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Abstract

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Methods: A custom made probe fitted to a dynamic mechanical analyzer was employed to measure stiffness values at 1 mm increments across equatorial sections of individual human lenses. Thermogravimetric analysis was used to determine the percentage water content in the nuclei of human lenses.

Results: There was a pronounced increase in lens stiffness over the age range from 14 to 78. In the nucleus, stiffness values varied almost 1,000 fold over this age range, with the largest change observed in lenses between the ages of 20 to 60. Nuclear stiffness values increased on average by a factor of 450. By contrast, in the cortex the average increase in stiffness was approximately 20 fold over this same time period. In lenses younger than age 30, the nucleus was found to be softer than the cortex. This was true for all six lenses examined. In contrast all lenses older than 30 were characterized by having nuclear values higher than those of the cortex. In lenses over the age of 50, the lens nucleus was typically an order of magnitude more rigid than that of the cortex. The crossover age, when the cortical and nuclear stiffness values were similar, was in the 30s. There was no significant change in the water content of the human lens nucleus from age 13 to age 82.

Conclusions: There is a marked increase in the stiffness of the human lens with age. This is most pronounced in the nucleus. Since in vivo data indicate that the nucleus must change shape significantly during accommodation, it is highly likely that these measured changes in physical properties will markedly diminish the ability of the lens to accommodate, and thus may be a major contributing factor to presbyopia. Since there was no measurable difference in the water contents of the nuclear regions of the lenses, this marked increase in stiffness is not due to compaction of the lens nucleus.

Massive increase in the stiffness of the human lens nucleus with age: the basis for presbyopia?

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The lens is unique in that there appears to be no protein turnover in the center throughout one's lifespan. This lack of turnover in the nucleus leaves it prone to age related changes. Mature lens fibers lack cellular organelles and contain a highly concentrated protein solution to enable light refraction.

Presbyopia, or "old man's eyes," affects almost all people by the age of 50. This disorder involves a loss of accommodative ability, where near focussing becomes impossible. Herman von Helmholtz [1] first expounded the basis for accommodation. In this scenario, contraction of the ciliary muscles leads to a change in the lens curvature. This optical system moves between two states; one where muscular force is applied to the zonules which flattens the lens and allows distant vision, and an accommodated state where zonular tension is released and the lens assumes a more rounded shape to allow focussing on near objects. The exact reason for the development of presbyopia is unknown, and many hypotheses have been proposed. One theory postulates that changes in the ciliary muscle are implicated [2-5] and another school of thought suggests that presbyopia is the result of age related changes to the lens or capsule [6-9]. In the latter hypothesis it is suggested that

changes with aging result in the inability of the lens to alter shape from the flattened form, to that of the more rounded accommoded state, when zonular tension is released. Presbyopia has also been described as a geometric disorder resulting from the increased size and volume of the lens and the angle of zonular insertion onto the lens [10]. The vitreous has also been proposed to contribute to accommodation [11].

It has been found that there is an increase in the lens cross sectional area in the accommodated form and that this difference in cross sectional area between accommodative states declines with age [12]. A recent in vitro study found that replacement of a presbyopic lens with a flexible polymer restores accommodative ability [13], strengthening the case that lenticular causes may be a major contributor to presbyopia.

It is now clear that the lens nucleus must change shape significantly to enable transition to the accommodated state [14-16]. The extent of change required by the nucleus is far greater than that of the cortex, which may not change shape to any great extent [16].

Sclerosis, or hardening, of the lens tissue would obviously hinder deformation and would significantly reduce the ability of the lens to undergo the changes required for accommodation. Preliminary data suggests that sclerosis of the human lens center might occur with age [17]. It has been suggested that the changes in physical properties may be due to fiber cell compaction in the nucleus [18], as has been found for

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several laboratory animal lenses. However, compaction of the lens center does not appear to take place in normal human lenses [19,20] although the number of lenses analyzed in these studies has been small.

