digital video camera and frame grabber produces a cross sectional image of the beam at each position of the mirror and the Scan Tox™ software then gives a measure of the back vertex distance and intensity for each beam (Figure 1). The analysis method of the output from the Scan Tox™ to derive measures of back vertex focal length and higher-order aberrations is given in the data analysis section below.

Prior to measurements, lenses were maintained overnight in M199 containing 5mM glucose. The lenses were then transferred to fresh medium for 30-60 minutes before being placed in the ScanTox laser scanning system. Repeatability of the Scan Tox™ laser scanning system was assessed by measuring 3 lenses 6 times at approximately 10 minute intervals with the lens holder remaining *in-situ* in the Scan Tox™ system.

Lenses maintained under hyperglycaemic conditions

To study the effects of hyperglycaemia on the optical properties of the bovine lens, culture media containing varying concentrations of glucose were prepared. The culture medium for the control group of lenses consisted of the standard supplemented M199 medium which contains a physiological concentration of glucose (5mM). This medium was designated 'normal' glucose. The medium representing 'moderate' glucose had further glucose added to a final concentration of 15 mM. Medium with a glucose concentration of 30mM was designated 'high' glucose. The three experimental conditions were chosen to reflect the levels of glucose in normals and the range of hyperglycaemia that can occur in patients with diabetes

Following an overnight incubation in normal glucose medium, lenses were transferred to fresh medium containing normal, moderate or high glucose and returned to the incubator. Measurements were taken with the Scan Tox™ system as described previously two to three times per day for five days. Media were replenished with a fresh solution at approximately 48hr intervals. A total of 12 lenses were measured for each level of glucose.

Lenses returned to normoglycaemia following hyperglycaemia

To model the effect of starting diabetic therapy when glucose levels return to near normal levels rapidly, seven bovine lenses were maintained in 'high' glucose (30mM) culture medium for five days before being transferred to normal glucose medium (5mM) and returned to the incubator. The lenses were scanned two to three times per day for five days to study the change in their optical properties when the glucose level is stepped down.

Data Analysis

All data handling was carried out in Excel 2007 (Microsoft Corporation, Waltham, MA). Data derived from the Scan Tox™ system first had unnecessary characters (brackets, comas etc.) removed using global research and replace commands and then the remaining numbers were converted from text into number format. The maximum beam scan was fixed at +/- 6.25mm and the central four data points were removed as advised by developers of the Scan Tox™ system. Data points were further screened to remove those affected by dust particles where a sudden large change in reading is occasionally evident and as additionally advised by the developers. To derive values for the back vertex focal length and primary longitudinal spherical aberration, the individual back vertex distance measurements were fitted with an aberration polynomial, representing the longitudinal ray aberration $L(y)$, of the following form,

$$L(y) = A + By + Cy^2 + Dy^4 \qquad (1)$$

where y is the normalised pupil coordinate (entry beam position), A is the back vertex focal length, B tilt, C the primary longitudinal spherical aberration and D secondary longitudinal spherical aberration. This polynomial is derived from the Hamilton wavefront expansion, which is differentiated to obtain the transverse ray aberration, before converting to a longitudinal ray aberration using trigonometry.[25] *Equation (1)* was programmed into the curve fitting routine in SigmaPlot 10.0 (SysStat Software Inc, Chicago, USA). The curve fitting software uses a Levenberg-Marquardt minimisation routine to adjust the polynomial coefficients. The routine stops when the difference on successive iterations between the absolute value of the norm of the residuals falls below 1e-10. Statistical analysis was carried out using SPSS 19 (IBM Corp, NY, USA) and MiniTab v14 (MiniTab Inc, PA, USA).

Results

Repeatability

The repeatability of individual data points produced by the Scan Tox™ was assessed by the average standard deviation of all measurement points from the three lenses tested since a one-way ANOVA showed no significant difference in standard deviation values between the lenses (*P = 0.11*). The median standard deviation for each measurement point was 0.26mm (Interquartile range 0.14 to 0.50mm) demonstrating good repeatability for the raw data points. The median value has been used since the data were slightly skewed with some larger values caused by possible dust particles or readings at the edge of the lens.

