

# Comparison of 1540-nm laser-induced injuries in *ex vivo* and *in vitro* rabbit corneal models

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**Abstract.** Despite the increasing use of infrared lasers in medical, industrial, and military settings, data on threshold radiant exposures and median effective dose ( $ED_{50}$ ) as they relate to laser-tissue interaction are limited. Our goals were to determine the  $ED_{50}$  for single-pulse, 1540-nm laser exposures in *ex vivo* and *in vitro* rabbit corneal models and to characterize the histopathological changes associated with the laser-tissue interaction. An erbium-glass laser was used to deliver single, 1540-nm wavelength pulses to 27 *ex vivo* and 24 *in vitro* rabbit corneal models. The *ex vivo* model was exposed to single pulses of 0.8-ms duration and radiant energies ranging from 17.61 J/cm<sup>2</sup> to 42.26 J/cm<sup>2</sup>. The *in vitro* corneal models were exposed to single pulses of 0.8 ms duration and had radiant exposures ranging from 14.87 to 29.72 J/cm<sup>2</sup>. Tissue exposure sites were observed for presence of a lesion immediately post-exposure and 24 h after exposure. Histopathological evaluations of tissue exposure sites were conducted 24 h after exposure. The  $ED_{50}$  was determined to be 21.24 J/cm<sup>2</sup> for the *in vitro* rabbit corneal models and 30.86 J/cm<sup>2</sup> for the *ex vivo* corneal models. Both the *in vitro* and *ex vivo* models displayed similar histopathological responses of tissue necrosis and epithelial cell proliferation. © 2007 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2822371]

Keywords: infrared laser; eye safe; tissue culture; median effective dose ( $ED_{50}$ ).

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## 1 Introduction

Lasers are common in everyday life and can be found in a variety of devices, including range finders, scanners, printers, welding tools, fiber optics, and medical instruments. While science, medicine, and society in general have enjoyed the benefits of lasers, there are also inherent ocular safety issues that should not be overlooked. Awareness of these dangers has fostered the use of lasers operating in the 1400- to 2200-nm region,<sup>1,2</sup> since these wavelengths are absorbed by the cornea and anterior chamber before they can cause permanent damage to the retina. However, this approach is not without risk. The cornea has a high density of pain receptors, so even small amounts of damage can cause severe pain.<sup>3,4</sup> The cornea provides approximately 85% of the focusing power in the eye,<sup>5</sup> so laser-induced damage that alters corneal shape or transparency can have a pronounced effect on vision.

The majority of studies reported to evaluate laser eye injuries and ocular safety issues rely on *in vivo* laser exposures of experimental animals.<sup>6</sup> The maximum permissible exposure (MPE) recommendation currently listed in the ANSI standards for 1540 nm is specifically based on median effective dose ( $ED_{50}$ ) data derived from *in vivo* exposures of non-human primate corneas to erbium lasers.<sup>7</sup> However, the

literature<sup>7-10</sup> provides several different  $ED_{50}$  values for the 1540-nm laser that range from 4.7 to 56 J/cm<sup>2</sup>. This difference has been suggested to correlate with pulse duration and the dependence on the spot size of the laser beam.<sup>11</sup>

Rabbits are a well-established, nonprimate, *in vivo* model for human eye research, and a robust database exists for laser-induced corneal injury in rabbits.<sup>10,12-15</sup> However, in order to reduce the number of experimental animals used in potentially painful studies, researchers continue to look for alternatives to traditional *in vivo* animal models.<sup>16-18</sup> Alternative testing methods such as those using *ex vivo* or *in vitro* tissues support the concept of reducing painful experimental procedures while allowing researchers to rapidly screen treatment techniques and develop safety standards for ocular laser exposure.

