Author's response to reviews

Title: Analysis of nuclear fiber cell compaction in transparent and cataractous diabetic human lenses by scanning electron microscopy

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Version: 2 Date: 10 Dec 2002

PDF covering letter
Response to Reviewer 1:

Include a statistical comparison between normal and age-related nuclear using the data from the 2001 EER study. All except the EN fiber fold value are significant, like those for the diabetic cataracts based upon the 2001 study. More data would be required to try and gain significance for the fold value. These data would add more weight to the conclusion that it is cataract formation per se that alters the lens nucleus characteristics.

As suggested by the Reviewer, the statistical comparison between transparent non-diabetic lenses and non-diabetic age-related nuclear cataractous lenses from our previous study has been added to Table 2. The authors agree with the Reviewer’s opinion concerning the importance of increasing the amount of fiber fold data to gain additional insight into its possible role in cataract formation. Current studies utilizing light and transmission electron microscopy of longitudinally cut nuclear fiber cells are underway to provide additional quantitative data for lenses with age and nuclear cataract.

Response to Reviewer 2:

1) The conclusions drawn are not adequately supported by the data shown due to the lack of information on the specific nature of the specimens examined and the lack of discussion on the numerous variables and potential morphological artifacts associated with comparisons made between postmortem and surgically extracted lenses.

The authors strongly feel that the evidence presented in this study supports the conclusion that compaction, rather than swelling, occurs in the nucleus of diabetic lenses despite cortical enlargement. This data also shows that the extent of nuclear compaction in diabetic lenses is similar to that of non-diabetics, suggesting that the drastic changes in lens osmolarity associated with diabetes do not affect the occurrence of nuclear fiber cell compaction with age. In compliance with several of the Reviewer’s points below, additional detailed information concerning the nature and handling of the specimens used have been included. However, as described in the manuscript, specific personal details concerning patient age, gender, diabetic type and treatment are not available from NDRI or the surgeon (Background: paragraph 4, sentence 2; Methods, Specimens: paragraph 1, sentence 4; paragraph 2, sentence 5). Hence, the authors have stressed that the results should be interpreted as trends between the tissues (Methods, Statistical Analysis: sentence 4; Discussion: paragraph 1, sentence 2). To minimize the occurrence of potential morphological variations between postmortem and extracted tissues, identical preservation methods were used between the groups, both temporally and chemically. A sentence to this extent has been added to the Methods (Specimens: paragraph 2, sentence 6).
2) **Background section:** Since this manuscript might have general medical or scientific interest, I believe the authors need to include in the background either a reference(s) describing or define in more detail the general growth, shape and nuclei of the healthy human lens.

The authors agree with the Reviewer’s suggestion, and references to works describing the development and cellular organization of the health human lens have been added (*Background: paragraph 1, sentence 1*).

3) **Background, Paragraph 2:** Within the context of this manuscript, the authors need to define normal aging.

In this case, normal generally refers to an aged, transparent, non-diabetic, non-cataractous lens, without a history of ocular pathology. However, the general term “normal” in referring to the lens and aging may mean different things to different readers. To avoid confusion, the use of the word normal has been changed to more descriptive terms.

4) **Background, Paragraph 2:** All though referenced, the authors should site what % change equals a slight increase and nuclear decrease (sentence 1). In addition, minor differences (sentence 2) and large hydration disparities (sentence 3) should be clarified. Please reference the information in sentence 4.

As requested by the Reviewer, the percent change per year of cortical and nuclear hydration have been added to the manuscript as described in the referred article (*Background: paragraph 2, sentence 2*). In addition, the entire paragraph has been rewritten for increased clarity.

5) **Background, Paragraph 3:** Since the main emphasis of this manuscript is compaction in the lens, the author need to fully define compaction within the context of this manuscript. In sentence 2, do the authors mean transparent lenses with senescence? I would not consider a senescent lens to be "normal" since they are morphological and physiological different from a young adult lens.

As defined in our previous study, we use the term compaction to describe the decrease in cell size and accompanying changes in nuclear shape with growth, age, and disease. A sentence to this effect has been added to the *Background* (*paragraph 3, sentence 1*). In sentence 2, the word “senescent” has been removed and the sentence reconstructed.

