Why Indentation Cannot Be Considered Exactly Equivalent to Non-contact Tonometry

We read the article by Ortillés et al. in the March 2017 issue that compared the indentation method with uniaxial extensiometry results in rabbit eyes with great interest.[1] Unfortunately, we observed that the analyses of the results did not match the conclusions. Further, the experimental methods were incomplete and lacked sound understanding of tissue mechanics. First, the authors have considered indentation to be similar to non-contact tonometry mechanically. This is incorrect because indentation applies a concentrated force over a small area.[1,2] Non-contact tonometry applies a distributed air-pressure over a significantly larger area.[3] The deformation characteristics of the two and duration of the test are vastly different.[2,3] Second, the data in Figures 3 and 4 are confusing. In Figures 3A and 3B, it is clear that post-corneal cross-linking (postCXL) (7d and 56d) corneas were more compliant than the preCXL state. Figures 3C and 3D agree with the results shown in Figures 3A and 3B. For example, the force versus U curves of 7d and 56d time points were to the right of the preCXL state, with 7d being the most compliant. The same is indicated in Figure 3C, which reports the stiffness or the slope of the curves plotted in Figure 3A. When other studies have reported stiffening after CXL using indentation,[3] it is unclear why the experimental data in this study was different.[1]

In Figure 4A, the stress versus stretch (expressed as [1.0 + uniaxial strain] in the direction of applied load) at different time points was presented. Here as well, the trends were the same (ie, both 7d and 56d were more compliant globally compared to preoperative time point of measurement). Third, it appears that the authors have evaluated the tangent moduli at one convenient stretch data point in Figure 4 to justify the end result, whereas the entire curve (from stretch 1.0 to maximum stretch) should have been analyzed with a regressed equation (eg, an exponential function relating stress to stretch and then taking the derivative of this curve to derive tangent moduli for a range of strains).[4] The study also does not describe the method used to calculate tangent moduli. If there were a sufficient number of sample points, then linear interpolation may suffice. However, Figures 3 and 4 show only a few sampling points per curve. This implies that linear interpolation, instead of regressed equation, could be inaccurate.[4] Last but not least, none of the samples was preconditioned before the actual stress versus stretch data were recorded.[5] Thus, extrapolation of the study results to the model proposed by the authors (Figure 6) is not supported by the analyses. In fact, the proposed compressive (negative) stress versus stretch curve (Figure 6) is a physical response commonly seen with mechanical testing of polymers but not tissue. This is because collagen fibers in cornea exhibit a crimping behavior and cannot bear compressive stress. In other words, the stress versus stretch curve (Figure 6) in the compressive (negative stress) zone would generally be linear (or a straight line). We believe that the authors need to reanalyze the data carefully before suggesting any conceptual model.

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Reply

We would like to thank Drs. Sinha Roy and Shetty for their comments regarding our article.[1] It is comforting to see that our research is appreciated by our colleagues. Nevertheless, we do not fully agree with their observations and provide detailed responses in consequence.

We agree that the area of the indenter is smaller than the actual "soft" area of influence associated with the air-puff, which cannot be precisely controlled during real experiments and, therefore, the loading acting on the cornea will be differentiated in both cases. However, saying that indentation applies a concentrated load on a very small area is not entirely correct because the "smallness" of the area

must be defined with respect to the surface on which the load is applied. In our case, the indentation to corneal area ratio was 1/10, which is similar to that for a Goldmann tonometer. Regarding the corneal deformation, the type of deformation that both loadings induce on the cornea is certainly comparable. The maximum corneal displacement is of the same order of magnitude (1 to 1.2 mm), and both loadings cause the cornea to deform beyond applanation, inducing a change of curvature in the corneal profile. Therefore, in both experiments, a flexion (bending) stress state is always achieved. Thus, mechanically, both experiments cause (up to a certain degree of stretch) compression in the anterior surface and tension in the posterior surface. However, we agree that the duration and nature of the loading is different. In the indentation test, load is applied at a low rate to achieve a quasi-static process during loading, whereas during air-puff the dynamic effects play an important role. Therefore, our protocol removes any possible bias on the measurements associated with dynamic effects.

