Effects of Diabetes Mellitus on Biomechanical Properties of the Rabbit Cornea

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Conflict of Interest

The authors indicate no financial conflict of interest.

Running title

Biomechanical Change in Diabetic Cornea of Rabbit

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Abstract:

To investigate the effects of diabetes on the biomechanical behavior of cornea in alloxan-induced diabetic rabbits. Diabetes mellitus (DM) was induced in 20 rabbits using alloxan, while another 20 age- and weight-matched non-diabetic rabbits served as controls. Eyes were enucleated after 8 weeks of inducing diabetes and the whole cornea was removed with a 3mm wide scleral ring and tested under inflation conditions with an internal pressure range of 2.0 - 30.0 mmHg to determine their stress-strain behaviour using an inverse analysis process. The blood glucose level (BG), advanced glycosylation end products (AGEs), central corneal thickness (CCT) and intraocular pressure (IOP) increased significantly in the DM group. There were statistically significant correlations between BG and AGEs ($r= 0.768$, $p= 0.00$), and between AGEs and CCT variation upon induction of DM ($r= 0.594$, $p= 0.00$). The tangent modulus (Et) of the cornea at four stress levels (1 to 4 kPa, equivalent to approximately IOP of 7.5, 15, 22.5 and 30 mmHg, respectively) was significantly higher in diabetic rabbits than in the control group ($p< 0.05$). Further, Et at stress of 2 kPa (which corresponded to the average IOP for the control group) was significantly correlated with BG ($r= 0.378$, $p< 0.05$), AGEs ($r= 0.496$, $p< 0.05$) and CCT variation upon induction of DM ($r= 0.439$, $p<0.05$). IOP, as measured by contact tonometry, was also significantly correlated with both CCT ($r= 0.315$, $p<0.05$) and Et at 2 kPa ($r= 0.329$, $p< 0.05$), and even after correcting for the effects of CCT and Et, IOP still significantly increased with both AGEs ($r= 0.772$, $p= 0.00$) and BG ($r= 0.762$, $p= 0.00$). The cornea of diabetic rabbits showed a significant increase in mechanical stiffness as evidenced by increases in corneal thickness and tangent modulus. The Et increase may be explained by a non-enzymatic cross-linking of collagen fibrils mediated by AGEs due to the high blood glucose levels in diabetes. The study also found significant IOP increases with higher blood glucose level even after controlling the effects of both corneal thickness and tangent modulus.

Keywords: Diabetes Mellitus, Ocular Biomechanics, Cornea

Introduction

Diabetes mellitus (DM) is a common disease, whose prevalence ranges between 8.3% and 11.6% of the general population in different ethnic groups (Geiss et al., 2014; Xu et al., 2013). DM is characterized by chronic hyperglycemia and an altered cellular homeostasis, which may lead to multi-organ dysfunction. 70% of DM patients suffer a number of debilitating complications affecting the physiology, morphology, and clinical appearance of the cornea (Didenko et al., 1999). These complications cause diabetic keratopathy in the form of structural and functional abnormalities resulting in impaired epithelial and endothelial function, punctate keratitis, decreased corneal sensitivity, recurrent corneal erosions and delayed wound healing (Gekka et al., 2004; Rosenberg et al., 2000; Schultz et al., 1984; Schultz et al., 1981).

The hyperglycemia caused by DM induces the formation and accumulation of advanced glycosylation end products (AGEs), which in turn are strongly associated with a number of pathological complications of DM (Brownlee, 2001). Studies have shown an increased levels of AGEs in the corneas of DM patients (Sady et al., 1995) that lead to an increase in collagen crosslinking in what is known as the Maillard reaction, which then results in the formation of Amadori products and create covalent cross-linking bonds (Krueger and Ramos-Esteban, 2007). As biomechanical behavior is dependent on the regulation and organization of structural components within the cornea, the formation of bonds, which is expected to accelerate in diabetes, leads possibly to a gradual stiffening of corneal tissue (Sady et al., 1995; van Heerebeek et al., 2008), and that is consistent with the observation that diabetic corneas are less susceptible to the development and progression of keratoconus (Seiler et al., 2000) and may behave differently in response to surgical procedures and IOP tonometric measurements (Abdelkader, 2013; Clemmensen and Hjortdal, 2014).