6) **Methods, Specimens:** My major criticism of this manuscript lies specifically with the lack of critical information supplied to the reader about the specimens
use in this study to make an informed judgment about the conclusions. I feel it is critical that the reader be supplied with the following information:

a. Did any of the diabetic eyes undergo any previous laser or intraocular surgeries? Previous interventions to the eye might have affected lens morphology.

To our knowledge, none of the specimens used had a history of laser or intraocular surgery. This information has been added to the Methods section (Specimens: paragraph 2, sentence 7).

b. Were the extracapsular extractions done by the same surgeon?

All of the extraction surgeries were performed by the same surgeon using the same techniques. This information has been added to the Methods section (Specimens: paragraph 2, sentence 3).

c. Were the extracapsular lenses nuclei extracted using the same technique and handled in a similar manner after extraction (forceps, cryo, placed in formalin, placed on sponge then in formalin etc...)? This variable could have played a major role in the final morphology of the lens and could account for the differences between transparent diabetic postmortem lenses and extracaps.

Each lens nucleus was extracted using the same operative techniques. Lenses were transported to our laboratories within 4 hours of removal in vials containing gauze moistened with balanced salt solution. These nuclei were promptly placed in primary fixative to mimic the treatment of the postmortem lenses. This information has been added to the Methods section (Specimens: paragraph 2, sentences 3 and 4).

d. With advances in cataract surgical techniques, why were extracapsular cataract extractions performed on nuclear cataracts from diabetic patients? Were these nuclei extremely hard? This information should be included in the manuscript. Was coherence laser interferometry used to determine the nuclear density?

The Reviewer raises a good point concerning the use of extracapsular extraction surgery when phacoemulsification has become the preferred technique for cataract removal in the US. Obviously, lenses that are liquefied are of very little value for morphological studies such as these. Our laboratory is fortunate enough to have a long-standing relationship with a local surgeon who continues to perform routine extracapsular extractions; he has been a valuable source of samples for over 6 years. The extracted cataractous nuclei were of the hardness expected of aged lenses, not overly so. Nuclear density was determined by an ophthalmologist using a slit lamp device. To our knowledge, laser
interferometry was not used.

e. Did any of the nuclear cataractous lenses have diabetic cataracts? If not, without knowing the history of the donor, would the authors please explain and reference what the difference is between the nuclear cataracts examined in this study and an age-related nuclear cataract.

No. Information concerning the presence of diabetes for a particular lens is provided with a simple yes or no answer. Aged lenses that were diagnosed as having nuclear cataract by an ophthalmologist without diabetes are termed age-related nuclear cataracts; in the presence of diabetes, the lenses are termed diabetic lenses with nuclear cataract. The non-diabetic, age-related nuclear cataract data used in this study is the same data produced in the previous investigation. Data from diabetic lenses with nuclear cataract was not included previously, and is new to this study.

f. How many hours postmortem were the NDRI lenses collected and how were they received (hours, temperature, in formalin, moist, dry etc...)?

NDRI lenses were enucleated and placed in primary fixative between 4 to 6 hours postmortem. Specimens were shipped by overnight courier at room temperature to our laboratories for further processing. This information has been added to the Methods section (Specimens: paragraph 1, sentences 2 and 3).

g. How were the numerous variables and potential morphological artifacts associated with comparisons between postmortem and surgically extracted lenses minimized so that a relative morphological comparison could be made possible?

All lens specimens (postmortem lenses and surgically extracted nuclei) were subjected to identical fixation and SEM preparation procedures.

7) The authors refer one group of lenses as "diabetic nuclear cataractous lenses". I'm not aware of this classification of a diabetic nuclear cataract. The authors need to include a reference. Do the authors mean a nuclear cataract from diabetic patients?

