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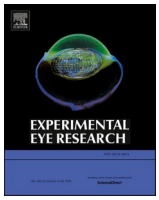

Short communication

Second harmonic generation microscopy of electromechanical reshaping on corneal collagen

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A B S T R A C T

Refractive errors remain a global health concern, as a large proportion of the world's population is myopic. Current ablative approaches are costly, not without risks, and not all patients are candidates for these procedures. Electromechanical reshaping (EMR) has been explored as a viable cost-effective modality to directly shape tissues, including cartilage. In this study, stromal collagen structure and fibril orientation was examined before and after EMR with second-harmonic generation microscopy (SHG), a nonlinear multiphoton imaging method that has previously been used to study native corneal collagen with high spatial resolution. EMR, using a milled metal contact lens and potentiostat, was performed on the corneas of five extracted rabbit globes. SHG was performed using a confocal microscopy system and all images underwent collagen fibril orientation analysis. The collagen SHG signal in controls is uniform and is similarly seen in samples treated with pulsed potential, while continuous EMR specimens have reduced, nonhomogeneous signal. Collagen fibril orientation in native tissue demonstrates a broad distribution with suggestion of another peak evolving, while with EMR treated eyes a bimodal characteristic becomes readily evident. Pulsed EMR may be a means to correct refractive errors, as when comparing its SHG signal to negative control, preservation of collagen structures with little to no damage is observed. From collagen fiber orientation analysis, it can be inferred that simple DC application alters the structure of collagen. Future studies will involve histological assessment of these layers and multimodal imaging analysis of dosimetry.

1. Short communication text

By 2050, it is predicted that half of the world's population will be myopic, and thus refractive errors remain a global health concern (Wolffsohn et al., 2020). The current standard of management includes glasses and contact lenses. Laser tissue ablation to reshape the cornea is very effective because the cornea provides approximately 2/3 of the refractive power of the eye. However, these ablative approaches are costly, not without risks, and not all patients are candidates for these procedures. A cost-effective alternative to reshape native corneal tissue would be compelling.

Electromechanical reshaping (EMR) has been explored as a viable cost-effective modality to directly shape tissues, including cartilage (Ho et al., 2003). In EMR, tissue is mechanically deformed by a jig or mandrel forged lens with either contact or penetrating electrodes. Then, an electrical field is applied, leading to the electrolysis of water. Evolved pH gradients transiently alter matrix components (glycosaminoglycans and collagen), leading to stress relaxation at a macroscopic scale. The degree of shape change depends upon dosimetry (applied potential and total charge) (Protsenko et al., 2006). Costs are low as the deformation jigs can be easily 3D printed or milled to specification.

Corneal shape change is intrinsically dependent on its collagen structure and organization. In this study, stromal collagen structure and fibril orientation was examined before and after EMR with second-harmonic generation microscopy (SHG), a nonlinear multiphoton imaging method that has previously been used to study native corneal collagen with high spatial resolution (Dilley et al., 2022; Bueno et al., 2011).

EMR was performed on the corneas of five extracted rabbit globes, with an untreated sixth globe serving as the negative control. A milled