The purpose of the present study was to determine the  $ED_{50}$  of 1540-nm laser-induced injuries in *ex vivo* and *in vitro* rabbit corneal models and compare the immediate and 24 h post-exposure tissue responses. Because of the correlation between  $ED_{50}$ , pulse duration, and laser beam spot size, the present study used analogous laser parameters to those reported by Clarke et al. for *in vivo* exposure of rabbit eyes to 1540-nm laser light.<sup>10</sup>

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## 2 Methods

### 2.1 Laser System

An erbium-glass laser (Laser Sight Technologies, Winter Park, Florida) producing 0.8-ms pulses at 1540 nm was used for all *in vitro* and *ex vivo* exposures. The laser is a free-running, optically pumped system, with pulse shapes that are roughly Gaussian. The laser beam was focused with a 50-mm lens (BK-7 lens material, LA1708, Newport, Irvine, California), and the spot size was determined using a knife-edge technique.<sup>19</sup> Laser energy was measured with a Moletron EPM-2000 meter and a J25 energy detector (Coherent, Santa Clara, California). Pulse duration was measured using a germanium photon detector (PDA 255, Thor Labs, Newton, New Jersey) connected to a Tektronix TDS 644B digitizing oscilloscope (Beaverton, Oregon). The epithelial surfaces for both *in vitro* corneal tissues and *ex vivo* eyeballs were positioned at a distance of 54 mm from the lens.

### 2.2 Ex Vivo Rabbit Corneal Model

Rabbit eyes for the *ex vivo* experiments were provided in a single shipment from a commercial source (Pel-Freeze, Rogers, Arizona). All eyes were obtained from rabbits approximately 6 months of age and shipped overnight, on wet ice, in bottles containing 0.1-M phosphate buffer, pH 7.2 supplemented with 1% fresh rabbit serum, 100 IU/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 0.25  $\mu\text{g}/\text{ml}$  amphotericin. Eyeballs that showed corneal opacity after shipment were excluded from the study. A total of 31 eyes were used for this study. There were four control eyes, which were handled in the same way as the exposed eyes except for laser exposure. The corneas from 27 eyes were exposed in four locations (1 marking lesion and 3 experimental; see laser exposure parameters below) for a total of 108 exposures. The *ex vivo* corneal models were maintained in an incubator at, 37°C and 5% CO<sub>2</sub> immediately prior to exposure and post-exposure. This approach was taken to mimic the natural environment of the *in vivo* cornea, where the optical absorption characteristics of the tissue are temperature dependent.<sup>20</sup> The maximum exposure duration of the *ex vivo* corneal model to the room temperature environment was 30 s prior to the first exposure, 2 min during the exposure sequence, and 1 min post-exposure. After laser exposure, each eye was placed in a separate well of a 6-well plate and incubated with F12/DMEM media (MediaTech) supplemented with 10% NuSerum (Collaborative Biomedical), 2 mM L-glutamine, 100 IU/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 0.25  $\mu\text{g}/\text{ml}$  amphotericin for 24 h at 37°C and 5% CO<sub>2</sub> to evaluate the tissue response to laser injury. The eyes were moved by grasping the optic nerve with forceps to ensure that the cornea was not injured during handling.

### 2.3 In Vitro Rabbit Corneal Model

Primary corneal epithelial cells and stromal keratocytes isolated from New Zealand White rabbits served as the seed cultures for the *in vitro* corneal models. All cell culture procedures were conducted using F12/DMEM media. Corneal equivalents were produced in two steps.<sup>21</sup> First, a liquid collagen/corneal keratocyte seed culture suspension was added to a Transwell (Costar) tissue culture insert, with a

porous polycarbonate supporting membrane, contained within a 12-well tissue culture plate. The collagen/keratocyte suspension formed a gel during incubation (37°C, 5% CO<sub>2</sub>) that was supported by the polycarbonate membrane, and the keratocytes were grown in culture for 5 to 7 days. Second, a seed culture suspension of corneal epithelial cells was plated upon the collagen/keratocyte gel and grown in culture for an additional 14 to 21 days. The tissue culture fluid levels were slowly lowered over the incubation period until an epithelial cell-air interface was established that allowed stratification of the epithelial layers into basal, wing, and superficial cells.