Most studies that investigated the effect of DM on corneal biomechanical response concentrated on using the Ocular Response Analyzer (ORA, Reichert, Depew, NY) and Corvis ST (CVS, Oculus, Wetzlar, Germany) (Perez-Rico et al., 2015; Scheler et al., 2012). Both these techniques provide useful measures of corneal biomechanical behavior, namely the ORA's corneal hysteresis (CH) and the corneal resistance factor (CRF), and the Corvis's several deformation parameters. However, while these parameters have shown promise in their ability to identify keratoconic corneas (Perez-Rico et al., 2015), (Goldich et al., 2009; Kotecha et al., 2010; Narayanaswamy et al., 2011), they do not link directly to the commonly

used and traditional mechanical properties of material such as the stress-strain behavior or the tangent modulus(Bao et al., 2015). Without this important link, it would be difficult to use the techniques' parameters quantitatively in applications such as planning of refractive surgery, design of corneal implants or optimization of cross-linking treatment of keratoconic eyes.

In this study, assessment of corneal mechanical behavior and how it is affected by DM is conducted using a direct measurement method, whereby the tissue is subjected to inflation pressure simulating the effect of intraocular pressure (IOP), and the resulting deformation of the cornea used to provide estimates of the tissue's stress-strain behavior through an inverse analysis procedure. Rabbit corneas have been used in this study for their similarity in biomechanical behavior to human corneas (Jue and Maurice, 1986) and the difficulties in obtaining human eyes with sufficient numbers.

Materials and methods

Experimental specimens

Forty male Japanese white rabbits (2-3 kg) were included in the study and randomly divided into two groups of twenty rabbits each, namely the diabetes mellitus (DM) group and the blank control (BC) group. The animals were outbred, 2-3 months of age, and obtained from the Animal Breeding Unit of the Wenzhou Medical University. The study was approved by the Animal Care and Ethics Committee of the University's Eye Hospital and all animals were treated in agreement with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. The rabbits were housed in individual cages where the temperature and humidity were well controlled, and each rabbit was fed a standard chow and water, and kept with a 12 hour light/dark cycle. Before the establishment of diabetic model, the rabbits were allowed to acclimatize for at least 1 week.

After 8 hours of fasting for solids and liquids (Lin et al., 2015), diabetes was induced in the DM group by intravenous injection of alloxan monohydrate (A7413, Sigma, USA) at a dose of 150 mg/kg body weight (O'Loughlin et al., 2013; Stables et al., 2014). After treatment, the rabbits were fed for 24 hours with a glucose solution and injected with molasses through the front feet to prevent hypoglycemia. To determine the hyperglycemic state of the animals, blood glucose levels (BG), as well as body weight (W), central corneal thickness (CCT) and intraocular pressure (IOP) were monitored 1 week post-alloxan treatment and each

subsequent week throughout the duration of the study. A glucose test strip (Roche, Germany) was used to measure the glucose level in blood samples obtained from the marginal ear vein of overnight-fasted rabbits. A blood glucose level of 12 mmol/L in three independent measurements was considered as manifest diabetes (Lin et al., 2015). Weight was measured by an electronic scale (SCRY-05, SEGA Corporation, China). After topical anaesthesia (single drop of 0.5% proparacaine), CCT and IOP were measured by a portable pachymeter (PachPen, Accutome Inc, PA, USA) and a Tono-pen tonometer (Reichert, Inc., New York, USA), respectively. For each eye, three measurements were made and the results were averaged. All examinations were made by the same operator (MLD) during the same hours (between 8 and 10 AM). On the other hand, the twenty rabbits included in the BC group did not undergo any treatment and were hence considered non-diabetic controls.

Specimen Preparation

Eight weeks following the treatment with alloxan, the rabbits in both groups were sacrificed by an intravenous injection of pentobarbital sodium overdose (Merck, Darmstadt, Germany) of 100 mg/kg body weight and one of the bilateral eyes were randomly selected and immediately enucleated. A 0.1 ml aqueous humour sample was collected from each included eye and preserved at -20℃ for AGEs measurement. AGEs levels in aqueous humour were measured by ELISA using a commercial kit (S-60223, TSZ Company, USA). The corneas were separated along with a 3-mm wide ring of scleral tissue before mounting them onto a custom built pressure chamber filled with Phosphate Buffered Saline (PBS, Maixin, China) (Ni et al., 2011; Yu et al., 2013; Yu et al., 2014). The pressure inside the chamber was controlled by a syringe pump whose movement was in turn controlled by a custom-built LabView software.