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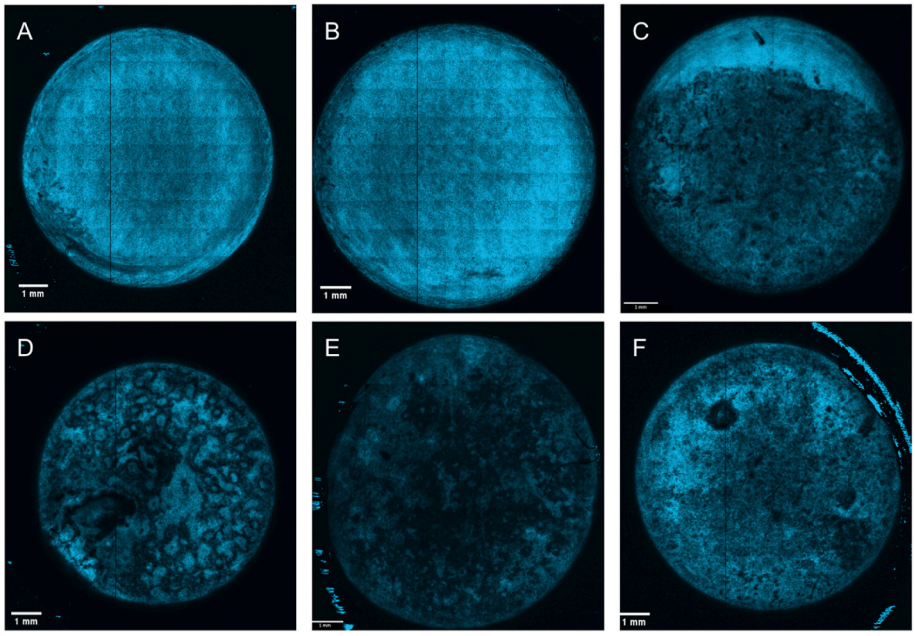

Fig. 1. Second harmonic generation imaging of negative control and treated rabbit cornea: A) negative control, B) 0.2C (pulsed), C) 0.1C (continuous), D) 0.15C (continuous), E) 0.2C (continuous), F) 0.3C (continuous). Total sample size: 6 rabbit globes.

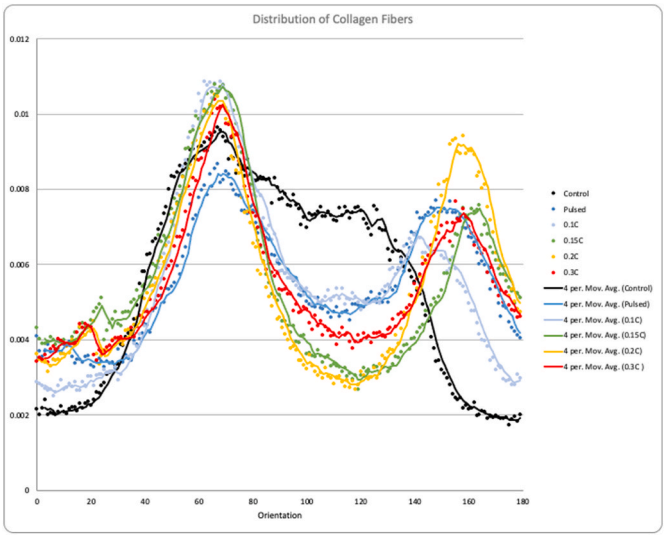

Fig. 2. Collagen fibril orientation analysis of negative control and treated rabbit cornea: negative control, 0.2C (pulsed), 0.1C (continuous), 0.15C (continuous), 0.2C (continuous), 0.3C (continuous). Total sample size: 6 rabbit globes.

metal contact lens served as an electrode and produced mechanical deformation while the globe was submerged in phosphate buffer solution. The technique of corneal reshaping using a contact lens has previously been described (Stokolosa et al., 2023). Total charge transfer, generated by a potentiostat (Model 650, CH instruments, Inc.), was specified at 0.1C, 0.15C, 0.2C, or 0.3C at a constant potential. A fifth sequence utilized pulsed voltage application (5 s on cycle, 30 s off) until 0.2C was delivered. SHG was performed using a confocal microscopy system (Leica LSM 510 Meta; Wetzlar, Germany) with a Ti:Sapphire femtosecond laser source (Chameleon; Coherent Inc.) ($\lambda = 850$ nm, 10x). Tilescans of the entire cornea (depth of ~150 μm) and Z-stacks (~500 μm thick, 2 μm/step) at the center of the cornea were acquired.

All images underwent collagen fibril orientation analysis and data was converted into frequency and was used to generate a fiber orientation angle distribution. Note that the peaks were aligned and normalized with unity area under the curve. Due to native cornea being anisotropic, in that the collagen fibers have a specific peak preference for orientation, the angle is essentially arbitrary in that there is no real origin. When these are modified, the fiber orientation changes, and the shape of the frequency distribution changes.