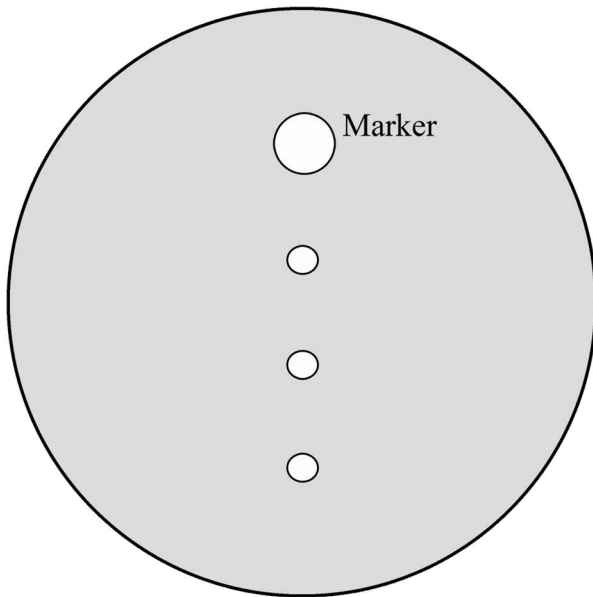
A total of 24 *in vitro* rabbit corneal models were used for this study. Four corneal models were used as controls, and twenty corneal models were exposed in four locations (1 position marker and 3 experimental; see laser exposure parameters below) accounting for 80 exposures. The *in vitro* corneal models were maintained in an incubator at 37°C and 5% CO<sub>2</sub> immediately prior to exposure and post-exposure in a manner analogous to the *ex vivo* corneal models. The maximum exposure duration of the *in vitro* corneal model to the room temperature environment was 30 s prior to the first exposure, 30 s during the exposure sequence, and 1 min post-exposure. The shorter time period during the *in vitro* exposure sequence, compared to the analogous *ex vivo* exposures, related to the flat surface of the *in vitro* tissues. After laser exposure, each corneal model was placed in a separate well of a 12-well plate and incubated with F12/DMEM media for 24 h at 37°C and 5% CO<sub>2</sub> to evaluate the tissue response to laser injury.

### 2.4 Laser Exposure Parameters

The spot size and calibration factor were calculated for each input energy voltage. Ten pulses were used to create a positional marking lesion at the edge of the *in vitro* and *ex vivo* corneal models. This marking lesion provided a reference point for histological analyses. The first experimental exposure was made using a single pulse, exactly 2.5 mm from the marking lesion. Two successive single pulse exposures were spaced exactly 5 mm and 7.5 mm from the marking lesion (Fig. 1). Because of the curvature of the cornea in the *ex vivo* model, the placement of the lens relative to the corneal surface was adjusted for each exposure to maintain a constant 54 mm distance. The *in vitro* corneal model was flat and received a consistent spot size and shape for all experimental exposures without continuous adjustment.

### 2.5 Evaluation for Injury

All experimental laser exposure sites were independently evaluated within 1 min post-exposure and 24 h post-exposure by two graders. Exposure site examinations were conducted with and without the aid of a handheld ophthalmoscope (Welch Allyn, Skaneateles Falls, New York). Lesions were graded on a nominal scale of present or absent, based on agreement by both graders. Fluorescein staining (Fluor-I-Strip-AT, 9 mg strips, Bausch & Lomb, Rochester, New York) of *ex vivo* exposure sites was evaluated with a Woods lamp and used to highlight laser lesions. Fluorescein staining was not necessary and not used to highlight laser lesions with *in vitro* exposure sites. The *ex vivo* corneal models were rinsed with sterile water after fluorescein application to reduce background staining. All corneal models were photographed be-



**Fig. 1** Cornea exposure diagram showing the orientation of the marking lesion and the experimental exposure sites.

fore laser exposure, within 1 min post-exposure, and 24 h post-exposure using a Nikon D1H digital camera with an F-50 Nikon macroscopic lens (El Segundo, California). After grading and photography, each corneal model was placed into an individual well of a 12-well culture plate containing F12/DMEM media and incubated for 24 h at 37°C and 5% CO<sub>2</sub>. After the 24 h post-exposure incubation, the corneal models were removed, graded, and photographed for presence or absence of a visible lesion.