An ultrasonic pachymeter (SP-3000, Tomey Inc, Nagoya, Japan) was used to take central and peripheral thickness measurements (the latter taken approximately 1.5 mm away from the limbus), and a Vernier caliper was utilized to measure corneal diameters in four directions (horizontal, vertical, and two 45^{o} diagonal directions). Side elevation images of each cornea were obtained from digital cameras (EOS 60D, Canon, Inc., Tokyo, Japan) positioned along the inferior-superior and temporal-nasal diameters. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was utilized to construct the anterior profile of the cornea based on the side images (Fig. 1A), and the thickness measurements were used to construct the posterior profile (Fig. 1B).

Biomechanical Inflation Testing

To ensure a fully inflated and wrinkle-free corneal surface, each specimen was first subjected to an initial inflation pressure around 2.0 mmHg. Then three cycles of loading and unloading, up to a pressure of 30 mmHg and with a rate of 0.41 mmHg/s, were applied to condition the tissue and stabilize its behavior. A recovery period of 90 seconds was allowed between each loading cycles to ensure the behavior was not affected by the strain history of loading cycles (Yu et al., 2014)' (Yu et al., 2013; Zheng et al., 2016). Finally, the specimens were subjected to a fourth loading cycle, the results of which were considered representative of the cornea's biomechanical behavior. PBS was sprayed on corneal surface to keep it hydarted during the recovery period between each two loading cycles. PBS was adequate to keep the corneas hydrated but without significant swelling during the test period; 1~2 hours (Yu et al., 2013; Yu et al., 2014). The present corneal inflation tests were completed within 2 hours including preparation time.

Inverse Analysis

Inverse analysis is the method used to provide estimates of the corneal material's mechanical properties based on the pressure-deformation experimental results. It is particularly suitable when a simple analytical solution is not available, such as where either the specimen geometry or material behavior is complex. In this study, the finite element (FE) solver Abaqus (Dassault Systèmes Simulia Corp., Rhode Island, USA) and the optimization software package LS-OPT (Livermore Software Technology Corp, CA, USA) were used to implement the iterative process of the inverse analysis procedure as described in a previous study (Zheng et al., 2016).

Forty FE models were developed representing all tested corneas. Each model had unique geometry based on the thickness, corneal profile and limbal diameter measurements, and constructed from 1728, 15-noded continuum elements (C3D15H) arranged in twelve rings and two layers. An encastre connection was assumed along the limbus to simulate connection to the mechanical clamps. A first order hyperelastic Ogden model (Yu et al., 2013; Yu et al.,

2014)' (Mulhern et al., 2001) was used to represent corneal material behavior using a strain energy density function in the form:

$$W = \frac{2\mu}{a^2}\left(\overline{\lambda_1}^{\alpha} + \overline{\lambda_2}^{\alpha} + \overline{\lambda_3}^{\alpha} - 3\right) + \frac{1}{D}(J-1)^2 \quad (1)$$

where W is the strain energy per unit volume and material parameters μ and α represent the strain hardening exponent and the shear modulus, respectively. $\overline{\lambda_k}$ is the deviatoric principal stretches = $J^{-1/3}\times\lambda_k$ (k=1, 2, 3), λ_1, λ_2, λ_3 the principal stretches, J = $\lambda_1\lambda_2\lambda_3$. D is a compressibility parameter $= \frac{3(1-2\nu)}{\mu(1+\nu)}$ calculated assuming corneal tissue was nearly incompressible (Grupcheva et al., 2001),(Dhaliwal et al., 2001) with a Poisson's ratio, ν. With reported values of ν for corneoscleral tissue between 0.46 and 0.5 (Battaglioli and Kamm, 1984), a value of 0.48 was assumed in this study, making D = 0.081/μ. (Yu et al., 2013). While λ, equals strain ε + 1, the stress, σ, is obtained by differentiating the strain energy. Finally, with the σ-ε relationship determined, the tangent modulus Et – a measure of material stiffness – can be determined as: Et = dσ/dε ≈ Δσ/Δε.

Statistical analysis

All statistical analyses were performed using PASW Statistics 20.0 (SPSS Inc., Chicago, USA). Comparison of biomechanical metrics and corneal shape parameters in the two specimen groups was performed using the independent T-test. In this study, P-values of less than 0.05 were considered to be statistically significant. The associations between various physical and biomechanical parameters of the specimens were determined by Pearson partial correlation analyses and the Spearman linear correlation factor.