Repeatability of the fitted values for the polynomial coefficients was also assessed since they are the main outcome measures used in the present study. Repeatability was high for back vertex focal length, A, (38.72 ± 0.24mm, 38.04 ± 0.18mm and 44.02 ± 0.22mm for the three lenses respectively; all values mean ± SE) and moderate for primary spherical aberration, C, (-12.42 ± 0.51mm, -5.74 ± 0.23mm and -2.15 ± 0.625mm for the three lenses respectively; all values mean ± SE). The repeatability within lenses was generally good and this gave us confidence in the ability of the Scan Tox™ to discern meaningful changes in the back vertex focal length although we note the inter-lens variation. Spherical aberration measures were much more variable with most lenses demonstrating negative spherical aberration but of varying magnitudes. In part this is due to dependencies between polynomial coefficients and in particular the primary and secondary spherical aberration coefficients, which can sometimes produce large values but opposite in sign for coefficients C and D.

Lenses maintained at normal, moderate and high glucose levels

Figure 2 shows the average variation in back vertex distance with beam entry position for lenses maintained at normal (5mM), moderate (15mM) and high (30mM) glucose. The measurements represent the average for 12 lenses for each glucose concentration and were measured at an average of 100.5 hours post immersion in the test medium (range 93 to 120 hours). The corresponding aberration polynomial coefficients A and C representing back vertex focal length and primary longitudinal spherical aberration together with their standard errors are shown in *Table 1*. The change in the A coefficient demonstrates that back vertex focal length decreases with increasing glucose concentration. This corresponds to a myopic shift although the confidence intervals are widely overlapping. Primary longitudinal spherical aberration is much more variable and shows no similar trend.

Variation in lens optical properties over time for normal, moderate and high glucose levels

Changes in back vertex focal length (polynomial A coefficient) and primary longitudinal spherical aberration (C coefficient) with time were modelled using linear regression in the absence of any evidence of a different relationship or underlying process. *Figure 3* shows the distribution of linear regression coefficients of all 36 lenses tested. Regression coefficients varied about zero and there was no apparent difference between the control, moderate and high glucose conditions. Similar results were obtained for primary longitudinal spherical aberration (coefficient C, data not shown) with only six lenses having regression coefficients that were statistically significantly different from zero ($P < 0.05$). A one-way ANOVA with lens group as factor demonstrated that there was no statistically significant

difference between the lens groups for either back vertex focal length (*P = 0.73*) or primary longitudinal spherical aberration (*P = 0.69*).

Step-down data

Changes in back vertex focal length (polynomial A coefficient) and primary longitudinal spherical aberration (C coefficient) when lenses are "stepped down" from a high to a normal glucose medium are shown in *Figure 4* along with their associated regression lines. The slopes are variable and close to zero although most are positive indicating a hyperopic shift as the lenses are returned to normal glucose concentrations. *Table 2* presents the slopes of the linear regressions for each of the seven lenses. The average regression slope for back vertex focal length was +0.0257 mm/h which was statistically significantly different from zero (*P = 0.031*; single sample t-test). The average regression slope for primary spherical aberration was -0.0211 mm/h, which was not statistically significantly different from zero (*P = 0.67*).

Discussion

The primary aim of the present study was to measure the effects of acute hyperglycaemia on the optical properties of the crystalline lens. These in-vitro conditions sought to replicate the environment of the lens in the early stages of diabetes mellitus where refractive fluctuations have been shown to occur. We know of no similar studies using near normal physiological levels of glucose. Lenses that were maintained in culture medium containing normal physiological levels of glucose (5mM) acted as controls and were compared with lenses that were exposed to moderate (15mM) and high (30mM) levels of glucose for periods averaging 100 hours. Although we identified a trend towards myopia, with the back vertex focal length decreasing in line with increasing glucose concentration, the differences between lens groups were not statistically significant. Similarly, there were no significant differences in primary longitudinal spherical aberration. Equivalent studies in humans that have investigated the short-term effects of acute hyperglycaemia in healthy young volunteers,[20,26] and patients with newly diagnosed diabetes mellitus have similarly failed to demonstrate a consistent change in refractive status.[3,11,16] Our results therefore indicate that even by controlling for some of the inherent variation in clinical studies by isolating the lens in an in vitro model, we have not been able to demonstrate an unambiguous and statistically significant change in the optical properties of the bovine lens.

