The effects of Nd:YAG laser cyclophotocoagulation on corneal sensitivity, intraocular pressure, aqueous tear production and corneal nerve morphology in the canine eye

by

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(ABSTRACT)

Corneal ulceration with prolonged healing following Nd:YAG laser cyclophotocoagulation in dogs is a frequent complication. It is hypothesized that these corneal ulcerations may be a form of neurotrophic keratitis due to laser-induced damage to corneal innervation. Fifteen clinically normal dogs had the neodymium:yttrium aluminum garnet (Nd:YAG) laser cyclophotocoagulation performed on the left eye. Each treated eye received 100 Joules of laser energy. Corneal touch threshold (CTT) and Schirmer I tear tests (STT) were performed before the surgery and on days 1, 3, 5, 7, 9, 11, and 13 post-laser treatment. Applanation tonometry was performed before surgery and twice daily for 14 days post-laser treatment. Eyes were enucleated after 14 days and corneal nerves were stained using a gold chloride technique. Major nerve bundles entering the cornea were quantitated by quadrant, using camera lucida
reproductions. Nerve bundle diameters were measured using NIH image computer software on computer-scanned images. Statistical methods included repeated values for analysis of variance for CTT, STT and IOP, and a paired t-test for nerve diameters and bundles. All laser treated eyes had significantly higher CTTs (P<0.05) compared to control eyes for all measurements. Six out of fifteen dogs had evidence of ulcerative keratitis. Intraocular pressure was significantly lower in laser treated eyes compared to control eyes in the a.m. on days 2-9, and 14, and in the p.m. on days 2-11 using a Bonferroni-corrected alpha level (P<0.0039). A significant decrease of one nerve bundle per corneal quadrant was found between the laser treated and control eyes. There was no significant difference in STT or nerve bundle diameters between laser treated and control eyes. Nd:YAG laser cyclophotocoagulation effectively reduces IOP while increasing CTT. The procedure also causes a significant decrease in the number of major nerve bundles entering the cornea, but has no effect on the diameter of those bundles. These findings support the hypothesis that nerve damage and corneal hypoesthesia are etiologic factors in ulcerative
keratitis following Nd:YAG laser cyclophotocoagulation.

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INTRODUCTION:

Canine glaucoma is a progressive disease that includes a variety of pathophysiologic processes related to each other due to an increase in intraocular pressure (IOP). This rise in IOP is responsible for pathological changes in the optic disk and corresponding defects in the field of vision. Normal IOP is dictated by the balance between production of aqueous humor by the ciliary body and the aqueous outflow, primarily through the iridocorneal angle.\textsuperscript{1,2}

One surgical treatment for glaucoma is neodymium:yttrium aluminum garnet (Nd:YAG) laser cyclophotocoagulation. In this procedure, the Nd:YAG laser is used to selectively damage the ciliary body to decrease production of aqueous humor.\textsuperscript{2-4} The laser beam is directed perpendicular to the sclera, 5-7mm posterior to the limbus, and passes through the sclera to be absorbed by melanin in pigmented tissue, causing direct destruction of the ciliary epithelial cells and indirect destruction by ischemia and inflammation.\textsuperscript{2-6} The optimal clinical effect is achieved with approximately 100-200J of energy delivered in short bursts.\textsuperscript{7}
At the VA-MD Regional College of Veterinary Medicine, this procedure has been used successfully on several canine patients to lower intraocular pressure (Table 1). Patients received between 79 and 250 joules of energy at 10-35 watts per application site. The number of application sites and watts delivered was extremely variable as an effective protocol was being developed. Nine out of fifteen patients treated between 1995 and 1998 with this protocol developed corneal erosions or ulcers which were slow to heal. Affected dogs were also observed to have a subjective decrease in corneal sensitivity and blink rate. Previous literature reports document such ulcers.8,9

