Title of Thesis: “Corneal Injury to Ex-vivo Eyes Exposed to a 3.8 Micron Laser”

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Date
**Report Documentation Page**

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Uniformed Services University of the Health Sciences
ABSTRACT

Title of Thesis: “Corneal Injury to Ex-vivo Eyes Exposed to a 3.8 Micron Laser”

Author: Lt. James G. Fyffe

Master of Science in Public Health

Thesis Directed by: Dr. Thomas Johnson

Assistant Professor

Department of Preventive Medicine and Biometrics

As a consequence of the significant expansion of laser use in medicine, industry and research, specific safety standards must be developed that appropriately address eye protection. The purpose of this study is to establish injury thresholds to the cornea for 3.8 µm 8 microsecond laser light pulses and to investigate a possible replacement model to live animal testing. Previous studies of pulsed energy absorption at 3.8 µm were performed using rhesus monkey cornea and were at pulse durations two orders of magnitude different than the 8 microsecond pulses used in this study. Ex-vivo pig eyes were exposed at varying energies and evaluated to establish the statistical threshold for corneal damage. Histologic evaluation was used to determine the extent of damage to the cornea. It is expected that the results will be used to assist in the establishment of safety standards for laser use and offer an alternative to future animal use in establishment of safety standards.

Keywords: Laser, corneal injury, replacement model, ex-vivo, pig, deuterium fluoride laser
CORNEAL INJURY TO EX-VIVO EYES EXPOSED TO A 3.8 MICRON LASER

BY

LT. JAMES G. FYFFE

Thesis submitted to the Faculty of the Department of Preventive Medicine and Biometrics Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirement for the Degree of Master of Science in Public Health, 2005.
DEDICATION

To my wife Elizabeth whose loving and Godly support makes me complete and to our children Jimmy, Annalise, and Sophia whose hugs, kisses, and giggles have gotten me through the past two years. I dedicate this thesis to you.

I thank God for the motivation and abilities that have resulted in this thesis. “I will praise You, for I am fearfully and wonderfully made; Marvelous are Your works, And that my soul knows very well.” Psalm 139:14
ACKNOWLEDGMENTS

I am grateful to Donald Randolph and Golda Winston for their invaluable contributions, which made this research possible. I am especially grateful to Dr. Tom Johnson for his unending energy and vision. I also thank Ms. Sue Pletcher, USUHS, and Tom Eurell, DVM, PhD, College of Vet. Med., University of Illinois for their histology support.
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CHAPTER ONE: INTRODUCTION

Statement of Problem

One of the most impressive new tools given to us by science and technology today is the laser. Society is using lasers more frequently and in more fields than ever before. Lasers are being employed in office equipment, survey equipment, rangefinders, communications devices, surgical equipment and other instruments. The spectral region for wavelengths greater than about 1.4 µm is often called “eye safe” because the cornea and aqueous humor have sufficient absorption to prevent damaging levels of radiation from reaching the retina. However, because it does absorb in the far infrared (IR) region, the cornea itself can sustain damage. The exact mechanism of damage to the cornea from IR wavelengths at short pulse durations (10^-6 to 10^-15 seconds in duration) is in dispute. [1-5] Damage from longer pulses (greater than 10^-6 seconds) follows the thermal model proposed by Takata et al; however, in a few cases the damage is clearly not thermal and appears to be due to acoustic effects. [2, 4, 6-8] There are several parameters that are contributing factors to the differences in damage such as wavelength, spot size, pulse width, and intensity. [5, 9] To date, there has not been an all encompassing investigation at one wavelength to determine how the variances in these parameters affects the estimated dose to 50% injury (ED50) as this would be cost and time prohibitive with the current method of live animal testing. [1, 10, 11]

Background

The military is rapidly finding new ways to employ lasers as target designators, communications devices, non-lethal weapons, and as anti-missile defense systems. This equipment emits either single pulses or sequences of pulses in beams of various
diameters and energies. With the increasing use of lasers, it is necessary to assess the health effects and hazards of non-ionizing radiation from such laser systems.\textsuperscript{[10, 11]} There are two problems with the current system of evaluating the safety of new laser systems. Laser systems are being developed at a rate too rapid to evaluate every system and the current method of evaluation, that is live animal testing, is increasingly unpopular and cost prohibitive. Due to these problems, current safety standards in the far IR region are not based upon experimental results at these wavelengths but are extrapolated from studies done at longer wavelengths (10 µm.)\textsuperscript{[12]}