Results

Experimental behavior and material constitutive models

As shown in Figure 2, a clear difference in pressure-displacement behavior at corneal apex was observed between the two specimen groups. Specimens exhibited nonlinear behaviour with an initial low stiffness increasing gradually until a stage at IOP of approximately 12-18 mmHg when the stiffness reached its highest level and remained almost constant thereafter.

Material parameters α and μ for each cornea were obtained through the inverse analysis process which provided the best possible fit (lowest RMS error) with the experimentally obtained pressure-displacement results (Table 1). The tangent modulus (Et, a measure of material stiffness) at different stress levels were determined using the relationship: $Et = d\sigma/d\varepsilon \approx \Delta\sigma/\Delta\varepsilon$, where σ and ε are the stress and strain, respectively. The stress-strain behavior determined using the inverse analysis procedure described above is presented in Figure 3. Although the stress-strain results displayed a nonlinear form, the relationship between Et and stress (σ) was close to linear as had been reported in previous studies (Elsheikh et al., 2007),(Elsheikh et al., 2008). At four stress levels (1.0, 2.0, 3.0 and 4.0 kPa), which were equivalent to internal pressures (IOP) of approximately 7.5 to 30 mmHg, Et was determined to quantify the effect of DM on behavior (Table 2). There were statistically significant differences between the Et values for DM and BC Group ($P<0.05$) at each of the four stress levels.

Correlation Analyses

Data obtained before the establishment of diabetes showed no significant differences between the DM and BC groups in blood glucose level (BG, p= 0.268), body weight (W, p= 0.564), central corneal thickness (CCT, p= 0.800), intraocular pressure (IOP, p=0.687) measured by the Tono-pen and advanced glycosylation end products (AGEs, p=0.319). Following the establishment of diabetes, eight weeks into the test, four of these parameters showed significant increases in the DM group compared to the BC group, including BG (23.9±5.8 mmol/L vs 6.3±0.9 mmol/L, t= 13.39, P<0.001), CCT (416.3±25.9 μm vs 385.2±28.8 μm, t= 3.58, P<0.001), IOP (25.7±2.9 mmHg vs 15.3±2.6 mmHg, t= 12.05, P<0.001) and AGEs (1314.8±153.0 pg/ml vs 454.0±154.3 pg/ml, t = 17.72, P<0.001). In contrast, there were significant reductions in W (2.06±0.35 kg vs 3.00±0.37 kg, t = -8.17, P < 0.01) in rabbits with DM.

Further, within the DM group, BG, AGEs, CCT and IOP increased significantly with the establishment of diabetes (P<0.001), while W decreased significantly (P<0.01). There was also a significant negative correlation between W and BG (r= -0.690, p= 0.00), and positive

correlations between BG and AGEs (r= 0.768, p=0.00), and between AGEs and CCT variation upon induction of DM (i.e. CCT after 8 weeks of inducing DM - CCT at start of study, r= 0.594, p=0.00).

The tangent modulus (Et) of the cornea at four stress levels (1.0, 2.0, 3.0 and 4.0 kPa) was significantly higher in diabetic rabbits than in the control group (p= 0.02, p= 0.00, p= 0.00, p= 0.00, respectively). Further, Et at stress of 2.0 kPa (which corresponded to the average IOP for the control group) was significantly correlated with BG (r= 0.378, p= 0.016), AGEs (p= 0.496, r= 0.001) and CCT variation upon induction of DM (r= 0.439, p= 0.005). IOP, as measured by contact tonometry, was also significantly correlated with both CCT (r= 0.315, p=0.048) and Et at 2.0 kPa (r= 0.329, p= 0.038), and after correcting for the effects of CCT and Et, IOP still significantly increased with both AGEs (r= 0.772, p=0.00) and BG (r= 0.762, =0.00).

Discussion:

The cornea is an important optical component of the outer ocular tunic, providing around 70% of the eye's refractive power in addition to acting as an efficient protective envelop for the ocular contents. Corneal mechanical behavior, essential for maintaining its dimensional stability and clear vision, depends on its geometric properties (thickness and curvature) and biomechanical properties (material stiffness) (Liu and Roberts, 2005). The ability to quantify corneal biomechanical behavior has several potential applications including early detection of keratoconus, planning of refractive surgery (Goldich et al., 2009), design of corneal implants and more accurate IOP measurement for glaucoma management (Sahin et al., 2009). Previous studies reported that glycosylation in DM patients increases collagen cross-linking (Sady et al., 1995), and hence increases the biomechanical stiffness of the cornea. However, most of the studies used ORA and CVS output parameters, which act as indicators of mechanical corneal stiffness, to assess the diabetes-induced changes in corneal biomechanical behavior (Goldich et al., 2009; Hager et al., 2009; Perez-Rico et al., 2015). The present study attempts instead to use a direct method to quantify the changes in corneal biomechanical behavior, and in particular the material stiffness as measured by the tangent modulus, associated with DM.