The cornea is innervated mainly by the ophthalmic branch of the trigeminal nerve.10 The ophthalmic branch divides within the orbital fissure giving rise to the nasociliary nerve which subdivides into the long and short ciliary nerves which innervate the anterior segment, including the cornea.11,12 The long ciliary nerves travel anteriorly through the suprachoroidal space at the 3 and 9 o’clock positions medially and laterally.10,13-17 Nerve axon bundles
are radially arranged and enter the cornea at the limbus through the middle one-third of the stroma.\textsuperscript{18} There are species differences as to the absolute numbers of corneal nerve trunks.\textsuperscript{12,18-22} There are 10-18 major stromal trunks innervating the dog cornea.\textsuperscript{18,22}

It was hypothesized that these clinical cases sustained nerve damage related to the Nd:YAG laser procedure that had resulted in neurotrophic keratitis. Corneal edema, ulceration and hypoesthesia have been reported in both humans and dogs following laser cycloablation.\textsuperscript{2,3,7,23-25} The purpose of this study was to study the effects of Nd:YAG laser cyclophotocoagulation on CTT in dogs, as well as the laser’s effects on the number and diameter of major nerve bundles in the canine cornea. In addition, aqueous tear production and IOP would be measured after Nd:YAG laser cyclophotocoagulation.

**LITERATURE REVIEW:**

*Anatomy and physiology of mammalian corneal innervation*

The anterior segment of the eye is innervated by neural processes from three ganglia; the trigeminal
(sensory), superior cervical (sympathetic) and ciliary ganglia (parasympathetic). The cornea is primarily innervated by the first trigeminal branch, the ophthalmic nerve, which originates in the Gasserian ganglion and then runs in the wall of the cavernous sinus. Before the ophthalmic nerve enters the superior orbital fissure, it divides into three terminal branches; the lacrimal nerve, the frontal nerve and the nasociliary nerve. The nasociliary nerve arises from the medial superior border of the trigeminal ganglion and enters the orbit through the orbital fissure. The branches of the nasociliary nerve supply the globe and the mucous membrane of the posterior ethmoidal cells, the nose, the upper eyelid, the nasal canthus and the lacrimal sac. The ramus communicans to the ciliary ganglion carries sensory fibers to the globe which divide into five to eight short ciliary nerves. The nasociliary nerve gives rise to two long ciliary nerves that course directly to the posterior pole of the eye, penetrating the sclera and
running anteriorly in the suprachoroidal space at the 3 and 9 o’clock positions.\textsuperscript{10,13-17}

As nerve bundles run through the suprachoroidal space, they branch and exchange axons until the bundles contain mixtures of sensory, sympathetic and parasympathetic fibers.\textsuperscript{17} Near the corneoscleral limbus, the nerves destined to reach the cornea separate and move beyond those supplying the anterior uvea.\textsuperscript{17} The nerves branch to form 8-30, depending on the species, circumferentially arranged branches at the corneoscleral limbus that also contain mixed populations of sympathetic, parasympathetic and sensory fibers.\textsuperscript{10,16,21} Sympathetic and parasympathetic innervation are considered less important as the number of autonomic axons are less than the number of corneal sensory axons.\textsuperscript{10,16}

The anatomy of corneal innervation has been documented in several species.\textsuperscript{18-21,26} Zander and Weddell published results of a comprehensive study on the innervation of the cornea in various species including
the dogfish, frog, white rat, guinea pig, rabbit, monkey and man. Large nerve bundles entering the cornea were studied using methylene blue, gold and silver stains, and a myelin stain. A radial, symmetrical arrangement of nerve bundles was generally observed across species, with some species differences in arrangement and density. Most nerve fibers penetrate at the midstromal level, although smaller nerve fascicles enter the cornea in episcleral and conjunctival planes to supply the superficial corneal stroma and peripheral corneal epithelium. The number of major stromal nerve trunks varies among species: human = 12-16, rabbit = 12-1628, rat = 8-1626, cat = 16-2021, and dog = 10-18.22