It is possible that inter-species differences may have accounted for the lack of response to increasing and decreasing glucose levels. Although the bovine lens has a similar sutural

system to the human lens, there are differences in fibre structure and organisation which may reduce its ability to undergo dynamic changes in curvature. [27] A previous morphometric study has shown that bovine lenses placed in solutions of widely varying tonicities (hypo and hypertonic) are largely refractory to osmotically-induced changes in curvature or thickness.[28] An alternative explanation is that, as with other mammalian lenses, the bovine lens possesses an efficient homeostatic mechanism to mitigate against osmotic challenge.[29] Experimental models using isolated lenses placed in anisotonic media have shown that the lens has an effective volume regulatory mechanism through the activation of membrane-bound ion channels e.g. potassium chloride co-transporters, sodium potassium ATPase and chloride channels.[13,14] A further osmotic counterbalance is provided by the organic osmolyte sorbitol.[30,31] Intracellular sorbitol accumulation increases lens osmolarity, thereby establishing an osmotic gradient that favours lens hydration which may potentially offset the dehydration induced by acute extra-lenticular hyperglycaemia.
Although the lens appears to be capable of maintaining its volume in the face of extreme shifts in tonicity, following repeated osmotic insults it is possible that these osmoregulatory mechanisms may become compromised. This could be further exacerbated by an increase in oxidative stress through depletion of the antioxidant glutathione.[32,33] These oxidative mechanisms have been implicated in the development of diabetic cataract,[29,32] which shows a unique damage phenotype in the outer lens cortex. The involvement of an osmotic mechanism in diabetic cataract formation is also implied due to the fact that tissue damage is preceded by swelling of lens fibres.

It is also possible that inter-lens variation may have masked a small but significant change in BVP. A post-hoc ANOVA was carried out on the control lenses to partition the intra and inter lens variation in back vertex focal length. The pooled intra-lens standard deviation was ±4.04mm which is higher than the values found in our repeatability measurements. The reason for the higher standard deviation could well be the longer time period over which the lenses were measured (120 hours compared to one hour for the repeatability test). The lenses were not controlled for rotational alignment and this could have added to the variability. However, the value for the pooled intra-lens variation would suggest that this source of additional variation and other effects when placing the lenses in the ScanTox™ were small. The inter-lens standard deviation is higher at ±7.34mm, which is unsurprising given that we can reasonably expect a range of lens powers. Both intra and inter lens variation are likely to affect whether results are statistically significant or not.

The impact of chronic hyperglycaemia on refractive status has been studied in populations of patients with type 1 diabetes mellitus. For example, a positive relationship was established in

a Danish population between poor glycaemic control and myopia.[4] Patients with an HbA1c above the median value (8.8%) were found to have a 60% increased risk of a myopic shift compared to those who are well-controlled. Although the relationship was not linear, the authors suggested that there could be a threshold value of HbA1c, above which myopia is likely to occur. It may also be that poor glycaemic control contributes additional stress to the volume regulatory channels affecting the lens over longer periods of time. The effect of fluctuating glucose levels on the lens in vitro has yet to be studied.

The most consistently reported relationship between plasma glucose concentration and refractive status is induced hyperopia following the initiation of diabetes therapy. Significant hyperopic shifts of a few dioptres have been reported.[8-10] The hyperopia develops over a period of a few days to a few weeks, followed by a more gradual return to the baseline refraction.[9] Okamoto and co-workers concluded that the magnitude of the refractive change depended on the pre-treatment plasma glucose concentration.[8] Our results for lenses stepped down from 30mM to 5mM glucose show an average change in back vertex focal length of +0.0257mm/h correlating to just over 3mm of change for a five day period. This is equivalent to an approximate 3D reduction (hyperopic shift) in back vertex power of the bovine lens, which is a similar order of magnitude to changes reported over the same time frame by.[9] There has been considerable debate regarding the source of this refractive change.[18] It is generally assumed that the thickness and curvatures of the lens remain unchanged and it has been hypothesised from optical modelling that the hyperopic shift is most likely due to a decrease in lens refractive index.[18]