The study showed a number of interesting and inter-related trends. While there have been no significant differences in blood glucose level (BG), advanced glycation end products (AGEs), CCT and IOP between the DM and BC groups before establishment of diabetes, this image changed with diabetes introduction, showing significant increases in BG (284%), AGEs (324%), CCT (9.6%) and IOP (71.3%) in the DM group, in line with an earlier study by Faried et al (Manar A et al., 2013). Interestingly, the increased level of glucose in the blood (284%) and the corresponding increase in AGEs (measured in the aqueous, 324%) appear similar and seem to be strongly correlated, as would be expected.

A related observation confirmed significant correlations between CCT, Et and IOP on one hand, and both of BG and AGEs, on the other, underlining notable increases in tissue thickness (7.5%), material stiffness (23.2 % at 2.0 kPa stress) and IOP measurements (68.5%) with the development of diabetes compared to control group.

The increase in corneal thickness could be caused by an edema due to the increased endothelial permeability, inhibition of the endothelial pump, increased stromal swelling pressure, and reduction in mean corneal endothelial cell density (ECD) that develop in diabetes. However, the first two reasons may be unlikely because of the normal endothelial permeability to fluorescein (Larsson et al., 1996) and the lack of change in endothelial deswelling rate (Su et al., 2008) associated with diabetes. The abnormalities in stromal matrix biology (Ni et al., 2011) resulting from the formation of AGEs (Kaji et al., 2000; Sady et al., 1995), and reduced ECD in DM (El-Agamy and Alsubaie, 2017) may be the cause of CCT increase in DM (Monnier et al., 1988). The increase in CCT with diabetes has been reported in earlier studies including increases of 1.5% in humans (Su et al., 2008),(Storr-Paulsen et al., 2014) and 84.3% in rats (Manar A et al., 2013). The large difference in CCT increases between different animal models and humans could be due to effective treatment to control diabetes in humans, in addition species differences between rat and rabbit.

The significant correlation of Et with both BG and AGEs points at tissue stiffening with diabetes development and tends to confirm the hypothesis that the collagen cross-linking caused by AGEs accumulation may be responsible for the biomechanical changes observed in diabetic tissue (Sady et al., 1995). This link is further supported by the fact that AGEs content in the aqueous humour is probably the most important factor influencing the pathophysiology of chronic diabetic complications and hence behaviour of diabetic tissue. In our study, although corneal swelling was expected to lead to stiffness (or Et) reduction (Kling and Marcos,

2013), the formation of collagen crosslinks by sugar aldehydes could compensate for the effect of corneal edema and lead to a higher overall stiffness.

Having discussed the individual correlation of CCT and Et with AGEs, it is interesting to note the strong, direct correlation between Et and CCT indicating that as the tissue thickens with diabetes, there is an accompanying stiffening effect, making the possibility of oedematous thickening of the tissue unlikely.

Analysis of trends related to IOP provides further insight into the ocular changes that accompany diabetes. Our results show that even after correcting IOP measurements for the effects of CCT and Et, IOP remained to be strongly correlated with both BG and AGEs. This result points at a probable increase in the actual IOP that is associated with diabetes development and independent of the errors caused in tonometry by changes in corneal stiffness. While the IOP increase in patients with diabetes had been reported, the mechanism relating diabetes to increased IOP is still unclear (Wong et al., 2011). Published hypotheses include (1) the development of an osmotic gradient that draws excess aqueous humour into the anterior chamber (Zhao et al., 2015), and (2) overexpression of fibronectin in the trabecular meshwork cells in patients with diabetes may cause resistance to aqueous outflow and contribute to the elevation of IOP (Sato and Roy, 2002).