The current body that sets laser safety standards in the United States of America is the American National Safety Institute (ANSI). Due to the nature of laser radiation, one standard cannot apply for all laser systems. Different wavelengths of laser radiation are absorbed, reflected, or transmitted differently by different tissues. The majority of laser safety studies have been conducted in the range of 0.4 µm to 1.4 µm due to the fact that at this wavelength, laser energy passes through the cornea, reaches the retina, and possibly causes damage leading to vision loss. The spectral region above 1.4 µm is generally considered “eye safe.” The cornea has sufficient laser absorption to prevent damaging levels of laser energy from reaching the retina. However, because of its absorption, the cornea itself can sustain damage. While the spectral region presenting potential hazard to the retina has been well characterized, there have not been many studies in the far IR region. Some investigators have found that the ANSI Maximum Permissible Exposure limit (MPE) has a safety factor ranging from 2 to about 100 times the damage threshold.\textsuperscript{[1]} This may be too conservative in some cases and not conservative enough in others. Further research on thresholds to validate or update the theory-based
standards is necessary. Using the current method of corneal research, the live rabbit model will be costly in time and money.

For a number of reasons, live animal research has become increasingly unpopular. For example, animal rights activism has promoted legislation to improve animal welfare in all aspects of society, from agriculture to laboratory research. One example is the Treaty of Amsterdam, in force since May 1999. This treaty lays out new ground rules for the actions of the European Union on animal welfare in a special “Protocol on the Protection and Welfare of Animals.” It asserts that animals are sentient beings and requires European institutions to pay full regard to the welfare requirements of animals when formulating and implementing local legislation.[13] In addition, these regulations have a potential economic cost. Trade barriers against products and services from the United States due to the differences in animal welfare practices have the potential to alter global trade.[13]

In the United States, the main legislation regulating the use of animals in the laboratory is 9 CFR 1 (also know as the Animal Welfare Act), which was signed into law in 1966. The original intent of the Animal Welfare Act was to regulate the care and humane use of animals used for research, experimentation, exhibition, and sale purposes. The Animal Welfare Act institutionalized the Internal Review Board (IRB) that now oversees and approves all animal research use. It has since been expanded to assure the humane treatment of animals during transportation in commerce and to protect owners of animals from theft by preventing the sale or use of animals that have been stolen.[14]

As noted earlier, research with live animals is both difficult and costly but it is also filled with institutional administrative and legislative barriers. Live animal research
requires special approval considerations through Institutional Animal Care and Use
Committees (IACUC) that can be difficult and time consuming. It also requires special
housing facilities and trained staff (basic care, feeding, veterinary care, etc.) throughout
the animals’ lifespan, not just during the experimentation period. Consideration also
needs to be made concerning the animals welfare during the experiment, such as pain
management (anesthesia) and comfort during transport. Due to these considerations a
specially trained veterinarian may be required for the experiment. In the case of laser
experimentation, additional costly support equipment such as restraint devices,
adjustment stands, and specialized tables are required to properly immobilize the animal.
Even a simple experiment conducted with one researcher and a few animals will require
as many as five other staff members, a veterinarian, cages and specialized rooms for
housing, bedding, food, water and medications for the life of the animal; all of this
significantly increases the cost and logistical support requirements of the experiment.[15]

Because live animal research is unpopular, administratively challenging, and
costly, there has been a major effort in both the regulatory and the scientific community
to consider and use alternatives (commonly called the three R’s: reduction, refinement,
replacement) to using live animals.[15] Notwithstanding the cost, live animal research is
still considered the most reliable and reproducible way of simulating a human response.
Since living systems are complex and have the ability to repair and regulate tissue, it is
still essential that some research be conducted using live animals. However, if the
number of live animals used during testing can be reduced to only those required to
verify the results supported by other models this would have a beneficial effect ethically,
financially and temporally. A computer model would be ideal and an in-vitro model
would also be an acceptable alternative to animal models from an ethical and financial standpoint. The complexity of certain tissues makes both of these options difficult, if not impossible, to exercise. The use of ex-vivo tissue is a reasonable alternative to both these other models and to live animals for many reasons. First, if properly harvested and preserved the complex structure of the tissue is maintained. At a minimum, the initial damage to the tissue from a laser pulse may be determined. Furthermore, with more time and resources, it is possible to examine corneal healing. However, dissipation of heat and other physiologic functions inherent in the live animal model would not be readily apparent. Second, since the tissue is from animals already sacrificed for other reasons, it will minimize the number of animals needed for a given experiment. Third, the use of ex-vivo tissue does not require a veterinarian or other highly trained individual to administer anesthesia, pain management or post procedure care. Fourth, the avoidance of animal protocols saves time, money and effort, allowing rapid performance of experiments with minimal external requirements.

