Original Article

The effects of vital dyes on mechanical properties of the human anterior lens capsule

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Purpose: The purpose of this study was to evaluate the mechanical effects of vital dyes on the anterior lens capsule via the nanoindentation method. **Methods:** Twenty anterior lens capsules of 20 different patients were dissected into four equal fragments. Each fragment was stained separately with dyes for intraocular surgeries, such as trypan blue 0.06% (TB), brilliant blue 0.025% (BB), and indocyanine green 0.05% (ICG), for 1 min. The remaining fragment was assessed as an untreated control group. The alterations on the mechanical characteristics of the anterior lens capsule were evaluated using a nanoindentation testing device with Oliver–Pharr and Martens hardness methods. **Results:** The mean values of elasticity were 7.842 ± 0.55 GPa for capsules fragments stained with TB ($P < 0.05$), 8.407 ± 0.82 GPa for capsules fragments stained with BB ($P < 0.05$), 8.557 ± 0.60 GPa for ICG ($P < 0.05$), and 6.09 ± 0.57 GPa for the untreated control group. The mean values of stiffness were 299.7 ± 47 MPa for TB ($P < 0.05$), 317.9 ± 34 MPa for BB ($P < 0.05$), 331.8 ± 48 MPa for ICG ($P < 0.05$), and 229.85 ± 44 MPa for the untreated control group. The elasticity of the capsules statistically decreased in comparison to the control group, and the capsule stiffness showed a statistically significant increase in comparison to the untreated controls. **Conclusion:** The mechanical characteristics of the human anterior lens capsule were affected in association with the alterations in the elasticity and stiffness properties of the capsule as a result of exposure to three different dyes.

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The lens capsule is the thinnest basement membrane in the body surrounding the entire lens of the eye; it is a viscoelastic membrane consisting primarily of collagen but also containing fibrillin, laminin, and heparin.[1] The capsule protects the lens structure, and it has an important role in accommodation. The loss of elasticity in the capsule leads to the inability of the lens to adapt to structural changes that are necessary for the accommodation of the lens.[2] It is possible to detect the impact of the lens capsule on accommodation by determining the mechanical properties of the capsule in detail.

Vital dyes are widely used to enhance visualization during intraocular surgical procedures, especially in cataract surgery.[3] Trypan blue (TB), indocyanine green (ICG), and brilliant blue (BB) are introduced as vital dyes through the anterior chamber to facilitate the visibility of the anterior capsule in the absence of a good red retinal reflex in senile cataract surgery, especially in the cases of a mature cataract.[4-6] TB is the most frequently used among these dyes, and the concentration used to stain the lens capsule is between 0.0125% and 0.1%.[7] These dyes are commonly used in completing the capsulorhexis stage during challenging cataract surgery procedures in which it is difficult to see the anterior capsule.[7,8] Although ICG is a rarely used agent in deep lamellar endothelial keratoplasty surgery, it enhances capsule visibility during cataract surgery.[9,10] BB is a relatively new dye that is used during the removal of the internal limiting membrane of the retina during vitreomacular procedures; however, it is also used as a capsule stain.[8,11] The kind of interaction between the dye and tissues is not exactly known. In macular surgery, it is the general opinion that the stained internal limiting membrane can be removed more easily and in large fragments in association with the changes in the stiffness and elasticity of the tissue.[11]

Nanoindentation is a popular technique that enables the measurement of mechanical properties, such as elasticity and stiffness, depending on the reaction of the tissue or the material against the applied load.[12] This study used conventional nanoindentation instrument to evaluate the potential effects of TB, BB, and ICG on the lens capsule.

Methods

Participants

We planned to examine the changes to the anterior lens capsules obtained during cataract surgery of the patients and exposure to different dyes at the mechanical level. Twenty anterior lens capsules of 20 different patients were included in the study.