The above findings of higher CCT, Et and IOP with AGEs can contribute to better understanding of how diabetes affects the risk to develop glaucoma. Several factors, with contradictory effects, should be considered in this discussion. These include (1) the increased CCT and Et lead to higher corneal stiffness and are therefore expected to lead to overestimation of IOP using tonometry techniques (Elsheikh et al., 2011; Tang et al., 2012), possibly resulting in false positives in glaucoma diagnosis and management (Gordon et al., 2002), (2) the likely increase in true IOP with diabetes could increase the risk to develop glaucoma, (3) the stiffening observed in the cornea may be taking place also in both the sclera and lamina cribrosa (the site of damage in glaucoma) (Goldich et al., 2009; Terai et al., 2012), with these changes causing respectively increases and decreases in lamina deformation and hence subsequent risks to develop and progress glaucoma (Kimball et al., 2014). These contradictory factors, which inevitably have different influencing levels, could make patients with diabetes more or less likely to develop glaucoma. In the published literature, a history of DM was shown to have a protective effect against developing primary open-angle glaucoma in the Ocular Hypertension Study (OHTS) (Gordon et al., 2002).

The study has a number of limitations. First, obtaining IOP measurements that were not influenced by corneal biomechanics was a challenge for two reasons. While the Tonopen used in this study tended to underestimate the IOP (Ma et al., 2016), the increases in corneal thickness and tissue stiffness (Et) were expected to lead to overestimations in IOP measurements (Elsheikh et al., 2011; Tang et al., 2012) – quantifying the overall effect was not possible. Second, the speed of sound, 1640m/sec, was used in the ultrasonic pachymeter, which may have affected the measurement of corneal thickness. However, since the study concentrated on comparing the biomechanical behavior of diabetic and normal corneas, having the thickness in both groups measured in the same way would not be expected to affect the overall comparison results. Third, the study adopted the notion of rabbit corneas being reliable models for human corneas in mechanical property characterization. This decision was necessary in light of the need to acquire statistically significant material property data – which is extremely difficult to obtain from human donor corneas – and justified by earlier studies demonstrating the similarity in biomechanical behavior of human and rabbit corneas (Bao et al., 2015; Ni et al., 2011).

In conclusion, this study has confirmed a number of important trends concerning the effects of diabetes on the biomechanical behavior of the cornea and subsequently on the measurement of IOP, the risk to develop glaucoma and other applications where knowledge of corneal biomechanics is important. The trends included significant increases in corneal thickness and material stiffness and in the value of IOP, all associated with the accumulation of AGEs in the aqueous humour and glucose level in the blood.

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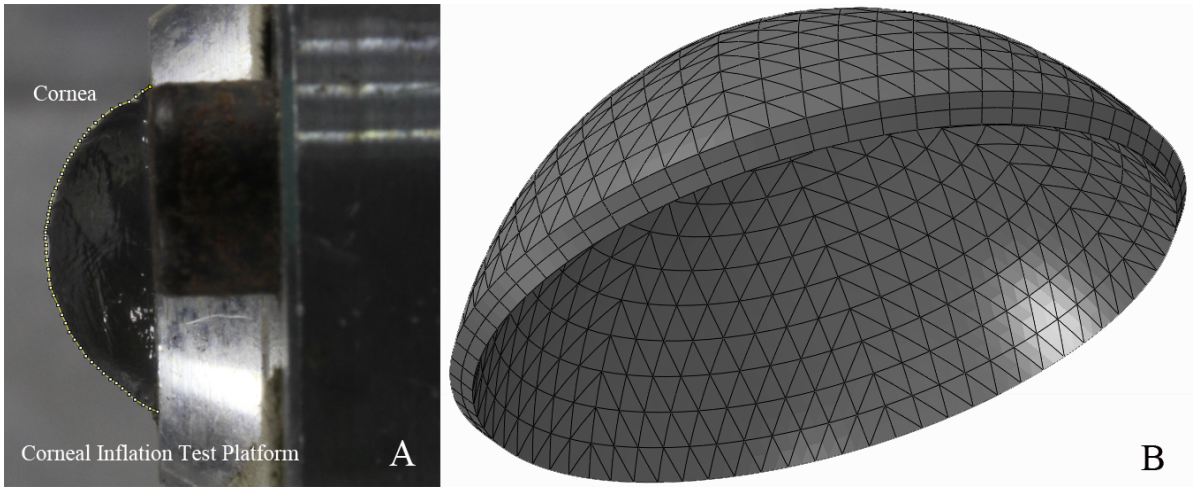

Fig.1 Corneal profile measured experimentally (A) and used to construct specimen-specific numerical models (B)