Research Goal

There are only a few previous cornea studies at or near 3.8 µm (listed in Table 1).\[1, 7\] These studies are different from the combination of parameters used here by more than two orders of magnitude. Current safety standards for wavelengths above 1.4 µm have been extrapolated from studies done at 10 µm.\[12\] Thus, safety standards for this wavelength and pulse duration may not be appropriate since they are not based on actual data generated from experiments at the wavelength of interest. It is the goal of this study to investigate the threshold for gross morphologic change to the cornea in an ex-vivo model of the eye for the 3.8 µm laser system to aid in setting laser safety standards. The
viability of ex-vivo pig-eyes as a replacement model for in-vivo testing is also investigated.

| Table 1. Summary of Corneal Studies Near 3.8 \( \mu \text{m} \)\[^{16-20}\] |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Wavelength      | Pulse Duration  | Spot Size       | Model           | Author          |
| 3.731 \( \mu \text{m} \) | 500 msec        | 0.72 mm\(^2\)   | Rhesus monkey   | Dunsky et al    |
| 3.698 \( \mu \text{m} \) | 125 msec        | 0.72 mm\(^2\)   | Rhesus monkey   | Dunsky et al    |
| 2.900 \( \mu \text{m} \) | 100 nsec        | 2.0 cm\(^2\)    | Rabbit          | Mueller et al   |
| 2.795 \( \mu \text{m} \) | 500 msec        | 0.53 mm\(^2\)   | Rhesus monkey   | Dunsky et al    |

**Research Hypothesis**

In the past it has been theorized that the cornea behaves much like water in absorption of laser radiation, thus absorption coefficients for water (127 cm\(^{-1}\) at 3.8 \( \mu \text{m} \)) have been used in theoretical calculations of safety standards.\[^{16}\] If this is a valid assumption, it is hypothesized that the observed maximum depth of damage will equal the maximum depth of penetration of water at a wavelength of 3.8 \( \mu \text{m} \). It is further believed that the ex-vivo pig eye will be an accurate model for testing laser radiation damage to the cornea.

**Specific Aims**

The specific aims of this research were to: (1) determine the ED\(_{50}\) of the cornea exposed to the 3.8 \( \mu \text{m} \) laser, (2) examine histology to determine the depth of damage at each fluence, and (3) compare results of the measurements against theoretical models to determine if previous assumptions hold true.
CHAPTER TWO: LITERATURE REVIEW

Structure of the Eye

The eye is a very complex structural extension of the central nervous system and as such is composed of many complex functional components. The overall anatomy of the eye can be seen in Figure 1. Light enters the cornea, which accomplishes the majority of the focusing in the eye. The light passes through the pupil which regulates the amount of light transmitting through the lens to the retina. At the retinal cells, incident photons are transformed to nerve impulses that are transmitted to the brain via the optic nerve. In laser research, the main concern of ocular damage has been the retina. The retina has limited regenerative capabilities; thus, damage to the retina results in near permanent loss of visual acuity (sight). The cornea, on the other hand, has some ability to repair itself. The epithelial layer in particular has a high capacity to regenerate while the other layers have a much reduced ability to repair damage.\textsuperscript{[21, 22]}

Figure 1. Structure of the Eye
Composition and Optical Properties of the Cornea

The cornea is very complex and provides a number of functions for the eye. The human cornea is composed of five different layers; epithelium, Bowman’s membrane, stroma, basement membrane, and endothelium. The animal model corneas similar to the human cornea except that the rabbit and pig cornea lack a Bowman’s membrane. Outside of primates, very few mammals have a Bowman’s membrane.

In the past, laser safety has mainly been concerned with damage to the retina because the retina has a limited capacity to regenerate after damage; whereas, the cornea has the ability to repair itself, particularly the outer epithelial layer. While the cornea may be able to repair itself it is also highly innervated and a small amount of damage can cause a great amount of pain or discomfort.[21-23]

To predict ocular damage, eye models require taking into account various thermal and refractive properties of the tissue in question. These factors include thermal conductivity, specific heat and coefficients of absorption and reflection. The thermal conductivity of ocular media appears to be closely represented by water and is fairly constant over various conditions.[16] The primary function of the cornea is focusing light on the retina. The cornea accomplishes about 85% of the focusing in the eye (Dr. M. Johnson, personal communication.) A change in corneal shape or transpanency can have a great effect on vision.

One of the most important factors in radiation safety is the absorption coefficient of the material, that is, the ability of the material to absorb a certain wavelength of radiation, which is given in inverse centimeters. The absorption coefficient is determined by measuring the amount of radiant energy that passes through a known thickness of
material. Using the Lamertian absorption coefficient $\alpha = \ln \left( \frac{1}{T} \right) \div x$, where $T$ equals the sample transmissivity and $x$ equals the sample thickness, we can now calculate the absorption coefficient of the material.\textsuperscript{[16]} In the case of the cornea, calculating the absorption coefficient above wavelengths of 1.4 $\mu$m is very difficult due to the almost complete absorption of the radiant energy at these wavelengths in the cornea. Some effort has been made to section the cornea to thinner slices to allow transmission, but these attempts were unsuccessful.\textsuperscript{[16]} For this reason and the fact that the cornea has a high water content, the absorption coefficient of the cornea at these wavelengths has been assumed to be that of water.\textsuperscript{[16, 24, 25]}