## 2.6 Embedding and Freezing of Corneal Tissues for Histological Evaluation

The corneas were recovered from the *ex vivo* models by sharp, circumferential dissection at the limbal margins. The *in vitro* corneal models were entirely removed from the transwell by sharp dissection of the underlying polycarbonate membrane. The marking lesion on each corneal tissue was highlighted using a surgical dye to facilitate positioning within the embedding matrix. Each corneal tissue was placed into a 5-ml disposable beaker filled with cryotome embedding medium (OCT, Sakura FineTech, Torrance, California) and oriented with the marking lesion at the 12 o'clock position and subsequent lesions along the center line. The corneal tissues were then placed in a freezing bath of hexane surrounded by liquid nitrogen. Once frozen, the 5-ml disposable beaker was removed, covered with aluminum foil, placed into a labeled cryostorage bag, and stored at -80°C until processed for histology.

## 2.7 Histology

Tissue sections were made using a motorized cryomicrotome (Bright Instruments, Huntingdon, England). The tissues were rapidly trimmed in the cryomicrotome until the highlighted marking lesion was revealed. The micrometer on the cryomicrotome was then set and the tissues trimmed to reveal the

exposure sites that were exactly 2.5 mm apart. The marking lesion and the exact spacing of the exposure sites allowed sectioning of the lesions with micrometer precision and accurately resolved the inside edge, middle, and outside edge of the laser lesion. Sections to be evaluated for histopathology were stained using Gill's #3 hematoxylin for 30 s, rinsed in tap water for 5 min, and counterstained using eosin/phloxine for 30 s. Stained sections were then dehydrated in a series of alcohols, dipped in xylene, and mounted using Eukitt (Electron Microscopy Services, Hatfield, Pennsylvania). Images for histopathological analyses were captured using a Leitz Orthoplan microscope equipped with a SpotRT digital camera (Diagnostic Instruments, Sterling Heights, Michigan). Criteria used to evaluate the tissue responses to laser exposure included alterations in the epithelial parameters (e.g., area of hyaline coagulative change versus area of granular coagulative change) and stromal parameters (e.g., area of coagulative necrosis and number and distribution of keratocytes). Comparisons were made between treatment groups and control groups to evaluate the 24 h post-exposure healing response.

## 2.8 Statistical Analysis of Laser Lesions

A probit statistical model was used to determine the ED<sub>50</sub> for all stochastic injury results.<sup>22,23</sup> The SAS 9.1 Probit Procedure (SAS, Inc., Cary, North Carolina) was used to determine the ED<sub>50</sub> with 95% fiducial intervals, as well as a  $\chi^2$  value. The goodness-of-fit test for the predicted data was determined using the Pearson's chi-square and likelihood ratio chi-square. If the Pearson goodness-of-fit chi-square test was applied and the *p*-value for the test was too small, a heterogeneity factor and a critical value from the *t* distribution were used to compute the fiducial limits. If the injuries followed a deterministic pattern, the technique described by McCally and Bargeron was utilized to determine the threshold of injury.<sup>24</sup>

## 3 Results

### 3.1 Laser Parameters

The laser produced an exposure spot size range of 2.62E-03 cm<sup>2</sup> to 3.16E-03 cm<sup>2</sup> at a distance of 54 mm above the epithelial surface of the rabbit corneal models. The spot size was oval in shape, with diameters ranging from 0.582 × 0.572 mm to 0.660 × 0.610 mm, respectively. The pulse duration was 0.8 ms. The *ex vivo* corneal models had radiant exposures ranging between 17.61 and 42.26 J/cm<sup>2</sup>. The *in vitro* corneal models had radiant exposures ranging between 14.87 and 29.72 J/cm<sup>2</sup> (Table 1).