Regarding the references provided by Sinha Roy and Shetty, only the nano-indentation publication[2] is related to cross-linking (CXL) treatment. However, comparing ex vivo in vitro nano-indentation measurements with our in vivo indentation tests is either not pertinent or must be done with great care. First, the diameter of the nano-indenter's tip is 10 nm, whereas the diameter of the indenter is 3 mm (a difference of six orders of magnitude). Second, the penetration depth is 4 µm, whereas the indentation is 1 mm (a difference of three orders of magnitude). In addition, the deformation induced in the cornea in both cases is vastly different. Whereas nano-indentation only causes a local deformation of the stroma, the indentation test causes an inversion in corneal curvature. Note also that, whereas nano-indentation only provides local force-displacement data, our indentation force-displacement curves provided average through thickness data in the cornea. All of these observations make it difficult to perform a direct comparison between these two experiments.

To answer the concern of Sinha Roy and Shetty regarding results on Figure 1 (p. 188), the deformation in the cornea during an indentation test (and also during an air-puff test) is not homogeneous. Therefore, the resulting force-displacement curve shown in Figure 1 (p. 188) obeys this heterogeneity of the deformation field. To support this assertion, we have simulated an indentation test (see panels b and c in **Figure 1**) by means of a finite element simulation (Abaqus; Dassault Systèmes, Paris, France). The model includes one-quarter of a spherical cornea (10 mm in diameter) and a scleral strip of 1 mm, preserving the curvature and the uniform thickness of a rabbit (ie, 380 µm). In addition, we have included the circumferential pattern of collagen fibers reported in rabbits[3] (panel a). We have used three different strain energy functions (SEFs) typically used in soft tissues and plotted both the radial and circumferential stress-stretch responses of the anterior and posterior stroma (panels d.1, e.1, and f.1), and the force-displacement mechanical response recorded at the indenter tip (panels d.2, e.2, and f.2). The SEFs that have been considered are: an isotropic Neo-Hookean material[4] (see panel d), an exponential isotropic Demiray material[5] (panel e), and an anisotropic Holzapfel-Gasser-Ogden response[6] (panel f). In addition, it must be pointed out that the SEF proposed by Holzapfel-Gasser-Ogden is the only one accounting for fiber crimping in compression. In all simulations, the cornea is pressurized at the physiological intraocular pressure (14 mm Hg) before initiating the indentation test (displacement of 1 mm). This configuration is defined by the black dots in **Figure 1**.

During an indentation test, the stroma of the anterior surface will reduce its in-plane initial tensional state toward an in-plane compression state (solid blue lines in **Figure 1**), whereas the stroma in the posterior surface will increase its traction state (solid red lines in **Figure 1**). This means that the fibers of the anterior surface will reduce their initial pre-stretch, and eventually crimp, after a minimum indentation displacement is reached (as Sinha Roy and Shetty have correctly stated). At this point, the ground matrix where the collagen fibers are embedded will be the only element contributing to load bearing in the anterior stroma. On the contrary, the posterior stroma always works in traction with the fibers increasing their stretching as the indentation load increases. Therefore, and considering that both the load registered during an indentation test corresponds to the global, or "homogeneized," response of the cornea and that the underneath material behavior is non-linear in nature, a non-linear response should be expected, as reported in the article.

It is not surprising that for an indentation of 1 mm (maximum stretch of 4%) the non-linearities are not significant. This fact is further demonstrated by the fibers in the anterior surface that, although they always work in traction, do not show the typical exponential behavior due to the low level of stretch. However, this does not mean that the fibers should behave linearly, but that the state of stress does not promote this phenomenon. In addition, non-linearities at the beginning of the force-displacement curve are related to the fact that the area of