A number of methods for analyzing the physical characteristics of lenses have been developed. These include testing using ultrasound wave attenuation [21], and the use of an automated guillotine [22-24]. The main focus of these studies has been the investigation of hardness changes in cataract lenses, since this is an important factor when phacoemulsification is to be employed during surgery. Few studies have looked at how normal lenses change with age. The bulk of studies on normal lenses have used techniques such as applied radial force [8,9,20], or compression/stretching [25-27] tests. These have shown that there is a considerable decline in the ability of the lens to deform with age. However, the use of these techniques is limited due to the inability to differentiate between capsular and lenticular changes, and a limited ability to detect differences throughout the lens. The use of a conical probe to examine changes within the lens showed that an increase in hardness does occur with age [17]. This method involved the use of a miniature dynamometer, in which a probe gradually penetrated the lens. The increasing friction on the probe as it encounters greater depths in the lens is a problem with this method of analysis, and there is also a possible effect of compaction as the probe is inserted deeper into the lens.

The aim of the present study was to generate a cross sectional map of stiffness across the lens and to examine how this profile changes with age. Instrumentation that has been used to analyze materials such as hydrogels was employed. The results of this study are surprising. Stiffness of the lens

center was found to increase by approximately 1,000 times and the outer region of the lens by a factor of 50. In addition we found that the nucleus of younger lenses was actually softer than the cortical region. The massive change in the physical properties of the lens center with age was not accompanied by a change in water content.

METHODS

Analysis of lens stiffness: Normal lenses covering a broad age range were collected from the Lions NSW Eye Bank at the Sydney Eye Hospital. These were transferred to Wollongong and stored at -80 °C. Lenses (18) between the ages of 14 and 76 years were analyzed with at least 2 lenses per decade. The work was approved by the human research ethics committee at the University of Wollongong.

Lenses were removed from the freezer, partially thawed and then sectioned equatorially. The lens halves were then cored using an 8.5 mm internal diameter trephine incorporating a removable 3.5 mm high steel ring which retained the lens sample (Figure 1 and Figure 2). Once the ring containing the lens was removed from the trephine, if necessary, a razor blade was passed across the top of the 3.5 mm high steel ring to produce a flat surface corresponding to an equatorial section roughly through the middle of each lens. This procedure provided support for the outer edges of the lens sections and also normalized the sizes of all the lens samples.

The lens sample held within the ring was then placed into a sample holder consisting of two pieces of Perspex. The top piece has a central indent in which to position the metal ring (Figure 1D,E), and two metal pins through it allowing it to locate into the base piece. The base piece has a 10x10 1 mm grid pattern drilled in it. This allowed movement of the sample holder by 1 mm. The bottom of the base piece of the sample holder was shaped to fit the stage of the penetration clamp of the dynamic mechanical analyzer (DMA).

The instrument used for the analysis was a Q800 DMA (TA Instruments, New Castle, DE). A custom made steel cylindrical probe was designed with a flat bottom and a diameter of 0.4 mm. This small probe enabled measurements to be taken every millimeter across the surface of the lens samples. All readings were performed at 22 °C.

Stiffness measurements on the lens sections were performed using a controlled force test. The probe was positioned on the lens surface and the force was ramped from 0 to a maximum force of 3 mN, at a rate of 1 mN per minute, while the change in displacement over the course of the run was monitored. Typically the displacement was of the order of 750 μm. These measurements commenced at the edge of the sample ring and then moved by 1 mm increments across the lens surface. Three runs across each sample were taken wherever possible. This consisted of a run across the center of the sample and then subsequent runs parallel to, but 1 mm above and below the initial traverse. It did not affect the results significantly if measurements were commenced in the lens center or the edge (data not shown). The nuclear value used for calculations of shear modulus was either the fourth or the fifth measure of stiffness across the lens, whichever was greater.
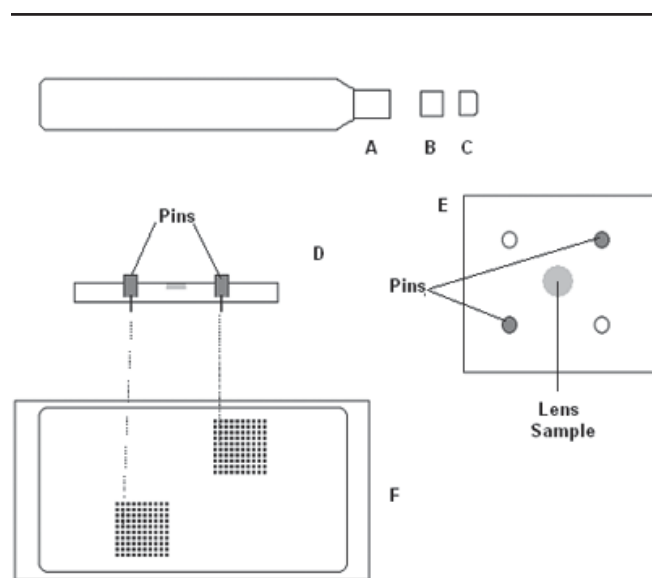