The authors agree with the Reviewer’s observation. The expressions “diabetic nuclear cataractous lenses” and “diabetic cataracts” have been removed and replaced with more accurate and descriptive terms.
Point-by-point changes:

- Abstract, Methods, sentence 1: “and nuclear cataractous lenses from” inserted after “Transparent”; “and diabetic cataractous human lens nuclei” removed; “patients” inserted following “diabetic”.
- Abstract, Results, sentence 1: “cataractous” removed after “Diabetic”; “with nuclear cataract” inserted following “lenses”; “diabetic” inserted following “non-cataractous”.
- Abstract, Conclusion, sentence 1: “the diabetic cataractous lenses” removed following “inner nuclei of”; “cataractous lenses from diabetics” inserted following “inner nuclei of”.
- Background, paragraph 1, sentence 1: References “[1,2,3]” added.
- Background, paragraph 1, sentence 2: “normal” removed following “years of age, the”; reference “[1]” changed to “[4]”.
- Background, paragraph 2: “Previous studies investigating the hydration of transparent human lenses revealed an age-related dependence between water content and lens region [5].” added to the beginning of the paragraph.
- Background, paragraph 2, sentence 1: “normal aging” removed following “With”; “advancing age,” added following “With”; “(+0.0087%/year)” added following “water composition”; “total” added following “nucleus decreases its”; “(–0.077%/year)” added following “water content”; “creating” removed and replaced with “setting up” prior to “a gradient of hydration; reference “[2]” changed to “[5]”.
- Background, paragraph 2, sentence 2: Sentence “This minor difference…normal lens.” removed. Replaced with “Studies of human diabetic lenses have revealed greater total water content in comparison to transparent, non-diabetic lenses, with swelling reported to extend into the adult and perhaps the fetal nuclei [6,7].”, which is now sentence 3.
- Background, paragraph 2, sentence 3: Sentence “However, larger hydration…persons with diabetes.” removed.
- Background, paragraph 2, sentence 4: Sentence “The osmotic imbalance…damaging the cells” has been moved to the end of the paragraph with reference “[9]” added.
- Background, paragraph 2, sentence 5: Sentence “Nuclear magnetic resonance…fetal nuclei [4].” removed.
- Background, paragraph 2, sentence 6: “age-matched normals” removed, replaced with “transparent, age-matched, non-diabetic lenses.;” Entire sentence moved to follow current sentence 3.
- Background, paragraph 2, sentence 7: Reference “[5]” changed to “[8]”.
- Background, paragraph 3, sentence 1: “human lens nuclear fibers…normal aging and cataractogenesis [1].” removed, replaced with “the innermost nuclear fibers of the human lens significantly decrease in size and change shape with age and cataractogenesis [4]; a process termed compaction.”
• Background, paragraph 3, sentence 2: Reference “[6]” changed to “[10]”;“lens” added following “the rate of”; “in the normal lens with senescence” removed; “over time” added following “compaction was not constant”.
• Background, paragraph 3, sentence 3: Reference “[7]” changed to “[9]”.
• Background, paragraph 4, sentence 1: “diabetic (further…”“diabetic”)” removed; “diabetic” removed following “clinically diagnosed”, replaced with “nuclear”; “from persons with diabetes” added following “cataractous human lenses”.
• Background, paragraph 4, sentence 3: “cataractous diabetics” replaced with “with nuclear cataract”.
• Background, paragraph 4, sentence 4: Reference “[1]” replaced with “[4]”.