### 3.2 Ex Vivo Rabbit Corneal Models

Analysis of the data using the probit statistical method yielded an ED<sub>50</sub> of 30.86 J/cm<sup>2</sup> for the immediate post-exposure observations and an ED<sub>50</sub> of 32.41 J/cm<sup>2</sup> for the 24 h post-exposure observations (Table 1). The Pearson chi-square and the log-likelihood ratio chi-square tests were not significant at the 0.1 level for the immediate *ex vivo* corneal model data. The immediate post-exposure *ex vivo* corneal model Type III analysis of effects for the dose gave a Wald chi-square of 11.95 and a *p*-value of 0.0005.

The *ex vivo* corneal models had 42 lesions out of 75 exposures apparent immediately after exposure. All lesions were

**Table 1** Median effective dose and fiducial limits of tissue response by experimental model.

Model	ED <sub>50</sub> (J/cm <sup>2</sup> )	95% Fiducial Limits	
		Lower Limit	Upper Limit
<i>In vitro</i>	21.24	20.59 <sup>a</sup>	21.89 <sup>a</sup>
<i>Ex vivo</i>	30.86	29.49	32.03
24 hr <i>Ex vivo</i>	32.41	30.63	34.31
<i>In vivo</i> <sup>b</sup>	56.00	54.70	57.60

<sup>a</sup>Note on fiducial limits. Doses below the lower limit did not produce injury, and doses above the upper limit always produced injury.

<sup>b</sup>See Ref. 10.

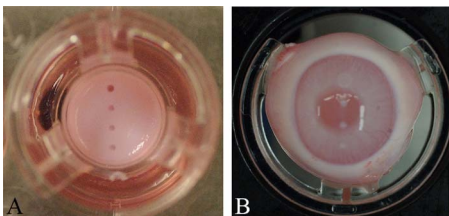
clearly identified immediately after exposure except for one exposure at 33.04 J/cm<sup>2</sup>, which had a visible lesion only at the 24-h examination.

The lesions produced immediately after laser exposures were clearly visible, with no disagreement between graders. The lesions appeared as circular, discrete, raised, opaque, white areas on the cornea (Fig. 2). Use of the ophthalmoscope and fluorescein staining did not reveal any lesions that were not visible with careful gross examination, although these aids did provide better detail of the lesion border. After 24 h of incubation, the gross appearance of the *ex vivo* lesions remained as circular, discrete, raised, opaque, white areas on the cornea. Histological analyses of tissues 24 h post-exposure revealed both tissue necrosis and a proliferative response (Figs. 3 and 4).

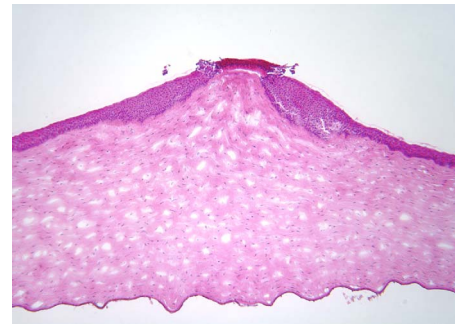
The *ex vivo* corneal model's immediate ED<sub>50</sub> fell within the 95% fiducial limits of the 24-h ED<sub>50</sub>, indicating that the two ED<sub>50</sub>s were not significantly different and that the ED<sub>50</sub> could be taken immediately post-exposure instead of waiting 24 h to determine the threshold (Table 1).

### 3.3 *In Vitro* Rabbit Corneal Model

Probit analysis was not used to evaluate the *in vitro* corneal models because the data was deterministic. McCally and Barger's technique to determine the threshold was applied.<sup>24</sup> The threshold exposure was defined as the midpoint energy between where the laser produced a minimal lesion and had no observable effect. Radiant exposures below



**Fig. 2** Digital photographs of corneal lesions immediately after laser exposures to *in vitro* (a) and *ex vivo* (b) corneal tissues. *In vitro*: Marking lesion and experimental exposures appear as discrete, round holes in the surface. *Ex vivo*: Marking lesion and experimental exposures appear as discrete, raised, opaque areas on the surface.