Figure 1. Lens cutting device and sample holder. **A**: Cutting device. **B**: Hollow trephine. **C**: Spacer cutter/sample holding ring. **D**: Side view of the top piece of the perspex sample holder. **E**: Top view of the top plate of the perspex sample holder. **F**: Top view of the base piece of the perspex sample holder.

Each measurement took approximately 3 min to complete, so three runs of eight measurements involved a total analysis time of 90 min per lens. To alleviate any possible sample dehydration, a plastic housing containing a large piece of moistened foam rubber was used to surround the probe.

Analysis of water content: Measurements of lens nuclear water content were performed using thermo-gravimetric analysis (TGA) at the University of Technology, Sydney. The instrument used was an SDT 2960 (TA Instruments).

Whole lenses were partially thawed in a petri dish, and then cored using a 4 mm trephine. The core had the top and bottom ends (approximately 1 mm) removed leaving a cylinder 4 mm in diameter and approximately 4 mm in height. This material was transferred to a platinum crucible, and immediately placed in the TGA for analysis.

TGA analysis involved heating from ambient temperature to 300 °C at 5 °C per minute. The loss in mass of the sample was monitored over time, and the total water loss was determined at the point where the derivative value of loss in mass dropped to a value near to zero. Typically this was at about 190 °C. The remaining mass was considered to represent protein.

RESULTS

This study was undertaken with a view to determining the stiffness profile across human lenses and to examine if there were any consistent alterations with age. A DMA was chosen as the instrument of choice. Since the cross sectional area of human lenses is quite small, a probe with a narrow (0.4 mm diameter) bore was designed and manufactured. A special base plate was also designed to enable measurements at 1 mm intervals across each lens, and a trephine constructed as shown in Figure 1 to produce an equatorial section of tissue constrained within an 8.5 mm metal ring that was independent of
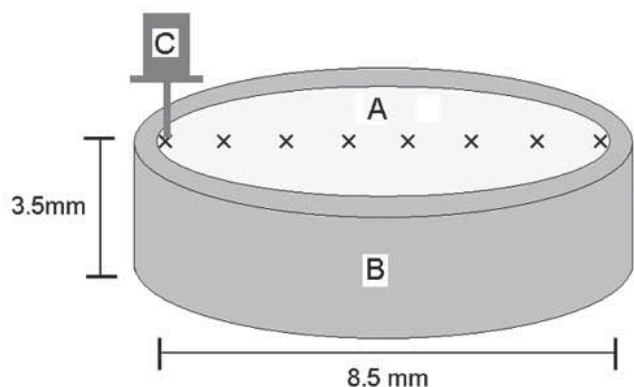
the original size of the lens. This apparatus allowed the measurement of 8 readings across each lens (Figure 2). In most cases three passes were made across the diameter of the lens sections and the results at each point were averaged. In order to measure the stiffness at each point, a protocol was adopted in which the displacement of the probe was determined following ramping of the force from 0 to 3 mN at 1 mN per min.

The results were converted into values of shear modulus using the equation:

$$P = \frac{4RdG}{1 - \nu}$$

where "P" is total load (N), "R" is radius of indentor (m), "d" is maximal depth of penetration (m), "G" is shear modulus (Pa), and "ν" is Poisson's ratio for the material [28]. For an elastic rubber like material, we assumed that $\nu = 0.5$ and that we were using a perfectly rigid flat punch in frictionless contact with a semi-infinite (large enough that edge effects are not encountered), fully elastic material.

The results of a typical analysis of a 64 year old lens are shown in Figure 3. It is clear that there is a substantial increase in the stiffness of the lens as the probe is moved closer to the lens center. As would be expected, the profile is roughly symmetrical about the central point.