Stromal fibers divide dichotomously and trichotomously to form a subepithelial plexus (Figure 1). Existence of a nerve plexus in the subepithelial layer of the cornea has been recognized since 1867. This subepithelial plexus and intraepithelial nerve terminals emerge between the epithelial cells and penetrate all layers of the corneal epithelium. Before entering
the corneal epithelium, stromal nerve bundles bend and redirect to run between the basal cell layer and the basement membrane of the corneal epithelium. As axons enter the epithelium, they lose their Schwann cell elements and continue into the epithelium as naked axons. Intraepithelial sensory axons have a variety of morphologies, including simple, branch-like terminations, and “epithelial leashes” (unique preterminal arborizations) which run parallel to the corneal surface deep in the basal epithelial layer. Fibers terminate in all layers of the cornea. The axons which terminate as free nerve endings have been characterized as A-δ and C nerve fiber sensory afferent neurons. These neurons are physiologically specialized as thermosensitive, chemosensitive and mechanosensitive fibers. The relative densities of these functional subtypes in the central and peripheral cornea are debated and reported differences may be due to species differences as well as differences in tissue handling and fixation.
The innervation density of corneal epithelium is reported to be 300-600 times that of skin and 20-40 times that of tooth pulp, based on the mechanoreceptor density in the skin of the monkey index finger pad and tooth pulp. Camera lucida drawings of axonal terminals enabled numbered counts of neural elements in gold impregnated serial corneal cross sections and revealed 1000 arborizations of each long ciliary axon. The cornea is so densely innervated that single intraepithelial terminals never innervate more than a few hundred square micrometers. However, a single axon can travel a great distance across the cornea and a single corneal sensory axon may innervate as much as 20-50% of the corneal surface.

**Canine corneal innervation**

The pattern of canine corneal innervation is similar to that previously described in human, cat, rabbit and rat corneas. It can be divided into a limbal plexus and major stromal nerve bundles. The limbal plexus is comprised of a dense, superficial nerve network.
arranged as a 0.8-1.0mm wide, ring-like band around the peripheral cornea. Origins of the limbal plexus include stromal and subconjunctival nerve fibers heading to the cornea, recurrent collaterals from the peripheral corneal plexus, and perivascular fibers associated with limbal vasculature. Nerve fibers enter the peripheral cornea at the corneoscleral limbus in a series of 11-18 prominent, radially directed, superficial stromal nerve bundles. These bundles are relatively uniform in size and are evenly distributed around the limbal circumference. Each large bundle contains approximately 30-40 axons visible under light microscopy, with small nerve fascicles between and superficial to those bundles. After entering the cornea, the main stromal nerve bundles branch dichotomously and trichotomously to form an elaborate axonal network throughout the anterior 0.4-0.5mm of the corneal stroma. The distal branches anastomose with each other to form a dense stromal plexus which extends diffusely throughout the anterior one-half of corneal stroma. The anterior stroma is especially
well innervated and has a delicate meshwork of thin, preterminal axons that occupy the region immediately beneath the epithelial basement membrane.\textsuperscript{22} The posterior corneal stroma is largely devoid of innervation, although a few nerve fibers can be seen in this region. The corneal epithelium is richly innervated by intraepithelial axons some of which have formed epithelial leashes comprised of 2–6 axons attached to a single subepithelial fiber.\textsuperscript{22} The leash axons give off thin, prominently beaded, ascending branches that divide extensively into a bulbous terminal expansion, throughout the basal, wing and squamous corneal epithelial layers.\textsuperscript{22}