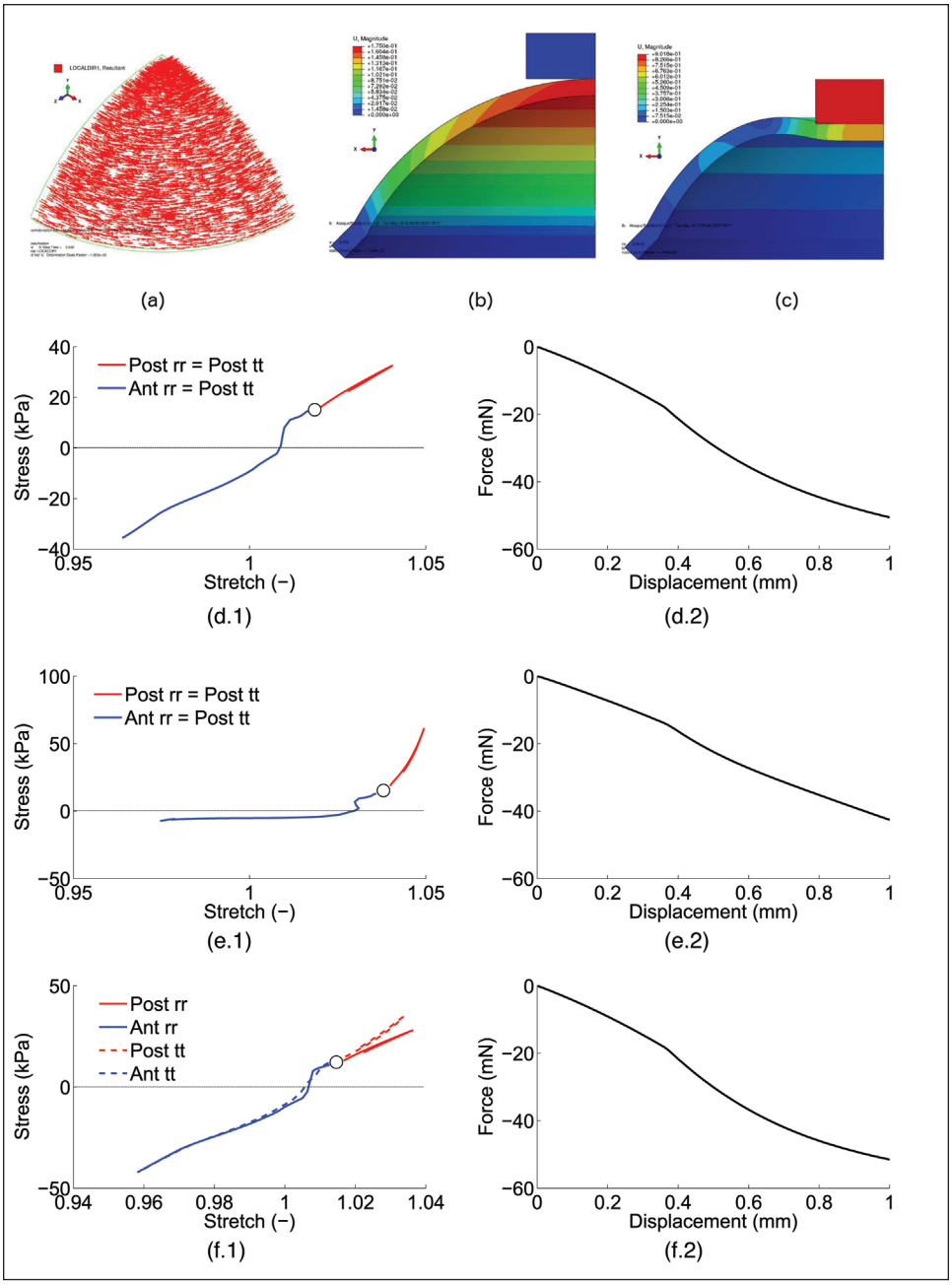

Figure 1. Stress-stretch response (rr = radial, tt = circumferential) of the anterior (Ant) and posterior (Post) stroma and force-displacement response of a rabbit cornea during an in silico indentation test. (a-c) Finite element simulation (Abaqus; Dassault Systèmes, Paris, France). The material models used are: isotropic hyperelastic (Neo-Hookean, d.1-2), exponential isotropic hyperelastic (Demiray, e.1-2), and anisotropic hyperelastic (Neo-Hookean + Holzapfel-Gasser-Ogden, f.1-2).

the indenter is not in full contact with the cornea. The corneal behavior described above occurs independently of the constitutive model used, as shown in **Figure 1**. In fact, the differences between the curves are associated with the degree of non-linearity of the SEF used for the computations only.