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All cataract surgeries were selected from scheduled operations. The study was approved by Health Sciences University Van Education and Research Hospital Ethics Commitee (number: 2018/05, date: 15.03.2018) and performed in keeping with the principles of the Helsinki Declaration. Patients included in the study had no systemic disease, no comorbid pathology except a cataract (all patients had a similar type of nuclear cataract without cortical spoke), and not received any ophthalmic drug therapy. Informed consent was obtained from all the patients after informational materials about the procedure including the risks and complications associated with the surgery and a study description were provided to them.

Sample preparation

Anterior lens capsules were removed entirely following the standard 5-mm capsulorhexis procedure performed by the same surgeon (O.G.) during the cataract surgery, and the samples were placed by a technician on 1 × 1-cm sterilized glass slides with the convex surface pointing down (only intact samples in one piece were used). Then, the samples were preserved inside a balanced salt solution (BSS Plus, Alcon Laboratories, Inc, Fort Worth, TX, USA) in standard plastic containers. The samples were transported to the central laboratory within 24 h and evaluated using a CSM instrument nanoindentation tester (CSM Instruments, Needham, MA, USA). In the laboratory, the samples were divided into four equal fragments (each fragment with an area of around 5 mm^2) stained with either TB (0.06%, Blue Rhexis), BB 0.025% (Brilliant Peel, Fluoron GmbH), or ICG 0.05% (Pulsion, Pulsion Medical Systems AG) for 1 min. The remaining fragment was assessed as control group [Fig. 1].

Indentation technique

Elasticity, stiffness, and morphological characteristics of the capsule surface were evaluated using a nanoindentation test device (CSM Instruments). A standard Berkovich diamond indenter with diagonal length of 100 mm was used (PR-CO10, Bruker Corp, Billerica, MA, USA). The indenter was connected to a rectangular console (Bruker Nano, Bruker Corp, MA, USA) with a width of 2 mm. The indenter geometry was an axisymmetric cone with a TETA apical half angle.

The indentation depth was adjusted to be lower than 3% of the sample thickness, 400 nm at the deepest point, to prevent any influence of the glass slide under the samples [Fig. 2].

In the loading–unloading mode, the maximum load value was adjusted with the residual loads. This maximum indentation load was introduced into the capsule sample material at a stable displacement rate (loading stage). When the maximum indentation load was reached, it was held for a while (holding time). Then, the load was removed, and the indenter was drawn back to the material (unloading stage). The force and depth (displacement) were measured and transferred to a "*P*-h" graphic using a software program performing addition/storage operations. When material reached displacement at a maximum depth, an elastic recovery occurred during the displacement of the load. The residual depth was formed at this point. The percentage of the total study area is the ratio of the residual depth to the maximum depth.[13] The elastic recovery occurring between the maximum depth and the residual depth (h) was also an elastic working area. The percentage of total working was the ratio of residual depth to maximum depth.

Measurement of elastic modulus and hardness

The values of elastic modulus (E) and micro-stiffness can be explained by the data of the loading and penetration depth.[13] The use of these data and relevant formulas were described in detail in our previous study. Additionally, E and H were determined according to our previously described protocol [Fig. 3].[14] Briefly, the elastic modulus (E) and micro-hardness (H) can be described by loading and penetration depth data. To determine E and H, key parameters must be measured as follows: maximum load (Pmax), indenter contact area at maximum depth (Ac), and loading displacement initial contact rigidity (S = dP/dh). As with conventional micro-hardness testing, dynamic micro-indentation hardness is also calculated by the division of indentation maximum load (Pmax) by the trace area (Ac).