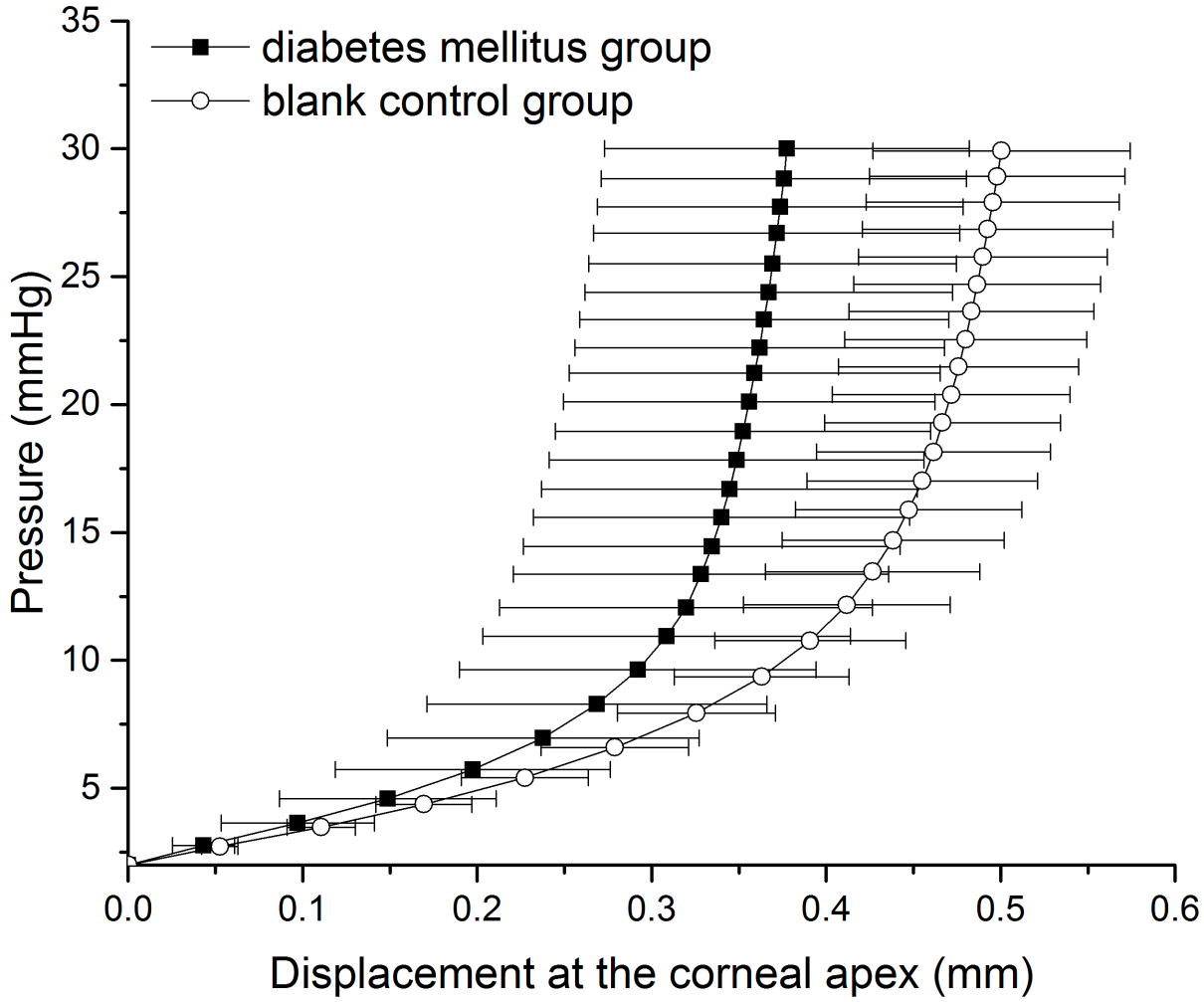

Fig. 2 Average pressure-displacement behavior at the corneal apex of diabetes mellitus group and blank control group. Error bars represent the standard deviation of displacement values.