In contrast the results of an analysis of a 19 year old lens are shown in Figure 4. The values for shear modulus were lower in this case and the central region of the lens was less stiff than the cortex. This was a surprising finding. Again the profile was symmetrical about the central point. Since these

Figure 2. A schematic diagram showing the method used for measurement of lens stiffness. The equatorial section of a lens (**A**), obtained as illustrated in Figure 1, was held in a metal ring (**B**) and the lens section was moved across and underneath the DMA probe (**C**) in 1 mm steps (see Figure 1). At each point a measurement of stiffness was made.
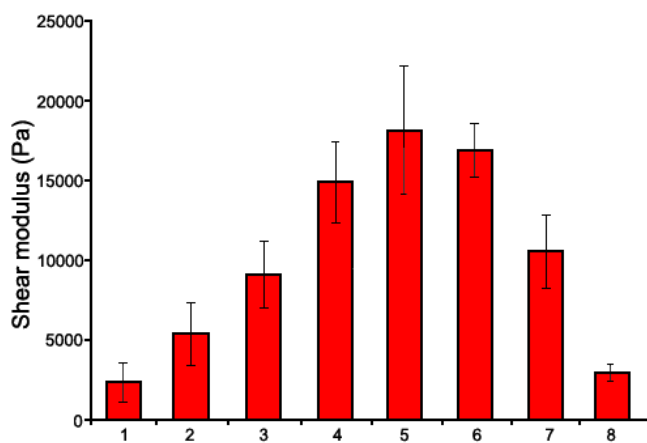

Figure 3. The stiffness profile of regions in an old human lens. Shear modulus measurements taken 1 mm apart across the equatorial section of a 64 year old lens (error bars represent standard deviations; n=3).

two lenses gave stiffness profiles that were quite different in appearance, we applied this DMA technique across an age range from 14 to 76 years. A summary of the results is depicted in Figure 5. Because the changes in lens stiffness were of such magnitude, it was only possible to compare them using log scales. These illustrate clearly that there is a consistent age dependent increase in stiffness in both the cortex and the nucleus of human lenses.

The magnitude of these changes in the two regions was, however, quite different. In the cortex there was, on average, an approximately 20 fold increase in lens stiffness over the age range from 14 to 76 (Figure 5 and Table 1). The lowest cortical value (48.5 Pa) was determined in a 14 year old lens and the highest (2,577 Pa) in a 76 year old lens. This corresponds to a 53 fold change. In the nucleus this increase in lens stiffness was much more pronounced, amounting to, on average, 450 fold in the space of just over 50 years (Figure 5). The values ranged from the softest nuclear value (25.7 Pa) measured in a 20 year old lens, to the hardest (23,954 Pa) in a 73 year old lens, a 930 fold increase.

The nuclear values of lens stiffness in younger lenses were consistently lower than those of the cortex. For the age group younger than 30 years, of which there were 6 individuals, the mean shear modulus value for the nucleus was 39.0 ± 13.8 Pa, compared to 98.3 ± 64.5 Pa for the cortex. Analysis of these results with a paired Student's t-test showed that this was statistically significant ($p < 0.05$). In lenses older than 60 years, the mean value for the nucleus was $17,400 \pm 4,900$ Pa, and $2,040 \pm 710$ Pa for the cortex. Analysis of these results with a paired Student's t-test showed that this was also statistically significant ($p < 0.02$). Since the nuclear values increase at a more rapid rate than those of the cortex, there must be a crossover point. As is illustrated in Figure 5, this crossover occurred at approximately age 30-35. Although more lenses need to be analyzed to confirm this trend, the largest changes seem to be observed in lenses between the ages of 20 and 60.

In order to confirm that the shear modulus values measured with the custom made probe were correct, the shear modulus values of a hydrogel of similar stiffness were also determined. The elastic modulus (E) of the dimethylacrylamide based hydrogel had previously been measured by compression testing on the DMA and was found to be 20 ± 5 kPa. The hydrogel was tested using the custom made indentation probe in the same manner as the lenses, and a shear modulus of 6.29 kPa was determined. When this was converted to elastic modulus by the standard expression

$$E = 2G(1 + \nu)$$

giving an elastic modulus value of 18.9 kPa. This is in very good agreement with the results found by compression testing.