Statistical analysis

The Shapiro–Wilk test and Levene's test were used to evaluate the compatibility of the variables to the normal distribution and homogeneity of group variations, respectively. As the assumption of parametric tests was provided, the mean values of groups were compared using the one-way analysis of variance and the multi-comparison Tukey's HSD (honest significant difference) tests. The association

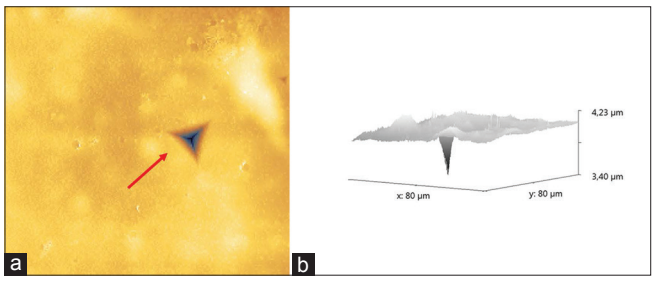


Figure 1: 3D Atomic force microscopy image of trace made by nanoindentation: (a) Surface image of measured capsule using ATM of indentation testing device. Trace made by the nanoindenter on a lens capsule (red arrow) seen via optical microscopy (bar = 10 µm). (b) 3D image of measured capsule

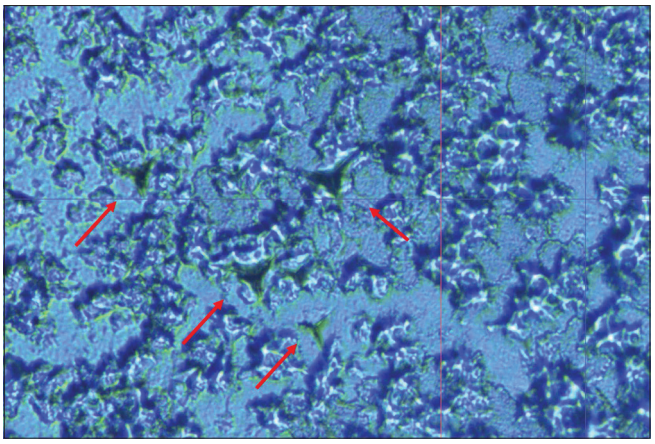
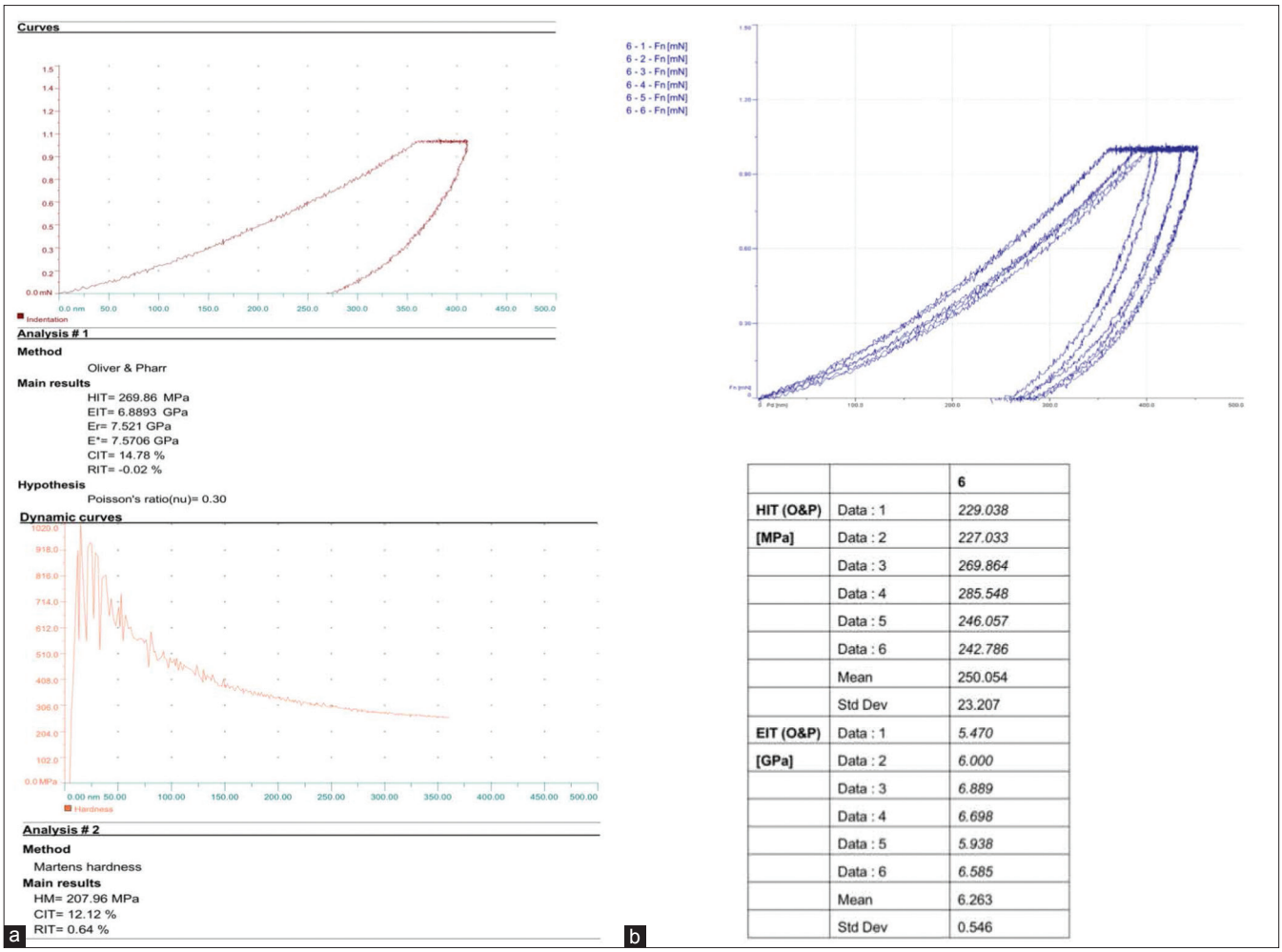