**Ex-vivo Studies**

Two previous studies have been performed by McCally et al in which they compared laser radiation exposure to the ex-vivo rabbit eye against the live rabbit eye model. They determined that freshly enucleated rabbit eyes were comparable to the live rabbit eye model.\textsuperscript{[10, 26]}

**Pig Eye as a Model**

The ex-vivo pig eye is often used as a replacement model for the cadaveric human eye in the engineering field for mechanical stress tests.\textsuperscript{[27]} The first question that must be addressed is how comparable is the pig cornea to the human cornea and the two most frequently used models, namely the rabbit eye and the rhesus monkey eye. For the purposes of laser safety studies, the most pertinent parameters have been summarized in Table 2.
Table 2. Comparison of Pig Eye to Human and Animal Model Eyes

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<th>Corneal Epithelium thickness</th>
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<td>770 µm</td>
<td>35 µm</td>
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<tr>
<td>Rhesus Monkey</td>
<td>20 mm</td>
<td>460 µm</td>
<td>30 µm</td>
<td>yes</td>
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<tr>
<td>Rabbit</td>
<td>18 mm</td>
<td>450 µm</td>
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<td>no</td>
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<tr>
<td>Pig</td>
<td>30 mm</td>
<td>1063 µm</td>
<td>47 µm</td>
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Estimated Dose to 50% Injury (ED$_{50}$)

The Maximum Permissible Exposure (MPE) limit is set by ANSI committee consensus. This committee is comprised of laser experts who review all available experimental data to determine the exposure threshold. There is a wealth of data contributing to the safety standards in the retinal hazard region (0.4 µm to 1.4 µm). The data is usually examined to determine the point at which the estimated dose would cause damage 50% of the time. This is refered to as the Estimated Dose to 50% injury (ED$_{50}$). There is some degree of uncertainty associated with all research that must be accounted for determining how much of a safety factor to build into the MPE. As a result, the factor can be as small as two, but most often is ten times the ED$_{50}$. This safety factor also depends on the nature of the radiation/tissue interaction.$^{23, 25, 28}$

Safety factors for laser radiation in the far IR region are determined in much the same way, but are not as well supported by experimental data. Lack of experimental data required extrapolation from existing data using theoretical models derived from studies done at other wavelengths. Many of the assumptions that have proven true in the retinal hazard region (0.4 µm to 1.4 µm) have been used in studies in the corneal hazard region (1.4 µm to 10.0 µm) even though the laser/tissue interaction is completely different at these wavelengths.
Deuterium Floride Laser

The term LASER is an acronym for Light Amplification by Stimulated Emission of Radiation. A laser has three basic components: an energy source, the lasing medium, and the optical cavity. The energy source pumps energy into the lasing medium to raise the excitation state of the medium. The medium is composed of atoms or molecules whose electrons are stimulated to a higher energy state by the energy source. As the lasing medium electrons fall into lower energy shells, the stored shell potential energy is released in the form of monochromatic photons that are coherent in both time and space. These photons are then reflected back and forth in the optical cavity, exciting the medium even more until a certain energy level is reached. These photons are released, resulting in a beam of coherent, monochromatic laser light. For this particular laser system, the lasing medium was a mixture of two gases, deuterium and fluorine.\[1\]

3.8 μm Laser Studies

The far IR spectral region (wavelengths above 1.4 μm) has generally been considered “eye safe” as the cornea has sufficient absorption to prevent damaging levels of radiation from reaching the lens or retina. There have only been a few studies done in the far IR region.

To date, there has been one corneal injury study done at 3.8 μm. Dunsky and Egbert examined corneal injury to the rhesus monkey eye caused by laser radiation at 3.8 μm. Their DF laser had a spot size of 0.72 mm\(^2\), a pulse duration of approximately 100 nsec and a maximum fluence of 7.7 J/cm\(^2\). They exposed a total of 11 rhesus monkey eyes and determined an ED\(_{50}\) of 0.377 J/cm\(^2.\)[1] Their parameters differ from this study by several orders of magnitude (spot size of 4 cm\(^2\), pulse duration of 8 μsec and maximum
fluence of $31.8 \text{ J/cm}^2$). There has yet to be a study investigating mechanisms other than thermal damage.\textsuperscript{[1]}

**Probit Analysis**

Probit analysis is a process that was originally developed for toxicology studies. In laser research, the basic principle is that many samples of tissue are exposed to laser radiation and examined to see if they show a response to a given endpoint. Outcomes are plotted as a frequency distribution of the response against the exposed dose. The plot should have a sigmoidal shape; the median point represents the ED$_{50}$ value of the radiation/tissue interaction.\textsuperscript{[23]} The Probit plot for this study serves as an example in Figure 2. Probit has been widely recognized as an appropriate tool for determining laser damage to the retina. The variance in parameters between studies, however, makes it difficult to directly compare one ED$_{50}$ to another.\textsuperscript{[23]}

