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Research paper

Simultaneous microstructural and mechanical characterization of human corneas at increasing pressure

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ABSTRACT

The cornea, through its shape, is the main contributor to the eye's focusing power. Pathological alterations of the cornea strongly affect the eye power. To improve treatments, complex biomechanical models have been developed based on the architecture and mechanical properties of the collagen network in the stroma, the main layer of the cornea. However, direct investigations of the structure of the stroma, as well as its link to the mechanical response, remained limited. We propose here an original set up, associating nonlinear optical imaging and mechanical testing. By using polarization resolved Second Harmonic signals, we simultaneously quantified micrometer (orientation of the collagen lamellae) and nanometer (local disorder within lamellae) scale corneal organization. We showed that the organization of the lamellae changes along the stroma thickness. Then, we measured simultaneously the deformation on the epithelial side of the cornea and the reorientation of the collagen lamellae for increasing intraocular pressure levels, from physiological ones to pathological ones. We showed that the observed deformation is not correlated to initial orientation, but to the reorganization of the lamellae in the stroma. Our results, by providing a direct multi-scale observation, will be useful for the development of more accurate biomechanical models.

1. Introduction

The cornea is the anterior part of the eye and is characterized by two main optical properties: transparency and focalization of light. It contributes to approximately two third of the optical power of the eye. Any modification in the shape or the mechanical strength of cornea induces a loss of vision. It is the case in several pathologies that are related to abnormal

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intraocular pressure (IOP), as glaucoma, or to variations of the structural and mechanical properties of the cornea, as keratoconus (Daxer and Fratzl, 1997; Meek et al., 2005; Morishige et al., 2014). Another clinical issue is corneal wound healing after laser refractive surgery procedures or corneal graft (Roberts, 2000). The mechanical strength and the shape of the cornea are tightly related to the highly organized three-dimensional architecture of the corneal stroma. This layer represents 90% of the corneal thickness, between the epithelium and the Bowman's membrane on the anterior side, and the Descemet's membrane and the endothelium on the posterior side (Krachmer et al., 2005). It is composed of hundreds of 2–3 μm-thick stacked collagen lamellae containing aligned nanometric collagen fibrils. Any disruption of the stroma microstructure is expected to affect the mechanical and optical properties of the cornea. It is therefore of great interest to develop specific and contrasted imaging tools to probe and quantify these hierarchical collagen structures in relationship with the corneal mechanical behavior.

The corneal microstructure has been first, to the best of our knowledge, investigated using electron microscopy (Beuerman and Pedroza, 1996; Radner et al., 1998). X-ray scattering patterns obtained by using Synchrotron radiation source (Meek and Boote, 2009) have also provided information about the anisotropic arrangements of collagen lamellae through the whole stroma in different regions of the cornea. Nevertheless, both techniques required tissue fixation preventing any mechanical assays. More recently, second harmonic generation (SHG) microscopy, a particular type of nonlinear optical microscopy, has proven to be an efficient tool for obtaining virtual biopsies in unstained fresh corneas (Aptel et al., 2010; Han et al., 2005; Latour et al., 2012b; Matteini et al., 2009; Morishige et al., 2014; Winkler et al., 2013, 2015; Yeh et al., 2002). All these studies have shown the complex and multiscale organization of human cornea, with lamellae mainly oriented along two orthogonal directions near the center, and mostly circumferentially near the sclera at the border (Quantock et al., 2015).

Elaborate biomechanical models (Nguyen and Boyce, 2011; Pandolfi and Vasta, 2012; Petsche and Pinsky, 2013; Pinsky et al., 2005; Studer et al., 2010; Whitford et al., 2015) have been developed recently to deal with both the stromal microstructure and the curved geometry of the cornea. They assumed a given distribution of lamellae and a hyperelastic behavior for the collagen fibrils. Calibration and validation of these models required experimental characterization of cornea mechanical behavior. Using controlled environment and loading conditions, *ex vivo* experiments have investigated various mechanical parameters through inflation (Boyce et al., 2008; Elsheikh et al., 2007, 2010; Hjortdal, 1995, 1996; Hjortdal and Jensen, 1995; Shin et al., 1997) or shear tests (Elsheikh et al., 2009; Petsche et al., 2012). In most inflation studies, the corneal surface strain was deduced from the apex displacement under increasing IOP (Elsheikh et al., 2007, 2010). Boyce et al. (2008) measured surface strains on bovine corneas using digital image correlation, whereas Hjordtal (Hjortdal, 1995, 1996; Hjortdal and Jensen, 1995) and Shin et al. (1997) reported pressure-induced mechanical strain on human corneas, using particle tracking on the epithelial side. More recent studies focused on *in vivo* mechanical characterization, using clinical devices (Pinero and Alcon, 2014), shear wave imaging (Nguyen et al., 2014) or full-field OCT combined with elastography (Nahas et al., 2013). Nevertheless, to our knowledge, all biomechanical models of cornea combined microstructural and mechanical information measured in distinct corneas. Therefore, significant progresses could be achieved on the validation and calibration of these models by being able to track the evolution of the corneal microstructure, and in particular of the lamellae orientation, during a mechanical loading.

To address this issue and determine in a reliable way the relationship between the collagen organization at nano- and micrometer scale in corneal stroma and the mechanical properties of this tissue, we present here semi-continuous observations of the evolution of the microstructure of the stroma during mechanical inflation tests on fresh human corneas. To that end, we combined SHG microscopy with mechanical assays and monitored simultaneously the stroma microstructure and the mechanical response. By tracking fluorescent micro-beads deposited on corneal epithelium, strains were measured on the anterior surface of the cornea for IOP varying from physiological to highly pathological levels. Quantitative structural information at both nanometer and micrometer scales were obtained by polarization-resolved SHG (P-SHG) microscopy, in particular the orientation of the collagen fibrils that form the collagen lamellae (Gusachenko et al., 2012; Stoller et al., 2002). By mapping this orientation at sequential depths within the cornea, we measured the probability density of fibril orientation along the whole depth of the cornea (Latour et al., 2012b). We then correlated structural and mechanical data obtained at different scales to get insight about the multiscale mechanics of each studied cornea. We showed that the distribution of fibril orientation changed from anterior to posterior stroma, indicating a transition from an isotropic to a more orthotropic microstructure. We also showed that the lamellae orientation evolved with pressure increase and that the surface strain was correlated to the observed changes in lamellar orientation.

2. Material and methods

2.1. *Cornea preparation*

Investigations were performed in accordance with the tenets of the Declaration of Helsinki and the French legislation for scientific use of human corneas. Human corneas provided by the French Eye Bank (BFY, Paris, France) were unsuitable for transplantation and assigned to scientific use (E.E.B.A., 2010). For this study, we used 14 human corneas that exhibited endothelial cell densities between 1400 and 2750 cell/mm^2 (mean value: 2330 cell/mm^2, higher than the viability threshold that is 2000 cell/mm^2 (E.E.B.A., 2010)) (see Table 1). The corneas were stored at 31 °C in a storage medium (StemAlpha2 #7002, StemAlpha) until the day before the experiment. They were then immerged for 24 h in a deswelling medium (StemAlpha3 #7003, StemAlpha) to recover a thickness close to the physiological one. However, they were slightly edematous, with a mean thickness of 630 μm, while the