• Methods, Specimens, paragraph 1: Sentence “Lenses were enucleated and placed in primary fixative (detailed below) between 4 to 6 hours post mortem. Specimens were shipped by overnight courier at room temperature to our laboratories for further processing.” added following sentence 1.
• Methods, Specimens, paragraph 1, sentence 3: Moved to the end of the paragraph.
• Methods, Specimens, paragraph 1, sentence 4: “Normal” changed to “Transparent”; Entire sentence moved to the end of the paragraph.
• Methods, Specimens, paragraph 1, sentence 5: “obvious nuclear scattering, history of cataract, or” added following “Lenses with”.
• Methods, Specimens, paragraph 1, sentence 6: Paragraph break inserted prior to sentence 6.
• Methods, Specimens, paragraph 1, sentence 6: “diabetic” replaced with “human” following “Six”; “nucleus” deleted following “cataractous lens”; “nuclei from patients with diabetes” inserted following “cataractous lens”.
• Methods, Specimens, paragraph 1, sentence 7: Deleted. Replaced by “These lenses exhibited significant nuclear light scattering diagnosed as cataract by slit lamp, and warranted removal by an ophthalmologist (grade 2-4 on a 0-4 scale [11]). The extractions were performed by the same surgeon using consistent techniques. Nuclei were transported to our laboratories in vials with gauze moistened with balanced salt solution at room temperature and were placed in primary fixative within 4 hours of extraction. As in the case of the non- cataractous diabetic lenses, detailed information concerning the diabetic type, disease duration, and treatment was not distributed.”
• Methods, Specimens, paragraph 1, sentence 8: Deleted. Replaced by “Potential variations in morphology between post mortem and surgically extracted lenses were minimized by using identical preparation techniques. All donated lenses had no history of laser or intraocular surgery, and were obtained according to the tenets of the Declaration of Helsinki.”
Methods, Sample Preparation, sentence 1: “Upon receipt, all lenses were fixed” replaced with “Lenses were preserved in a primary fixative of”; reference “[8]” replaced with “[12]”.

Methods, Sample Preparation, sentence 3: Reference “[9]” replaced with “[13]”.

Methods, Morphometry, sentence 1: “diabetic and diabetic cataractous” removed following “compaction between”, replaced with “transparent and cataractous diabetic lenses”; reference “[1]” changed to “[4]”.

Methods, Statistical Analysis, sentence 1: “or diabetic cataractous groups based...nuclear cataract” replaced with “transparent diabetic or nuclear cataractous diabetic groups for statistical analysis.”

Methods, Statistical Analysis, sentence 2: Reference changed from “[11]” to “[15]”.

Methods, Statistical Analysis, sentence 6: Reference changed from “[1]” to “[4]”.

Results, paragraph 1, sentence 2: Reference changed from “[7]” to “[9]”.

Results, paragraph 1, sentence 3: “normal and cataractous lenses” replaced with “transparent and cataractous diabetic lenses”; references “[12,13,14]” replaced with “[16,17,18]”.

Results, paragraph 2, sentence 1: “in the diabetic (Fig. 2A) and diabetic cataractous” changed to “in the transparent diabetic (Fig. 2A) and cataractous diabetic”.

Results, paragraph 2, sentence 2: “normal” deleted following “Due to the”.

Results, paragraph 2, sentence 3: “Diabetic” replaced with “Transparent”; “diabetic” deleted following “while the”.

Results, paragraph 2, sentence 4: “decreased 11% in diabetic cataracts as compared to the transparent diabetics” replaced by “was 11% smaller in diabetic nuclei compared to transparent tissue”.

Results, paragraph 2, sentence 6: “transparent” inserted after “On the average, the”; “diabetic” removed following “while the”.

Results, paragraph 2, sentence 8: “The” replaced by “Transparent”; “diabetic cataracts” replaced with “diabetics with nuclear cataract”.

-Results, paragraph 4, sentence 1: “Comparisons between diabetic and cataractous diabetic lenses.” changed to “Comparisons between transparent and nuclear cataractous diabetic lenses.”

-Results, paragraph 4, sentence 2: “between diabetic and diabetic cataractous lenses” changed to “between transparent and nuclear cataractous diabetic lenses”.

Results, paragraph 5, sentence 2: “aged normal human lenses (ages 59-81; further referred to as “normal”) and age-related” changed to “aged transparent human lenses (ages 59-81) and non-diabetic age-related”; reference “[1]” changed to “[4]”.

Results, paragraph 5, sentence 3: “transparent” inserted following “and those from”. 
• Results, paragraph 5, sentence 4: “transparent” inserted following “angles of the”; “larger than that of normal lenses” changed to “larger than that of non-diabetic transparent lenses”.
• Results, paragraph 5, sentence 5: “normal” following “smaller than the” replaced by “non-diabetics”.
• Results, paragraph 5, sentence 6: “A comparison of the age-related nuclear and diabetic cataractous lenses yielded insignificant small” changed to “A comparison of non-diabetic age-related nuclear cataractous lenses and nuclear cataracts from diabetic patients yielded insignificantly small”.
• Results, paragraph 5, sentence 7: “angles of the diabetic cataract were” replaced with “angles of the cataractous diabetic nuclei were”.
• Results, paragraph 5, sentence 8: “smaller than the diabetic cataract (p=0.855)” replaced with “smaller than in the diabetics with cataract (p=0.855)”.