Conclusions

The current study found no evidence for a consistent change in either the back focal length or longitudinal spherical aberration of bovine lenses at any time point in response to acute hyperglycaemia. This is may be due to the ability of the lens to osmoregulate in the short-term to offset any change in lens shape or refractive index. We identified a trend towards myopia with increasing hyperglycaemia, which while not statistically significant, might suggest that a more pronounced change could occur in the longer-term. A trend towards hyperopia was also observed for lenses stepped down to normal glucose levels in line with clinical data supporting the argument that lens changes are the cause of refractive variation. The difficulties of maintaining lenses in long-term organ culture mean that the current bovine model may not be suitable for investigating the effect of chronic hyperglycaemia on the optical properties of the lens. Consequently, further work could investigate the use of lenses

extracted from an experimental model of diabetes e.g. streptozotocin-induced diabetes in rodents.

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Disclosure

The authors report no conflicts of interest

Figures

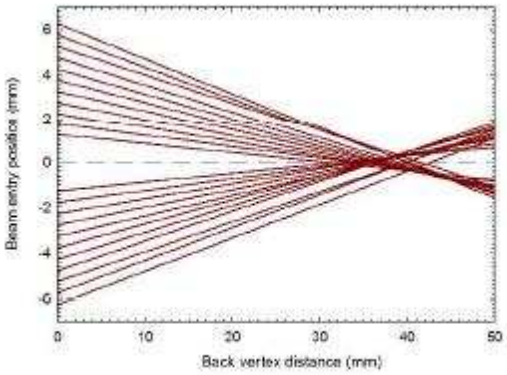

Figure 1: Scanning laser profile for each refracted beam (central four beams removed – see text) in a control lens.

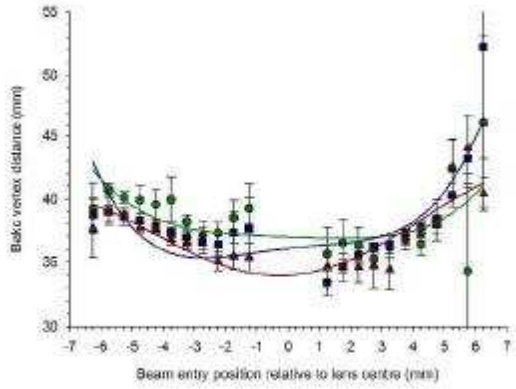

Figure 2. Average back vertex distance variation for bovine lenses (N=12 each group) immersed in normal (green circles), moderate (blue squares) and high (red triangles) glucose concentrations. Solid lines represent the respective fitted aberration polynomials. (Error bars all ± 1 SE)