These are remarkable changes in physical properties of the human lens. The reasons for these are not yet known, but some features can be ruled out. There was, for example, no evidence for lens compaction. If lens compaction were taking place with age, the percentage of protein in the nucleus should
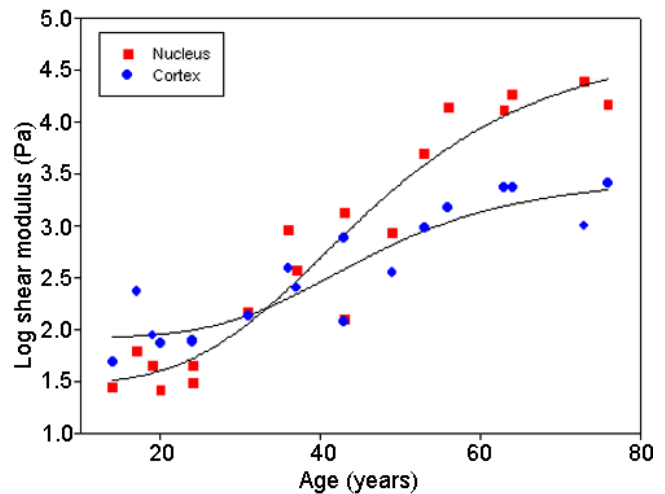
Figure 5. Stiffness values of cortex and nucleus as a function of lens age. Nuclear and cortical shear modulus values plotted on a log scale as a function of age for individual lenses. Nuclear values are shown in blue and cortical values in red. The curves were fitted using SigmaPlot (Systat Software Inc., Richmond, CA), these curves were nonlinear regressions using a Hill 4 parameter sigmoidal function:

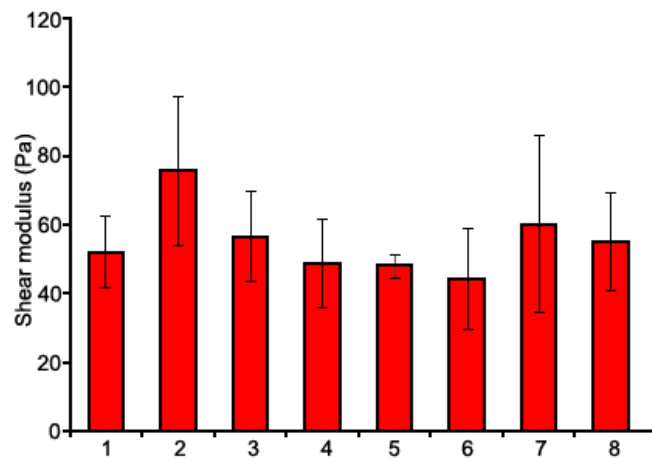$$y = y_0 + \frac{ax^b}{c^b + x^b}$$

Figure 4. The stiffness profile of regions in a young human lens. Shear modulus measurements taken 1 mm apart across the equatorial section of a 19 year old lens (error bars represent standard deviations; n=3).

For the curve of nuclear data, $R^2 = 0.9215$ with standard error of mean=0.3436. For the curve of cortical data, $R^2 = 0.8364$ with standard error of mean=0.2617. Mean and standard errors of coefficients are listed in Table 1.

increase. As shown in Figure 6, no significant change in the percentage of protein was noted over the whole age range. It should be noted that the lenses used for analysis of stiffness were, in several cases, the contralateral lenses of those used for the determination of nuclear hydration. These are indicated on the graph (Figure 6).

The finding of no apparent change in percentage protein with age in the human lens is in agreement with that of van Heyningen [19]. Others have found variable results on water content with age [29,30], in this regard human lenses are clearly different from those of common experimental animals. Rats [19], rabbits [19,20], and cattle [19] all show a clear increase in lens protein content as the animals age.

DISCUSSION

In this study large changes in the biophysical properties of normal human lenses as a function of age have been demonstrated. The observed increase in stiffness was much more evident in the center of the lens.