Another aspect that is worth mentioning is that even for the Neo-Hookean material, which gives an almost linear response at small strains, a slightly curving out of the linear behavior is observed. This means that, despite the low stretches (4%), the Neo-Hookean material is already presenting a slight non-linear response in compression. This behavior is also observed with the anisotropic model that accounts for collagen fiber crimping. In fact, and due to the low level of stretch, the only difference between including or not including the fibers is that the circumferential response (along the fiber, denoted by tt) is stiffer than the radial response (denoted by rr) as can be observed in panels d.1 and f.1 (**Figure 1**). Hence, although the non-linear response in compression is discrete (and dependent on the level of stretch), it supports our illustrative example depicted in Figure 4 in the article (p. 191).

In addition, we would like to stress that we do not perform either simulations or models in our study, but we only report experimental evidence. Figure 4 in the article (p. 191) is used in the article only for the sake of clarity, aiming at illustrating conceptually how the cornea is working to those not familiar with mechanics, and in an effort to better explain the observed experimental results. Furthermore, and to the best of our knowledge, experimental characterization of separate extracellular corneal stroma and collagen fibers has not been performed yet. Therefore, no experimental evidence supports that the corneal stroma should follow a linear response in compression.

The arguments given in the previous paragraph can be used to explain the results obtained after CXL treatment (Figure 1 in the article, p. 188). First, CXL treatment mainly affects the collagen fibers located in the anterior stroma, (ie, approximately 50% but dependent on the treatment, as demonstrated by immunohistochemistry [Figure 3 in the article, p. 189]). Because an indentation test, and similarly a non-contact tonometry test, primarily induces compression in the anterior stroma, these tests are expected to have a reduced ability to detect changes induced by the treatment. This, on the contrary, will not be the case for an inflation test or, as also demonstrated in our experiments (Figure 2 in the article, p. 189), a uniaxial tensile test where the tissue only experiences traction. If, on the contrary, CXL treatment would be hypothetically applied to the posterior surface, the effect of the treatment could be more easily detected as the fibers experience traction in the posterior stroma during an indentation test (or an air-puff). As Sinha Roy and Shetty have stated,[7,8] the effect of the fibers will only be detected in tension (uniaxial, biaxial, or both due to inflation), supporting our findings. We developed our study based on scientific evidence that reported the poor ability of bidirectional applanation tests (air-puff tests) in detecting differences between corneas before and after CXL. This has been suggested by Gatinel,[9] one of our main references in the study, and supported by several publications.[10-13] Finally, Sinha Roy and Shetty fall into a conceptual error when comparing our trends in uniaxial and indentation results because the stress state is not equivalent.

Regarding the third concern of Sinha Roy and Shetty, the explanation of the tangent moduli is graphically explained in Figure B in our article. As Sinha Roy and Shetty should be aware, there is not a single tangent modulus when a hyperelastic material is considered in general. For these reasons, we have taken the maximum tangent modulus before damage starts. This approach has been used by other authors.[14] Therefore, and contrary to the opinion of Sinha Roy and Shetty, the point has not been "conveniently chosen."

Regarding specimen preconditioning and data processing, in the Materials and Methods section we stated that all of the experiments were done with an Instron machine, with more than 100 points being recorded during each loading cycle of the experiment. For the sake of clarity, not all of them were plotted when representing the uniaxial traction curve. However, this fact does not prevent the use of graphical, interpolating, or fitting approaches to evaluate the slope. In our case, and because we had enough sampling points, we performed an interpolation and a numeric derivative. In addition, all of the samples were preconditioned, both in indentation and uniaxial tests. We refer Sinha Roy and Shetty to the following sentences extracted from the article: "Five cycles of loading (N) were applied on the corneal center using an indenter. . ." (p. 186, In Vivo Indentation Tests); "Three cycles of loading (N). . . (p. 187, In Vitro Uniaxial Tensile Tests); "Only the last cycle of each test (of each level for the uniaxial tensile tests) was kept for the analysis: (p. 187, Statistical Analysis); "average stretch-stress curves for the last cycle. . ." (p. 189, caption of Figure 2); or "average stretch-stress curves for the three groups for the last cycle of the in vitro uniaxial tensile tests" (p. 189, Mechanical Characterization). We always refer to the last cycle of stretch (ie, after preconditioning) and the average of the last cycle (ie, the average of the last cycle for all of the specimens).

Last but not least, we acknowledge the concern of Sinha Roy and Shetty about the reevaluation of our data. However, after 2 years of work, different rounds of reviews, and the arguments given above, we feel confident that our conclusions match our experimental results and also support previous reported data.

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