The collagen SHG signal in controls is uniform and high intensity (Fig. 1A) and is similarly seen in samples treated with pulsed potential (Fig. 1B); consistent textural patterns are also observed between the control and pulsed sample. EMR specimens treated with continuous potential have reduced signal and may be nonhomogeneous (Fig. 1C–F). Large changes in structure and pattern are evident.

Collagen fibril orientation in native tissue demonstrates a broad distribution; it exhibits a peak and nearly bimodal distribution (Fig. 2). The wide distribution of the collagen fibrils, meaning that fibers are in various directions with no specific uniform pattern, allow for the cornea to withstand diverse forces as well as maintain transparency. It is also important to note that the cornea robust and acts as a scaffold when it comes to change; it can even withstand drastic environment changes, such as dehydration and rehydration, while maintaining very similar mechanical properties (Romano et al., 2019). With increasing charge transfer due to EMR, this bimodal characteristic becomes readily evident (0.1–0.3C). Approximately 90° away from the first peak, there is development of a second peak. Note that this distinct second peak is not observed in native tissue; however, about 50° away there is suggestion of another peak evolving. With pulsed application, the second peak is broader than that of continuous application and is oriented at a value that is approximately 155°.

Corneal EMR performed with constant potential resulted in loss of SHG signal and disorganized fibril orientation. Fig. 1C–F showed SHG signal loss, suggesting a loss of native collagen tertiary structure, which may lead to opacity (Ávila et al., 2019). However, pulsed EMR (0.2C) showed no reduction in SHG signal compared to negative control, indicating preservation of collagen structures with little to no damage. While this suggests that pulsed potential application may leave corneal collagen unmodified, fiber orientation analysis showed a dose-dependent orientation change between pulsed and continuous treatment (Fig. 2). Hence, the strong SHG signal combined with the change in fiber orientation suggests that EMR of corneal collagen may be

feasible without damaging tertiary structures. Therefore, it can be inferred that simple DC application alters the structure of collagen.

As previously mentioned, corneal EMR performed with pulsed potential does not largely change the appearance of collagen as seen in the SHG tilescans; however, there are subtle changes in fiber orientation (Fig. 2). More importantly, the changes in fiber orientation that is observed in continuous EMR samples may only represent the collagen fibers that still give off SHG signal. We believe that the regions of the image that lack SHG signal have collagen fibers that have undergone chemical denaturation. Hence, the lack of fluorescence may indicate that SHG may underestimate the overall orientation change of the collagen fibrils in samples treated with continuous EMR.

Corneal tissue manipulation poses a unique challenge due to the underlying layers such as Descemet's membrane and corneal endothelium, which are critical for maintenance of corneal transparency. Future studies will involve histological assessment of these layers, aimed at understanding the effects of EMR at different depths of the tissue. Other avenues include multi-modal imaging analysis of dosimetry studies to determine the optimal parameters to reshape corneal tissue without extensive tissue disruption. Moreover, correct parameters will allow us to determine the optimal pH to reach during pulsed EMR treatment; this will ensure the ideal amount of hydrogen ions diffuse throughout the cornea without overtreating and creating too many peroxides. To help determine any potential changes in the treated corneas' mechanical properties, future studies will include the use of optical coherence elastography, which uses an external force to visualize deformation as a function of time. It is also important to investigate whether changes in the frequency or waveform of the pulsed application method can control alterations in fiber orientation.

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CRediT authorship contribution statement

Katelyn K. Dilley: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Karthik R. Prasad:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Theodore V. Nguyen:** Conceptualization, Formal analysis, Investigation, Supervision, Writing – original draft. **Anna Stokolosa:** Conceptualization, Formal analysis, Investigation, Writing – original draft. **Pamela A. Borden:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft. **J. Martin Heur:** Formal analysis, Investigation, Supervision, Writing – original draft. **Sehwan Kim:** Investigation, Project administration, Supervision, Writing – original draft. **Michael G. Hill:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft. **Brian J.F. Wong:** Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

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