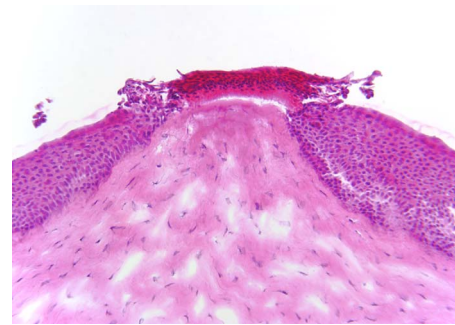


**Fig. 3** Representative image of damage to an *ex vivo* rabbit cornea 24 h after 1540-nm laser exposure at 37 J/cm<sup>2</sup>. The epithelium within the beam path was markedly damaged. The stroma directly underlying the epithelial layer within the beam path showed an increased basophilia typical of thermal denaturation. The epithelial cells outside the beam path and adjacent to the damaged epithelium demonstrated a marked proliferation. Minimal cellular response was observed in the adjacent stroma. Original magnification=63×.

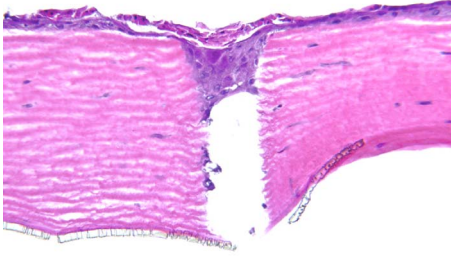
20.59 J/cm<sup>2</sup> did not produce a visible lesion, even when viewed using the ophthalmoscope, while all exposures over 21.89 J/cm<sup>2</sup> produced a visible lesion. The data did not change for the immediate versus 24 h post-exposure; therefore, the threshold exposure was 21.24 J/cm<sup>2</sup>. All lesions appeared immediately after laser exposure and were clearly visible (without fluorescein staining), with no disagreement between graders (Fig. 2). Fluorescent staining was not useful in the *in vitro* model, as the entire tissue was dyed pale yellow.

The lesions appeared as discrete holes in the tissues. There was no color change in the tissue. Lesions did not appear to change grossly after the 24-h post-exposure incubation. Histological analyses revealed both tissue necrosis and a proliferative response (Figs. 5 and 6).

The *in vitro* corneal model ED<sub>50</sub> of 21.24 J/cm<sup>2</sup> was not within the *ex vivo* corneal model's ED<sub>50</sub> 95% fiducial limits of 29.49 to 32.03 J/cm<sup>2</sup>, indicating that the two ED<sub>50</sub>s were different from each other (Table 1). However, the two models showed similarity in their histopathological responses with



**Fig. 4** Higher magnification image of the *ex vivo* rabbit cornea shown in Fig. 3. The epithelial cells within the beam path demonstrated a distinct coagulative necrosis. There was a decreased number of stromal keratocytes within the area of increased stromal basophilia. The marked epithelial proliferation is primarily restricted to the cells of the *stratum basale* located outside the periphery of the beam path. Original magnification=160×.

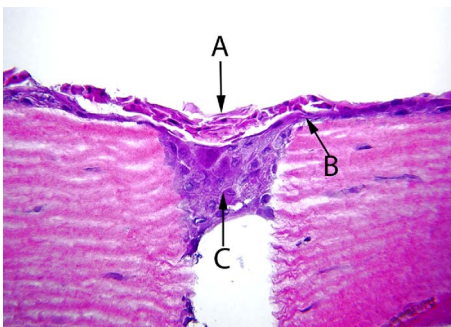


**Fig. 5** Representative image of damage to an *in vitro* rabbit corneal model 24 h after 1540-nm laser exposure at 28 J/cm<sup>2</sup>. The most superficial layer of the epithelium within the beam path was markedly damaged. However, the underlying epithelial cells showed a proliferative response that was analogous to the *ex vivo* rabbit cornea. The stromal matrix in the model tissue was affected more severely than the *ex vivo* rabbit cornea with a distinct tissue ablation within the beam path. Original magnification=160 $\times$ .

tissue necrosis and epithelial cell proliferation.