![Figure 2. Probit Plot for ED$_{50}$ of 3.8 µm Laser Study](image)

**Equation:**

$$y = 1.01177 + (0.03727 - 1.01177) \left(1 + \exp\left(\frac{x - 6.69579}{0.31152}\right)\right)$$

**Model:** Boltzmann

$\chi^2$/DoF = 0.00001

$R^2 = 0.99993$
CHAPTER THREE: MATERIALS AND METHODS

Eye Preparation

A total of 24 pig eyes were enucleated from 12 pigs (5 Yucatan mini-pigs, Sinclair Research Inc., Auxvasse, MO; and 19 Yorkshire, Charles River PharmServices, Southbridge, MA). These animals were not sacrificed for this experiment but were used under other animal use protocols approved by the Uniformed Services University of the Health Sciences (USUHS). The eyes were obtained under a tissue sharing protocol. The eyes were placed in Dulbecco's Modified Eagle Medium (D-MEM) with 10% fetal bovine serum (FBS) media (Invitrogen, Carlsbad, CA) and then placed in CO₂ bags and refrigerated at ~10 ºC. The eyes were stored 24 - 48 hours before being exposed. Due to laser system difficulties, four eyes were stored for approximately 92 hours. These four eyes were unsuitable for use because the eye developed mold, the corneal tissue began to break down and the ocular globe softened and lost its shape. Preceding laser exposure, the eyes were brought to room temperature, washed in excess 0.9% saline, and placed on a gauze support. An identification marker was written in pencil with the date and exposure number on a slip of paper. The use of pencil was important as pen ink dissolves in formalin, which was the preservation solution. Prior to exposure the eye was photographed with the identification marker and placed on an exposure platform.

Laser Exposure

Once the eye was in place, a 1 cm thick sheet of aluminum was placed over half of the eye to create a control region. The eye was then wet down with 1-2 mL 0.9% saline solution approximately 20 seconds prior to the laser exposure. The detail of the experimental setup is shown in Figure 3.
The laser used for all exposures was a 3.8 µm deuterium fluoride (DF) chemical laser with a square, top-hat (uniform energy density) profile and a spot size of 4 cm². The spot size was precisely measured at the beginning and end of each experimental day. During each exposure, fluence and total energy were measured and recorded. The exposures delivered to the tissue samples ranged from 4.0 J/cm² to 55.3 J/cm².

After exposure to the laser, three different observers determined if there was any damage to the eye. The criterion used for minimal damage was identical to that used by Brownell and Stuck[29], namely the presence of a superficial gray-white spot that develops within 30 minutes of the exposure. The eye was photographed once a consensus on the presence/absence of damage was reached. Initially, the eye was marked for orientation with a silk suture at the 12 o’clock position to mark the point of masking. With the suture at the top of the eye, all eyes had the right half exposed. After the initial
exposures not only was it determined that this process was time consuming, but it was also realized that the eye could be consistently oriented without a suture. The eye was then wrapped in gauze with the identification marker and placed in 10% buffered formalin for preservation. Based on these observations, and ED$_{50}$ was determined using Probit analysis.$^{[30]}$

**Post Exposure**

Post exposure, the eyes were prepared for histological evaluation. The eyes were blocked in paraffin, cut to a thickness of 8 µm, stained with hematoxylin and eosin, and mounted on a glass slide. Each cornea was completely photographed via a microscope using a Leica Microsystems microscope in conjunction with QCapture software (version 1.68.6, Quantitative Imaging Corporation, Burnaby, BC, Canada). Each photographed section of the cornea was then reoriented to as near horizontal as possible for uniformity purposes in taking measurements. A 50 µm × 100 µm grid overlay was applied to the photograph to ensure that measurements were taken accurately and consistently. Measurements were taken on the control side from the endothelium to epithelium and on the exposed side from endothelium to the bottom of vacuole formation. Each cornea served as its own control, thus increasing the power of this study. Measurements from the exposed side were compared to the measurements from the control side to determine the laser radiation penetration depth. Initially, measurements were taken every 50 µm across the width of the cornea, but this was too time consuming and difficult at lower magnifications. Data points from every 100 µm across the width of the cornea were eventually used and approximately 7000 measurements were taken. The full diameter of the laser spot size (4 cm) was not captured in every exposure, therefore only the center
most 3.6 cm were used for comparison. The measurements were then graphically charted for comparison with theoretical models.
CHAPTER FOUR: RESULTS

Gross Morphology

Immediately after exposure, the eyes were classified as either damaged or undamaged based on the presence or absence of clouding in the cornea. At higher energies, the observed damage included pitting and ablation of the cornea extending to the stromal layer. The general trend of increasing damage correlating to increasing fluence can be seen in Figure 4.