Figure 2: Representative optical microscopy image of traces made by the nanoindenter on lens capsule: Detection of area in which the measurement is taken at 50× magnification under optical microscope. Traces made by the nanoindenter on a TB applied lens capsule at 50 magnification (bar = 4 µm)



		6
HIT (O&P)	Data : 1	229.038
[MPa]	Data : 2	227.033
	Data : 3	269.864
	Data : 4	285.548
	Data : 5	246.057
	Data : 6	242.786
	Mean	250.054
	Std Dev	23.207
EIT (O&P)	Data : 1	5.470
[GPa]	Data : 2	6.000
	Data : 3	6.889
	Data : 4	6.698
	Data : 5	5.938
	Data : 6	6.585
	Mean	6.263
	Std Dev	0.546

Figure 3: Graphic representation of an anterior lens capsule flexibility and hardness measurement: (a) Anterior lens capsule hardness measured via nanoindentation using Oliver–Pharr and Martens hardness method. (b) Curves obtained by six difference measurements performed on the same capsule when different elasticity measurements taken from the same capsule were transferred to the electronic environment

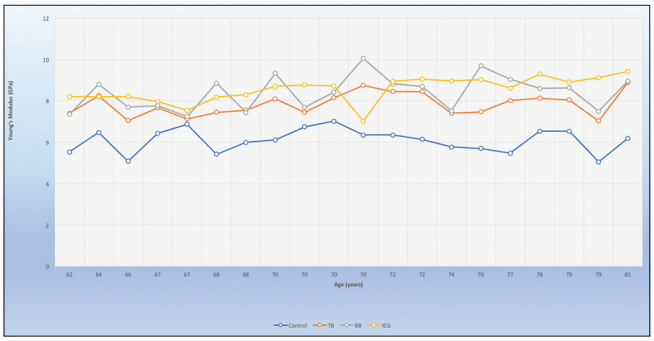


Figure 4: Elastic modulus values of the anterior lens capsule: Alterations of elasticity values with vital dye applications in the human anterior lens capsule. Note the notable increase in elasticity scores in the human anterior lens capsule fragments after application of three different vital dyes ($P < 0.05$)