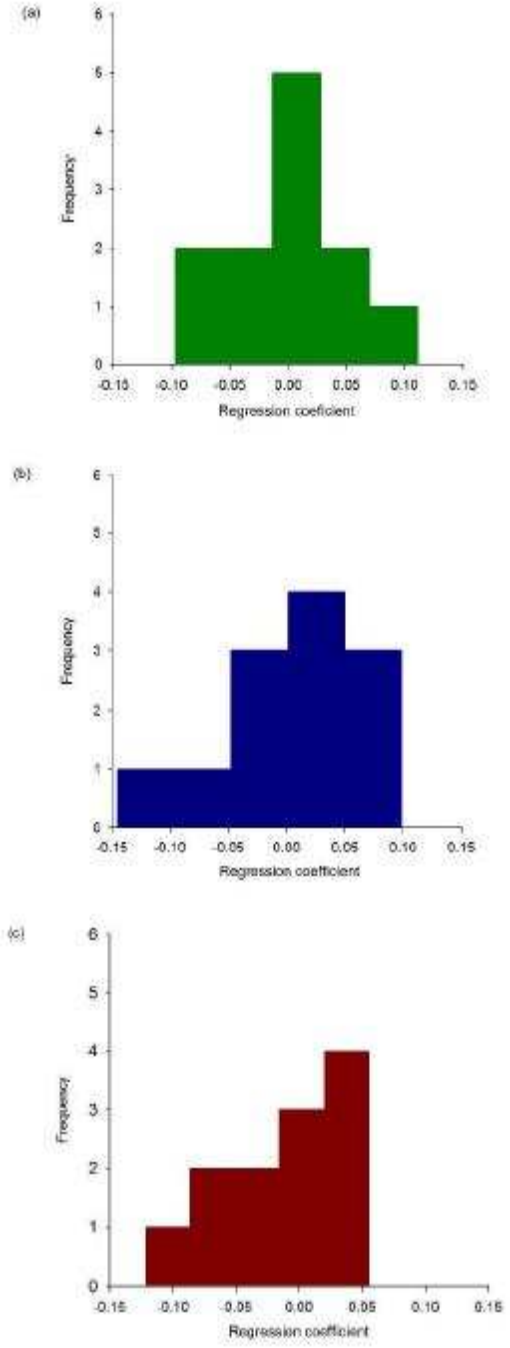

Figure 3. Distribution of linear regression coefficients for lenses at (a) normal, (b) moderate and (c) high glucose demonstrating the change in back vertex focal length with time.

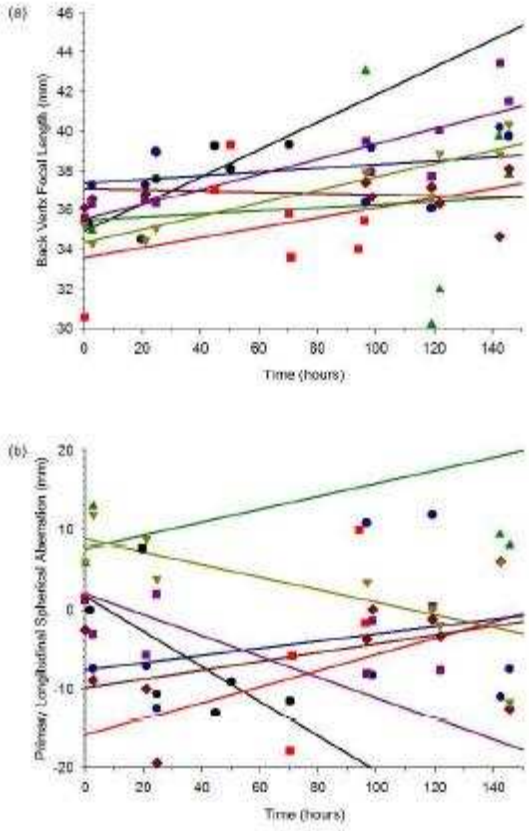

Figure 4. Variation in back vertex focal length, (a), and primary longitudinal spherical aberration, (b), with time for seven lenses stepped down from high to normal glucose concentrations (see Methods for details). Lines represent the corresponding linear regression slopes.

Tables

Glucose concentration	Back vertex focal length (mm)	Primary spherical aberration (mm)
Normal (5mM)	36.99±1.24	-0.39±6.30
Moderate (15mM)	36.10±1.18	+1.89±6.04
High (30mM)	33.98±0.80	-9.78±4.08

Table 1. Fitted aberration polynomial coefficients for the average data curves shown in figure 2 for lenses maintained at normal, moderate and high glucose concentrations. (All values mean ± SE).

Table 1. Fitted aberration polynomial coefficients for the average data curves shown in *Figure 2* for lenses maintained at normal, moderate and high glucose concentrations. (All values mean ± SE).

Lens	Back vertex focal length		Longitudinal spherical aberration	
	Slope (mm/h)	SE (mm/h)	Slope (mm/h)	SE (mm/h)
1	+0.0693*	0.0222	-0.2218	0.1232
2	+0.0252	0.0357	+0.1027	0.1892
3	+0.0078	0.0315	+0.0816	0.1662
4	-0.0030	0.0069	+0.0554	0.0392
5	+0.0093	0.0107	+0.0453	0.0659
6	+0.0383*	0.0076	-0.1320	0.0602
7	+0.0333*	0.0051	-0.0789*	0.0297
Average	+0.0257		-0.0211	

Table 2. Slope for the regression lines showing the trend in all 7 lenses stepped down from high to normal glucose concentrations for change in back vertex focal length and primary longitudinal spherical aberration with time. *indicates statistically significant at P < 0.05.

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