• Discussion, paragraph 1, sentence 1: “Comparisons of the transparent diabetic lenses to those of the diabetic cataracts” changed to “Comparisons of transparent diabetic and diabetic lenses with nuclear cataract”.
• Discussion, paragraph 1, sentence 3: “As observed in the aged normal and” changed to “As observed in non-diabetic aged transparent and”.
• Discussion, paragraph 2, sentence 2: References “[8,9]” changed to “[12,13]”.
• Discussion, paragraph 3, sentence 2: Reference “[15]” changed to “[19]”.
• Discussion, paragraph 3, sentence 3: References “[5,6]” changed to “[8,20]”.
• Discussion, paragraph 3, sentence 5: “normal” following “between aged” replaced with “transparent”; reference changed from “[1]” to “[4]”; “nuclear” added following “here between transparent and”.
• Discussion, paragraph 3, sentence 9: Reference changed from “[1]” to “[4]”; “for the diabetic cataracts group” changed to “for the cataractous diabetic group”.
• Discussion, paragraph 3, sentence 10: References changed from “[17,18,19,20,21,22]” to “[21,22,23,24,25,26]”.
• Discussion, paragraph 3, sentence 11: References changed from “[23,24,25]” to “[27,28,29]”.
• Discussion, paragraph 4, sentence 1: “The exact details concerning the cause of compaction remain unknown, but appear to be multifactorial in nature.” changed to “Although the exact details concerning the cause of compaction remain unknown, some features of the multifactorial process are understood.”
• Discussion, paragraph 4, sentence 4: References changed from “[26,27]” to “[30,31]”.
• Discussion, paragraph 4, sentence 5: References changed from “[2,28]” to “[5,32]”.

• Competing interests: Changed from “None” to “None declared”.

• Author’s contribution section added.

• Acknowledgements, sentence 3: “, EY-05722 (Duke),” added following “EY-08148 (MJC)”.

• Figure 1 caption, sentence 5: Reference changed from “[10]” to “[1]”.
• Figure 2 caption, sentence 2: “The” changed to “Transparent”.
• Figure 2 caption, sentence 3: “angle of the diabetic cataractous lenses (B)” changed to “angle of nuclear cataractous diabetic lenses (B)”;
  “diabetic” following “12% from the” changed to “transparent diabetics”.
• Figure 3 caption, sentence 2: “The” changed to “Transparent”; “while the diabetic cataractous lenses” changed to “while the cataractous diabetic lenses”.
• Figure 4 caption, sentence 2: “The” changed to “Transparent”; “while the diabetic cataracts (B)” changed to “while the cataractous diabetics (B)”.
• Figure 5 caption, title: “Stereo imaging of a diabetic cataractous lens” changed to “Stereo imaging of a cataractous diabetic lens”.

• Table 1, column 1 title: “Aged normals [1]” changed to “Aged transparent lenses [4]”.
• Table 1, column 2 title: Reference “[1]” changed to “[4]”.
• Table 1, column 3 title: “Diabetics” changed to “Transparent diabetic lenses”.
• Table 1, column 4 title: “Diabetic cataracts” changed to “Diabetic lenses with nuclear cataract”.

• Table 2, column 1 title: “Diabetic vs. Diabetic cataract” changed to “Transparent diabetic lenses vs. Diabetic lenses with nuclear cataract”.
• Table 2, column 2: Entire column of data added from previous study, as requested by the Reviewer.
• Table 2, column 4 title: “Age-related nuclear cataract [1] vs. Diabetic cataract” changed to “Age-related nuclear cataracts [4] vs. Diabetic lenses with nuclear cataract”.

• References: The entire section has been re-ordered and re-numbered.
The following articles have been added:


FA Bettelheim, L Li, F Zeng: **Do changes in the hydration of the diabetic human lens precede cataract formation?** *Res Commun Mol Pathol Pharmacol* 1998, **102**: 3-14