### 3.4 Discussion

The *ex vivo* and *in vitro* corneal models allow for alternatives to the gold standard *in vivo* model when exploring damage thresholds for the infrared wavelengths. The *ex vivo* and *in vitro* corneal models exposed to the 1540-nm wavelength also provide new data to aid in the determination of the infrared laser safety MPE. These models have certain benefits and disadvantages. Both the *in vitro* and *ex vivo* model's median effective dose thresholds are different from the thresholds reported for *in vivo* laser exposures in rabbits using analogous exposure conditions.<sup>10</sup> The *in vitro* model uses far fewer animals to produce the corneal models for experimentation, but the model takes time to produce and requires personnel and equipment for cell culturing. The *ex vivo* tissue model uses the same number of animals, but the globes may be obtained through tissue sharing or from approved tissue sources, and the number of animals experiencing pain during experimental procedures is reduced. The *in vitro* corneal model allows for exact spot size duplication while moving the exposures along



**Fig. 6** Higher magnification image of the *in vitro* corneal model shown in Fig. 5. (a) Superficial layer of epithelial cells damaged by laser and not able to repair. (b) Proliferating cells arising from the basal layer of the corneal epithelium. (c) Proliferating cells seen filling the ablated tissue space produced by the laser energy. Original magnification=250 $\times$ .

a horizontal axis due to the flatness of the tissue. This is important, as it reduces the time to expose the tissue, compared to the *ex vivo* tissue, and ensures reproducibility for each exposure point. Both models allow researchers without lab animal facilities to conduct laser tissue experiments more readily.

The *in vitro* and *ex vivo* corneal models were incubated for 24 h to encourage healing, but only the *ex vivo* corneal model showed a change in the number of grossly visible lesions after 24 h and subsequently a change in the ED<sub>50</sub>. This change in the *ex vivo* ED<sub>50</sub> was not significant. While the grossly visible changes in the *ex vivo* model were not seen in the *in vitro* model, histology revealed a different picture, with both models showing a proliferation of the epithelial cells, indicating that a healing process was initiated.

The ED<sub>50</sub> of both the *ex vivo* and the *in vitro* models were much higher than the ED<sub>50</sub> that was reported by Lund, Avdeev, and Stuck for the 1540-nm laser.<sup>7-9</sup> We propose that this difference is related to the spot size and pulse duration of the laser beam. The spot size reported by Lund, Avdeev, and Stuck ranged from 1 to 2 mm in diameter. The beam spot size determined in the present study was oval in shape and ranged from 0.582 to 0.660 mm in diameter, similar to Clarke's work,<sup>10</sup> where the diameter of the beam was 0.418  $\times$  0.598 mm. McCally et al. reported that both spot size and pulse duration affect the median effective dose for the 1540-nm wavelength. They also reported that at shorter pulse durations, the 1540-nm laser does not follow a modified critical temperature model.<sup>11</sup>

Both the *in vitro* and the *ex vivo* ED<sub>50</sub>s determined in the present study were well below the ED<sub>50</sub> of 56.0 J/cm<sup>2</sup> reported for analogous *in vivo* infrared laser exposures (Table 1).<sup>10</sup> This increased sensitivity of the model systems may be useful in rapid screening of laser-tissue interactions but should be taken into account when extrapolating experimental data. Since the biological effects of laser exposure depend on tissue chromophores and the absorption properties of the exposed tissue, the higher *in vivo* ED<sub>50</sub> most likely indicates differences in the structural and chemical characteristics of the corneal models compared to the living native tissue.

Factors that could contribute to the elevated threshold for laser injury following *in vivo* exposures would include: the thickness and composition of the tear film layer, the relative deturgescence of the cornea, and the homiothermic temperature of the tissue.<sup>25</sup> The tear film layer provides nutrients to the cornea, and the different nutrients may contain chromophores that alter the laser energy before being absorbed by the corneal tissue. The inherent light-absorbing characteristics of the corneal models and the native tissue may also differ. An obvious difference is the coloring of the three tissues. The *in vivo* exposure data were reported using Dutch Belted rabbits,<sup>10</sup> which have a dark brown iris, while the *ex vivo* models in the present study used a New Zealand White rabbit with a pink eye, and the *in vitro* model was light pink throughout. The different coloring of the three models may affect the laser absorption and influence the laser damage threshold.