Although nuclear compaction does not appear to be associated with the huge changes in stiffness that we documented,

TABLE 1. COEFFICIENTS FOR CURVES FITTED TO NUCLEUS AND CORTEX DATA

Parameter	Coefficient	Standard error	t	p
Nucleus				
a	3.3715	0.9355	3.6041	0.0029
b	3.8748	1.7377	2.2299	0.0426
c	46.4359	6.1631	7.5345	<0.0001
y0	1.4744	0.2428	6.0725	<0.0001
Cortex				
a	1.5646	0.5292	2.9566	0.0104
b	4.6281	2.8053	1.6498	0.1212
c	46.0387	7.1049	6.4799	<0.0001
y0	1.9175	0.1499	12.792	<0.0001

Mean (coefficient), standard error, and probability (t is the Student's t-test statistic) values of the nonlinear regression fitted to the data sets in Figure 5. In the "Parameter" column, y0 is y_0.
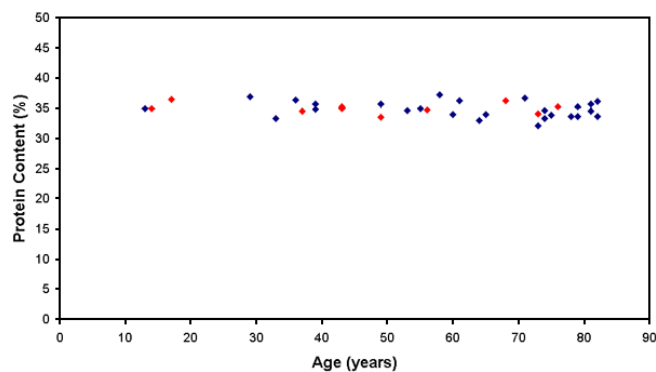

Figure 6. Protein content in the nuclei of individual lenses. Protein, expressed as a percentage of lens wet weight was calculated by measuring water content and is plotted as a function of lens age. Values in red indicate the contralateral lenses of those used for determination of shear modulus.

there is not yet a clear picture of the molecular changes that are responsible. It is clear, however, that there is a marked parallel between the findings of increased lens stiffness described here, and the well known age dependent alterations in accommodation [1,3,4,26,31]. This is illustrated in Figure 5 and Figure 7. In addition, the maximal change in lens power clearly shows a marked change from teenage years, such that by age 60 human lenses show essentially no change in focal length with stretching [26]. As has been summarized in recent articles by Croft, Glasser and Kaufman [31,32], the basis for presbyopia remains unclear but has been largely ascribed to two possible causes involving either the ciliary muscle or the lens. Other theories have also been advanced. These include a geometric theory based on the age dependent alteration in the sites of attachment of the zonules [10,33] coupled with lens growth and multifactorial [34] postulates. The vitreous has also been suggested to play a part in facilitating asymmetric alterations in lens dimensions and forward movement of the lens during accommodation [11]. It is well established that the consistency of the vitreous alters with age [35].

It is clear that our results support changes to the properties of the lens as being implicated. As documented by Koretz and coworkers [16] and others [14,15] normal accommodation in humans involves changes to the shape of the lens nucleus. If the nucleus becomes progressively stiffer with age, it is not surprising that the lens becomes much more difficult to deform. This is especially true if the change in stiffness involves nearly 3 orders of magnitude.

Of considerable interest is the fact that these results reveal changes in lens hardness starting from the teenage years
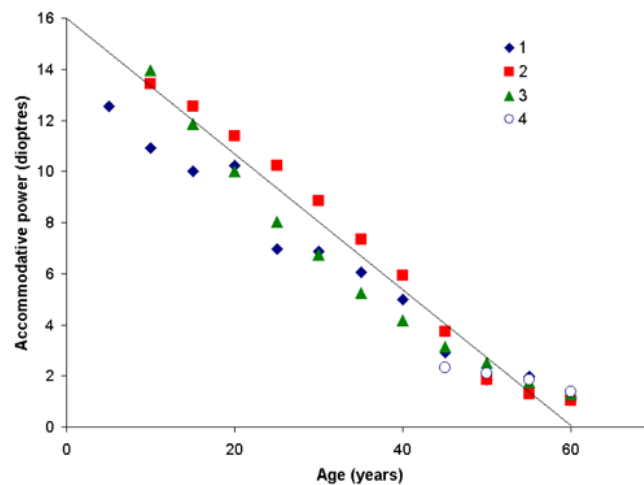

Figure 7. Change in accommodative power (diopters) as a function of age. Accommodative power of human subjects expressed as a function of age. The data points on the graph represent a summary of four separate studies on human accommodation as indicated by the following references. Data were taken from [49] for curve 1 (blue diamond), from [50] for curve 2 (red square), from [51] for curve 3 (green triangle), and from [52] for curve 4 (open circle). This includes the regression of a straight line as modified from Pierscionek and Weale [53].

and continuing into old age. These are most dramatic in the nucleus. The shortest attainable focal length of the human lens increases almost linearly as a function of age [31]. The recent in vitro demonstration that accommodation can be restored to human lenses by implanting intraocular lenses made from a flexible polymer [13] also supports a key role for the lens in presbyopia.