Figure 5: Martens hardness values of the anterior lens capsule: Alterations of hardness values with vital dye applications in the human anterior lens capsule. Note the notable increase in hardness scores in the human anterior lens capsule fragments after application of three different vital dyes ($P < 0.05$)

between the variations was evaluated using the Pearson correlation coefficient. Identifying statistics were indicated as mean ± standard deviation. A P value of < 0.05 was accepted as statistically significant. Data were analyzed using the SPSS Statistical Software Program (Chicago, IL, USA, Version 20).

Results

Twenty lens capsules were obtained from a single eye of 20 male patients. The age distribution of patients ranged between 62 and 81 years; the mean age of patients was 71.5 ± 5.39 years,

and the age distribution of patients was homogeneous. The mean elastic modulus value (Young's modulus) was 7.72 ± 1.13 (range: 5.05–9.42) GPa and the mean value of Martens hardness was 294.81 ± 45.25 MPa (range: 150–417). A positive correlation was observed between the ages and elastic modulus values and the mean values of Martens hardness ($R^2 = 0.81023$, $P < 0.05$).

The mean values of elasticity were 7.842 ± 0.55 GPa for capsules fragments stained with TB ($P < 0.05$), 8.407 ± 0.82 GPa for capsules fragments stained with BB ($P < 0.05$), 8.557 ± 0.60 GPa for ICG ($P < 0.05$), and 6.09 ± 0.57 GPa in the control group. There was a statistically significant decrease in the capsule elasticity of the three groups in comparison to the control group [Fig. 4].

When the control group of tissue samples that were stained with TB, BB, and ICG vital dyes were compared with Martens hardness values, a statistically significant difference was detected between the groups. The mean values of stiffness were 299.7 ± 44 MPa for TB ($P < 0.05$), 317.9 ± 48 MPa for BB ($P < 0.05$), 331.8 ± 48 MPa for ICG ($P < 0.05$), and 229.9 ± 44 MPa for the control group [Fig. 5].

Samples stained with vital dyes were compared with each other in terms of Young's modulus and Martens hardness values. There was no significant difference between the elastic modulus ($P > 0.05$) and the stiffness values ($P > 0.05$) of the vital dyes.

Discussion

Atomic force microscopy (AFM) is a common technique that has been used to measure the mechanical properties of the tissues via the indentation method in ophthalmology; however, traditional nanoindentation technique has recently been widely used in ophthalmology. Another advantage of the AFM measurement over the indentation method is the measurement of the contact area and the depth using same tip by means of a force–displacement curve.[15] Ah-Young *et al*. measured the mechanical properties of various polymers using AFM and nanoindentation techniques, and, when the outcomes were evaluated by Oliver and Pharr and image analysis, the results of stiffness and Young's modulus were found to be associated with each other in both measurements. The researchers reported that the outcomes of traditional nanoindentation and AFM nanoindentation techniques revealed similar mechanical values.[16]

The indentation measurement of the biological materials was very difficult when it was compared with the metals. Biological materials usually have a small elastic modulus, and their mechanical behavior changes according to the time and hydration.[17] This condition might cause significant difficulties in mounting the tip on the indentation location.[18]

This study was evaluated the effects of vital dyes that have been used to make the human anterior lens capsule visible via nanoindentation technique. We did not encounter a similar study in the literature. In our study, anterior lens capsules of the 20 male patients were examined by dividing them into four fragments. Vital dyes of TB, BB, and ICF were introduced to anterior lens capsules, and the samples were compared with the control group in terms of Young's modulus and Martens hardness values, separately. There was an inverse relationship between the elasticity coefficient of Young's modulus and the elasticity of the substances. While the high value of elasticity coefficient means lower elasticity, the larger the stiffness means the higher the rigidity of the biological material. In this study, we compared the values of Young's modulus and Martens hardness in TB, BM, and ICG groups and separately in the control group, and we found a significant difference. In the previous study by Haritoglu *et al*., vitrectomy light was applied after staining, and they reported no difference in the outcomes.[19] In our study, we did not expose the stained capsule fragments to a light source.