Figure 4. Corneal Damage at Increasing Fluence (in J/cm²)

One point of interest is at 10 J/cm². The damage to the cornea increased as fluence rose until 10.3 J/cm² where a decrease in corneal damage was seen. Beyond this point, the correlational fluence-damage relationship resumed. This observation was further validated with histologic evaluation. This demonstrated a decrease in depth of penetration and suggested a change in the mechanism of damage.
Each of the exposed eyes was coded based upon a binary code (0 = no damage, 1 = damage). This data was evaluated using Probit analysis. The results indicate an ED$_{50}$ of 6.7 J/cm$^2$ (with a slope of 31.9 and a Chi-square probability of 0.9977). Fiducial limits could not be calculated using this data set. The results are summarized in Table 3.

There was a question as to whether or not a full power exposure would collapse the ocular globe; therefore, a full exposure was made at 55.3 J/cm$^2$. The globe did not collapse but did sustain extensive corneal damage with almost complete removal of the epithelial layer. However, no meaningful data could be collected for this exposure because there was no control region for comparison.

<table>
<thead>
<tr>
<th>Classification</th>
<th>$\Phi$ (fluence) in J/cm$^2$</th>
<th>Average Depth of Damage in $\mu$m</th>
<th>Maximum Depth of Penetration in $\mu$m</th>
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*ED$_{50}$=6.7074 J/cm$^2$

+Negative numbers indicate corneal swelling
Histology

There were three regions of interest in the data when changes to the cornea and depth measurements were considered. Region A contained the two lowest energies of 4.09 J/cm$^2$ and 4.3 J/cm$^2$, which were below zero indicating swelling in the epithelial layer of the exposed region. Region B ranged from 5.5 J/cm$^2$ to 9.9 J/cm$^2$ and was linear with an exponential component at the upper end. Region C included all fluences above 9.9 J/cm$^2$, which had a dramatic drop in radiation penetration depth and again followed a general linear trend upward with wide variation (see Figure 5).

**Figure 5. Average Depth of Penetration**

![Average Depth of Penetration](image)

The first region of interest was Region A, an example of which can be seen in Figure 6. There was no gross observable damage to the naked eye at the time of exposure. There was an observable ridge running down the center of the cornea. It was
first assumed that this was a corneal abnormality that was missed prior to exposure. However, a subsequent exposure demonstrated the same finding and tissue histology revealed swelling in the epithelial layer. It was not possible to measure depth of laser radiation penetration because there was no definable cellular damage other than the swelling. It was clear that energy absorption caused changes, but it was difficult to make a quantitative evaluation since there was no vacuolation. The epithelial swelling averaged approximately 20 µm.

**Figure 6.** Histology of Exposed and Control Cornea from Region A

![Histology of Exposed and Control Cornea from Region A](image)

Corneal damage at 4.30 J/cm² (400 magnification; left side exposed / right side control)

The next region of interest was Region B with fluences from 5.5 J/cm² to 9.9 J/cm², an example of which can be seen in **Figure 7**. While not readily apparent in this photograph, significant gross damage (i.e. the corneal clouding) was clearly visible to the naked eye immediately post-exposure. Histologic evaluation showed that the damage was contained within the epithelium. Damage to the epithelial layer was characterized by
vacuolation, slight nuclear condensation, and cellular swelling. In both regions A and B, the damage seemed to be localized at one layer with all damage at the depth of penetration leaving the tissue above unchanged.

**Figure 7. Histology of Exposed and Control Cornea from Region B**

![Figure 7](image)

The last region of interest was Region C with fluences above 9.9 J/cm², an example of which can be seen in **Figure 8**. The average penetration of laser radiation at the center of the cornea at this fluence was 129 µm. Histological evaluation showed major cellular damage in the epithelial layer and vacuole formation in the substantia propria (stroma). Damage to the epithelial layer was characterized by vacuolation (possibly caused by steam generation), cellular deformation, nuclear condensation, cellular destruction in some locations, and cellular swelling in other locations. The main action of damage appeared to be extreme surface heating and gas/steam disruption at the epithelial/stromal boundary. Damage to the stromal layer was characterized by
vacuolation, nuclear condensation, and cellular destruction. The damage to the stromal layer was not uniform. In some locations, there was no evidence of laser radiation penetration past the epithelium. In other locations, the damage ranged from a few micrometers to greater than 100 $\mu$m into the stroma. The overall mechanism of damage seemed to have changed in Region C when compared with Regions A and B. There seemed to be a great deal of heat generation on the surface with tissue damage down to the depth of penetration, not just in one layer. There was also separation of the epithelial layer from the stromal layer, presumably caused by the rapid formation of gas/steam.