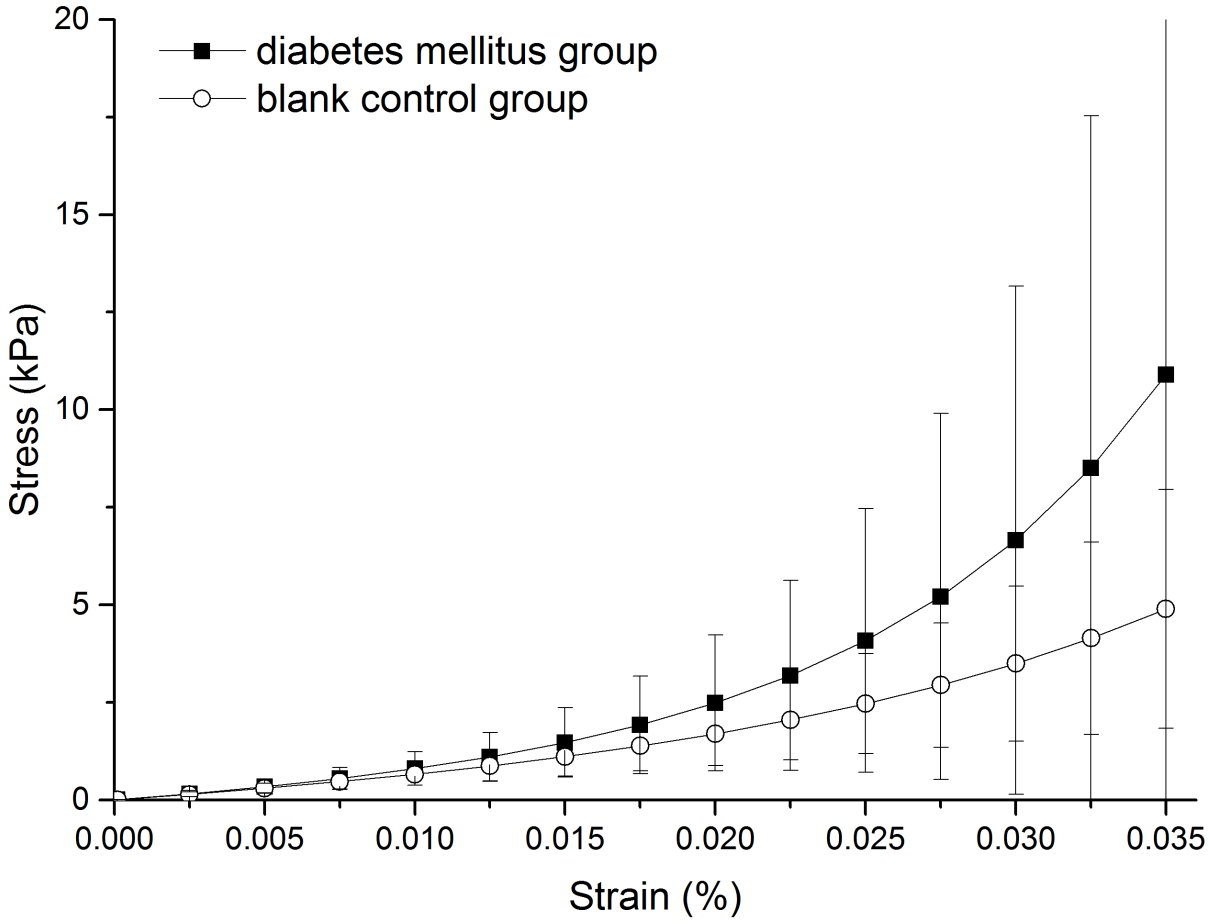

Fig. 3 Average stress-strain behavior of diabetes mellitus group and blank control group. Error bars represent the standard deviation of stress values.

Table 1 Constitutive parameters α and μ in two test groups

Group	α	μ	RMSE, mm
DM	0.0202±0.0096	83.467±20.976	0.0023±0.0023
BC	0.0184±0.0074	61.548±12.381	0.0011±0.0012

DM = diabetes mellitus group, BC = blank control group

Table 2 Average and standard deviation values of tangent modulus in DM and BC groups at different stress levels

Biomechanical Parameters	Stress (kPa)	DM	BC	p	Et_{DM}/Et_{BC} %
Tangent Modulus, Et (MPa)	1.0	0.12±0.03	0.1±0.02	0.02	123.4
	2.0	0.19±0.05	0.15±0.03	0.00	130.2
	3.0	0.26±0.07	0.2±0.04	0.00	132.9
	4.0	0.34±0.08	0.25±0.05	0.00	133.4

DM = diabetes mellitus group, BC = blank control group; Et-DM/Et-BC = Ratio of tangent modulus among biabetes mellitus group (Et-DM) and blank control group (Et-BC)