Last, *in vivo*, *ex vivo*, and *in vitro* ocular systems have inherently different levels of tissue complexity that may be reflected in the complexity of the tissue chromophores. The *in*

**Table 2** Fixed factor correlation relative to *in vivo* data applied to median effective dose and fiducial limits of tissue response by experimental model.

Model	Fixed Factor	ED <sub>50</sub> (J/cm <sup>2</sup> )	95% Fiducial Limits	
			Lower Limit	Upper Limit
<i>In vitro</i>	2.64	56.07	54.36	57.79
<i>Ex vivo</i>	1.81	55.86	53.38	57.97
24 h <i>Ex vivo</i>	1.73	56.07	52.99	59.36
<i>In vivo</i> <sup>a</sup>	–	56.00	54.70	57.60

<sup>a</sup>See Ref. 10.

*vivo* tissue would have the most complex system, as it gains nutrients from both the tear film layer and the aqueous humor, which are dynamic systems. The *ex vivo* would be less complex, and the *in vitro* the least complex.

The *in vitro* and *ex vivo* ocular tissues appear not to have an overlap of fiducial limits, indicating a significant statistical difference in the ED<sub>50</sub>. This statistical difference cannot be explained by spot size or pulse duration, because these laser parameters were held constant during the study, and most likely is related to inherent differences between the model tissues. However, there is a relationship between the data from the *in vitro* and *ex vivo* models used in the present study and the *in vivo* data reported by Clarke et al. that should provide a method to extrapolate *in vitro* and *ex vivo* data to predict the response of *in vivo* exposures. We determined that the *in vivo* ED<sub>50</sub> reported by Clarke et al. is approximately 2.64 times that for the *in vitro* corneal model and 1.81 times that for the *ex vivo* corneal model (Table 2).<sup>10</sup>

The tissue reaction in the first 24 h post-exposure appeared to primarily involve the epithelial cells. The histopathological response of the *in vitro* corneal tissue was analogous to the response of the *ex vivo* rabbit corneas with essentially identical nuclear and cytoplasmic patterns of coagulative necrosis. The marked epithelial proliferation observed with the *in vitro* model was primarily restricted to the cells of the *stratum basale* in a similar manner as observed with the *ex vivo* tissue. These results indicate that from a histopathological perspective, the *in vitro* rabbit corneal tissues developed in the present study are appropriate models for the acute post-exposure response of native corneal tissue following 1540-nm laser exposure. In addition, we believe that this is the first report of acute, post-exposure corneal epithelial cell proliferation induced by single-pulse, 1540-nm laser light.

The histopathological responses of the *ex vivo* and *in vitro* corneal models appear to bracket the histological observations reported by Clarke et al. in a manner that may reflect the radiant exposure deposition and highlight an increased sensitivity of the *in vitro* model to radiant exposure. The representative histological image shown in Clarke's publication revealed a full-thickness stromal response 24 h following a single, 1540-nm and 0.8-ms pulse with a radiant exposure of 145 J/cm<sup>2</sup>. The *ex vivo* model used in the present study showed only a small stromal effect with a radiant exposure of

37 J/cm<sup>2</sup> (Fig. 3). The *in vitro* model used in the present study showed a total ablation of the stromal matrix within the beam field with a radiant exposure of 28 J/cm<sup>2</sup> (Fig. 5).

The combination of a fixed factor that can be applied to *in vitro* and *ex vivo* experimental data and the similarity of the histopathological response between *in vitro* and *ex vivo* corneal models will improve the extrapolation of experimental data to *in vivo* results and help fill the gap of research data appropriate for infrared laser safety standards. In addition, the apparent increased sensitivity of the *in vitro* model to laser effects (supported by ED<sub>50</sub> and histopathological observations) may help detect subtle laser-tissue interactions.

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