**Figure 8.** Histology of Exposed and Control Cornea from Region C

![Histology of Exposed and Control Cornea from Region C](image)

Corneal damage at 15.90 J/cm$^2$ (200 magnification; left side exposed / right side control)

Approximately 7000 data points were collected in measuring corneal damage and were then graphed, as shown in **Figure 9**. This graph is a three dimensional model representing all depths of damage at all fluences. Two regions of interest are noted in
different colored bands. The region above the yellow band represents the overall corneal thickness of the rhesus monkey and the rabbit.

**Figure 9. Depth of Penetration Across the Cornea at All Fluences**

The region above the black band represents the overall corneal thickness of the human. This means that points recorded below these bands corresponds to laser radiation that might have passed through the respective corneas. Thus, potentially damaging levels of radiation might have gone unnoted if these models had been used. Furthermore, radiation that would pass through the human cornea, possibly causing damage to the retina or lens, was captured and quantified in this pig cornea model.
CHAPTER FIVE: DISCUSSION AND CONCLUSIONS

Comparison Against Theory

Results from this study indicate that previous assumptions of corneal transmission and absorption properties being the same as water at 3.8 µm may not be true. Previous studies have based conclusions on temperature calculations and theory. Most of this theory is a direct result of retinal studies where these assumptions have proven true, as they have considered the ocular media as a whole unit. In retinal studies, this is appropriate since the radiant energy must first pass through the ocular media (cornea, aqueous humor, lens, and vitreous humor) before reaching the retina. The energy absorbed in the ocular media (cornea, aqueous humor, lens, and vitreous humor) must be accounted for before determining the incident energy causing the retinal damage. The absorption and transmission properties of water and ocular media (cornea, aqueous humor, lens, and vitreous humor) are very similar when taken as a whole. However, when comparing the cornea alone to water, it absorbs radiant energy similarly in the near IR region only, as seen in Figure 10.[16]
Determining absorption coefficients for the cornea above 2.4 \( \mu \text{m} \) is very difficult because most of the radiant energy is absorbed in only a few micrometers of tissue. If the cornea and water do in fact have the same absorption characteristics for wavelengths above 2.4 \( \mu \text{m} \), then it should be equally difficult to determine the absorption coefficients for water. However, it has not proven difficult. In fact, absorption coefficients for water have been experimentally derived to at least 10 \( \mu \text{m} \).\textsuperscript{16} This is important because previous theory has assumed that the cornea absorbs and transmits laser energy in the same manner as water. If this is indeed the case, then the experimental results should match with theoretical predictions.

The experimental results from this study were compared against theoretical predictions. First, the maximum possible temperature for each exposure was calculated. Using a simplified approach to energy absorption, assuming all energy was absorbed and
not reflected or re-radiated, the average energy densities (J/cm$^3$) at each fluence were calculated by dividing each of the experimental incident fluences (J/cm$^2$) by the average depth of penetration (cm).

\[ P(\text{J/cm}^3) = \frac{\phi(\text{J/cm}^2)}{\text{average depth (cm)}} \]

Using the energy density for each exposure, it was then possible to calculate the average temperature change achieved at each exposure using the formula:

\[ P(\text{J/cm}^3) = \rho(\text{g/cm}^3) \times C(\text{J/g }^0\text{C}) \times \Delta T( ^0\text{C}) \]

in which $P$ is the energy density required to reach the desired temperature change (calculated above), $\rho$ is the density of water (0.9982 g/cm$^3$ at 20 $^0$C) and $C$ is the specific heat of water (4.184 J/g $^0$C).[16] The change in temperature was calculated for each exposure and summarized in Table 4.

<table>
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<th>Energy Density in J/cm$^3$</th>
<th>$\Delta T$ in $^0$C</th>
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The temperature changes ranged from 53 to 1082 °C. These temperature changes fall between the required temperature changes to denature protein (40 °C) and vaporize water (2361 °C). It is important to note that the lowest energy density and, thus, lowest temperature change corresponds with the maximum depth of damage at a fluence of 10.0 J/cm² in region B shown in Figure 5.

The theoretical maximum penetration depths were calculated based on this range under the assumption that the temperature change in the cornea was uniform to the penetration depth and that all photons penetrated to this depth and no further. In reality, the temperature distribution in the tissue would probably have a Gaussian distribution. However, by making the assumption that it was uniform, the maximum penetration depths would be less conservative than if accounting for the Gaussian distribution. This allows for a worst-case analysis. First, the energy densities required for temperature changes (required temperature minus room temperature) to denature protein, boil water, and vaporize water (40, 80, and 2361 °C respectively) were calculated. The experimental incident fluences (J/cm²) were then divided by the required energy densities (J/cm³) providing the maximum theoretical penetration depth (cm) for each of the fluences. These results were then graphed in comparison with the experimentally observed values in Figure 11.
The graph indicates that up to the ED$_{50}$ value of 6.7 J/cm$^2$ the experimental data is reasonably close to the theoretical model; however, above the ED$_{50}$ fluence the experimental results do not follow this model, indicating some other mechanism of damage may be in operation. This too, was noted by Takata who proposed two possible explanations. He first suggested that thermal damage proceeds more rapidly in the cornea than in the retina and, thus, does not fit the theoretical models developed from retinal studies. The second and more plausible explanation suggested was that other contributing factors such as acoustic or shock waves may contribute to the increased damage. Furthermore, he noted an even higher degree of damage with pulse durations less than $10^{-7}$ seconds.$^{[6]}$