Quite remarkably, DMA analysis of the younger lenses showed that the nuclear region was softer than the cortex in lenses younger than age 30. This is in contrast to those lenses aged over 30. This finding is in agreement with those of Fisher [9] but at variance with those of Pau and Kranz [17], although these authors did not show separate data for cortex and nucleus.

It should be noted that while this study was underway, Weeber and Eckert in the Netherlands were conducting a similar investigation also using DMA analysis [36]. Although their methodology was slightly different from ours, the results that they obtained seem to mirror those obtained here. For example, they reported, using a smaller number of lenses, an approximately 10,000 fold increase in shear modulus in the nucleus over a similar age range, and in their abstract describe that the nuclear values for stiffness in young lenses were less than the cortical values.

All lenses used in this study had been stored frozen. It is possible that fresh lenses may behave differently and this will be investigated in a future study. Analysis of fresh lenses is more difficult technically due to their "stickiness" making coring and lens sectioning more difficult.

While our results demonstrating increased hardness of isolated human lenses mirror those found by others for decreased accommodative power measured in the eyes of volunteers of different ages, several caveats should be noted. First, the process of accommodation involves stretching, rather than the compressive forces that were used for DMA analysis. Second, we cannot discount a contribution from proteolysis. Proteases may become active after we section the lenses prior to DMA measurements, although it would be expected that levels of endogenous proteases in the nucleus will be low, especially in older lenses. In addition as noted above, freezing may affect the results and our calculations of lens stiffness in the nuclei of the youngest lenses may be an overestimate due to the limitations of the current methodology.

It is conceivable that the physical changes we have measured in these lenses are part of a spectrum of age related ocular alterations, all of which may contribute to presbyopia. The huge increases in stiffness could also be the result of some other factors that over time may affect lens biochemical processes. For example, lens transport [37,38] could be influenced by the regular lens deformation associated with accommodation and, if this process is compromised could, in turn, affect the biophysical properties of the lens.

It is not understood what factors determine lens stiffness and why these should change with age. In very young lenses this could be a reflection of the initial difference in protein composition in the nucleus compared with the cortex [39,40]. For example, γ-crystallins are concentrated in the nucleus since they are predominantly synthesized prenatally. It is clear however that both cortex and nucleus show similar alterations over time, although the magnitudes are quite different.

A quite unexpected finding was that the nuclei of young lenses (prior to age 30), were less stiff than those of the cortices. Given the requirement for substantial nuclear deformation during accommodation, one is tempted to suggest that such a difference may be present to facilitate the process of lenticular shape change.

Protein oxidation can also be ruled out as the reason for the large increase in human lens hardness with age. Analysis of protein sulfhydryl (data not shown, [41]) and protein methionine sulfoxide contents [42] have revealed no significant oxidation of human lens proteins past age 60. Once again, laboratory animals such as rodents appear to be poor models for aging of the human lens since in these animals there is evidence of considerable protein oxidation with age [43,44].

Progressive losses in soluble α-crystallin with age [45,46] could be implicated in the physical changes noted, since this protein is a molecular chaperone and has been proposed to play an important role in maintaining optical clarity by binding to, and sequestering, other lens proteins as they denature over time. This may also contribute to the activities of other key proteins, for example channel proteins such as connexins and aquaporin 0. Since protein aggregation within the cell may also lead to changes in the relative amounts of free and bound water, this factor may also be implicated. Age dependent changes in the composition of fiber cell membranes [47] could also influence lens stiffness. These factors are to be investigated in the next phase of this study. This work is to be repeated with a higher sampling frequency so as to correlate the size of the zone of stiffness with the barrier to diffusion that also develops at middle age. This barrier appears responsible for the later onset of age related nuclear cataract [48].

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