In the study by Jaber *et al*. performed on cadaver eyes with 0.06% TB solution, they reported no difference in the values of resistance to laceration, unlike our study.[20] It is important to consider that AFM provides measurements of the surface of the tissue with a penetration of <1 mm. It is known that TB does not penetrate the tissue but rather stains the surface. Therefore, a stiffening effect measured by AFM might be more pronounced at the surface of the stained tissue and may not necessarily correlate to the tear resistance of the whole capsular bag. In addition, in this study, TB 0.06% provided the weakest stiffening effect as measured by AFM, and one may, therefore, hypothesize that this effect might not be detectable in the experimental setting used by Jaber *et al*.[20] Wollensak *et al*. investigated the association between ICG and TB and the lens capsule. They introduced 0.1% TB to porcini eyes for 30 s, 1 min, and 30 min. According to a hypothesis that explains the positive effect of TB, the staining of the lens capsule with TB modifies the biomechanical characteristics of the lens capsule. The stiffness and relative extensibility of the anterior lens capsules that were kept in dye for 1 and 30 min were significantly increased and decreased, respectively. This impact was correlated to the changes in the elastic behavior of the capsule in consequence of cross-linking between TB and free oxygen radicals between the collagen fibrils due to the photosensitivity of the dye.[21] Dick *et al*. studied the effect of 0.12% TB solution on the capsule elasticity, and they reported no difference between the measurements obtained at the first and fifth seconds after staining; however, a significant decrease was found in the elasticity values after the tenth second.[22] In the study by Wollensak *et al*. investigating internal limiting membranes that were stained with ICG, they reported easy laceration associated with increased stiffness. Increased oxidative damage was proposed as a reason.[23]

An anterior lens capsule with increased mechanic stability causes significant outcomes in ophthalmic surgery. Capsule dyes might enhance the change of success in complex cases, such as a white cataract, by increasing capsule stability, as well as contrast. On the other hand, the possible chemical reactions in the lens capsule caused by dyes should not be ignored. Recently, the nanoindentation technique has been commonly used for detecting the biomechanical properties of vital tissues in ophthalmology. The data obtained by nanoindentation technique will be a significant source for the development of techniques in cataract surgery.

Further *in vivo* studies should also focus on the effects of vital dyes on the anterior lens capsule, especially in complicated cases such as pediatric cataracts, subluxated lenses, or zonular weakness in which it is challenging to complete a curvilinear capsulorhexis.

In parallel to our study, in the study by Haritoglu *et al*. that inspired our methodology, they showed via AFM that the exposure to these three dyes resulted in significant differences in the capsule stiffness.[19] In another study performed using traditional nanoindentation technique, we showed that the staining with TB vital dye did not alter the values of capsule elasticity and stiffness.[14] The reason of this difference was due to the *in vitro* exposure of the dyes while they were applied *in vivo* in our previous study.[14] It was concluded that antioxidative mechanisms had an impact on the oxidative conditions of the lens capsule. The main reason for the differences between our study and other studies were the ratio of dyes, the amount of dye penetrating into the capsule, the duration of oxidative damage, and the differences between the measurement techniques.

Conclusion

The clinical and surgical implications of our study is that reduced hardness due to vital dye application in eyes can cause ocular structures to become weak and vulnerable to injury, and decreased elasticity may cause capsule ruptures easily.[21,22] Performing bigger capsulorhexis in vital dye stained eyes with caution may be useful for avoiding capsular complications in cataract surgery.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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