Granted, this is a very simplistic approach. There are several elegant and complex thermal models available that might more accurately approximate our
experimental results. However, as stated before, this approach was used to determine a worst-case analysis. Further research is needed to precisely define the reasons for the unexpected results beyond the theoretical model predictions. Since the current thermal model does not accurately predict experimental results, a new theoretical model needs to be developed based upon experimental observations and applied to reconstruct current safety standards.

**Study Limitations**

There were several limitations identified with this study:

1. There are some questions concerning the use of ex-vivo eyes that could influence the results. The pig eye was chosen because it was available and its similarity to the human eye. The major disadvantage to using ex-vivo eyes is that the tissue is not living and there is no way to determine the cornea’s ability to recover from laser damage. It is possible that the live cornea may have been able to repair some of the damage we noted within 24 hours; therefore, the true ED$_{50}$ could be higher than what we observed. It is also possible that storing the eyes permits absorption of the DMEM solution changing the absorptive or reflective properties of the eye.

2. Some previous corneal studies have used a slit lamp to better observe lesion formation in the cornea (Bruce Stuck, personal communication.) It is possible that some lesions were not visible to the naked eye that would have been apparent with the use of a slit lamp. If so, our damage threshold would have been lower than observed.
3. Another factor in this study difficult to account for was the spot size. Most studies use lasers with spot sizes of a few millimeters or less. This study used a spot size greater than a hundred times more than the usual spot size. With spot sizes of millimeters there is the potential for thermal distribution to surrounding tissue. This is not possible with the large spot size used in this study.

4. Another aspect of having such a large spot size is the non-uniformity of laser energy over the area of the spot size. This is caused by a number of reasons such as atmospheric attenuation and turbulence. These effects are magnified with large spot sizes and are difficult to control and to take into account.

Based on this study, the ex-vivo pig eye has potential as a replacement model for live animals. Further experimentation is necessary to confirm the utility of this model as compared to the rabbit eye. However, it is clear that the use of ex-vivo eyes could reduce the number of live animals needed to establish safety standards. Indeed, at the very least, ex-vivo eyes could be used to set the starting point for in-vivo studies.

**Recommendations**

The MPE set by ANSI in this wavelength regime is based on theory.\[^5\] Research in the IR region has found that the safety factor ranges from 2 to 100 times the experimentally determined ED\(_{50}\).\[^1\] Typically, a safety factor of 10 times the ED\(_{50}\) is used to ensure safety.\[^12\] As might be expected, the safety factor is too high in some cases and not high enough in others. When a corneal ED\(_{50}\) is established experimentally, it is usually based upon gross morphological changes, not on histological examination.
However, considering the results at 4.30 J/cm\(^2\) which demonstrated swelling damage, perhaps histology might be necessary to document cellular damage invisible to the naked eye.\(^{[5]}\). The current MPE for a 3.8 \(\mu\)m 8 microsecond laser is 0.03 J/cm\(^2\).\(^{[12]}\) Assuming that the ex-vivo pig eye is a suitable model for establishing the ED\(_{50}\), this is a safety factor of 225, which could needlessly limit the use of this laser system.

Further research is needed to validate this study. These studies should include live animals and ex-vivo eyes using the same laser system. Some aspects of this study should be changed in follow on studies:

1. A light box should be used for photographing the ex-vivo eye to eliminate artifacts caused by the curved reflective surface of the cornea.
2. The cornea should be removed after exposure and preserved for histologic evaluation rather than preserving the whole ocular globe.
3. Freezing might be a better method of preservation than formalin. This would help reduce the number of artifacts caused by sectional geometry.
4. The slit lamp must be evaluated for its ability to detect corneal damage not visible to the naked eye.
5. The exposure doses should be done in triplicate with a concentration just above and below 10.0 J/cm\(^2\).

Conclusion

Results from this study indicate that assumptions made in previous theory for wavelengths greater than 2.4 \(\mu\)m may not be true. Further research needs to be conducted to determine the mechanism for the unexpected results and a new model derived based on
these results. Results from this study also indicate that there is some value in using the ex-vivo pig eye in laser safety studies.
BIBLIOGRAPHY