\textit{Neurochemistry of corneal nerves}

Corneal innervation has sensory, sympathetic and parasympathetic origins.\textsuperscript{16,35-38} Their respective neuropeptides found in the cornea were identified by ocular denervation studies of the trigeminal, superior cervical and ciliary ganglia.\textsuperscript{16,35-38} The relative contribution of each component varies with species.\textsuperscript{16,35-38}
Corneal sensory nerves may exert efferent functions through the release of trophic neuropeptides such as substance P and calcitonin gene-related peptide (CGRP). Substance P is an 11 amino acid polypeptide which increases formation of inositol phosphate and diacylglycerol via G protein activation.\textsuperscript{39,40} Substance P is thought to be the mediator responsible for a local ocular axon reflex implicated in ocular neurogenic responses to noxious stimuli.\textsuperscript{11,41,42} Nerve fibers that stain positively for substance P are found throughout the corneal stroma, epithelium and limbus.\textsuperscript{35} Calcitonin gene-related peptide is a neuropeptide which activates adenylate cyclase and cAMP through G protein coupling.\textsuperscript{40} Calcitonin gene-related peptide is found throughout the cornea and its distribution is dense and complex.\textsuperscript{43} The distribution of these two peptides is similar suggesting co-localization of substance P and CGRP in most corneal nerve fibers.\textsuperscript{35,44,45} Calcitonin gene-related peptide is released in response to corneal trauma and is thought to be involved in the resultant neurogenic ocular inflammation which manifests as
lacrimation, miosis, vasodilation and breakdown of the blood-aqueous barrier. Substance P and CGRP have been shown to increase the rate of mitosis of corneal epithelial cells in vitro. They have been found to modulate other aspects of corneal epithelial cell behavior, including adhesion and cellular migration. A smaller number of corneal nerve fibers contain CGRP alone but never contain substance P alone. Denervation of the ocular components of the trigeminal nerve results in a disappearance of substance P labeled axons in the cornea. Clinical effects associated with denervation of the trigeminal nerve include a decreased healing rate of standardized epithelial abrasions and a high incidence of spontaneous, recurrent epithelial erosions.

Corneal sympathetic and parasympathetic fibers have been implicated in modulating corneal sensitivity and epithelial wound healing. Beta-adrenergic receptors have been identified on corneal epithelial cells. Sympathetic corneal fibers exert trophic effects on corneal epithelium by stimulating mitotic activity and promoting wound healing. Corneal sympathetic nerve fibers in rabbits
modulate ion transport processes in the corneal epithelium.\textsuperscript{52} Loss of corneal sympathetic innervation significantly decreased epithelial proliferation in normal and wounded corneas.\textsuperscript{51} Regulation of corneal physiology and metabolism by sympathetic fibers may be more important in some species than others, as monkeys and humans do not appear to have many corneal sympathetic nerve fibers.\textsuperscript{16,37} Tyrosine hydroxylase (TH) is a mixed function oxidase found in all cells which synthesize catecholamines and is the rate limiting enzyme in catecholamine synthesis.\textsuperscript{40} Tyrosine hydroxylase has been found in about 30\% of canine corneal nerves.\textsuperscript{22} Interspecies differences in the organization and density of TH immunohistochemically positive nerve fibers may reflect species variation in the level of sympathetic control over corneal physiology and wound healing.\textsuperscript{37}

The parasympathetic nervous system also plays a role in corneal innervation.\textsuperscript{38} Surgical transection of rat ocular sympathetic and sensory nerves reduces, but does not eliminate, corneal nerve fibers stained immunohistochemically against acetylcholinesterase and select
neuropeptides.\textsuperscript{35,37,38} A decrease in acetylcholine (ACh) is found after prolonged eyelid closure which supports a role for ACh as a mediator of tactile sensitivity in the cornea.\textsuperscript{53} Some investigators hypothesize a role for ACh in the regulation of Na and Cl transport in the triggering of nerve impulses.\textsuperscript{54} Acetylcholine has also been implicated in contributing to pain associated with corneal injury due to activation of corneal C-fibers by ACh.\textsuperscript{55} Vasointestinal peptide (VIP) is contained in parasympathetic nerves and may exert regulatory effects on corneal epithelial cell proliferation.\textsuperscript{35}

Quantitative analysis of immunolabeled corneal nerve fibers has been performed on the central cornea in dogs. This analysis demonstrated that substance P, CGRP, TH and VIP are expressed in >99%, >99%, 29.7% and 0%, respectively, of all corneal nerves labeled with a pan-neuronal marker, protein gene product 9.5.\textsuperscript{22}

\textbf{Methods of measuring corneal sensitivity}

In 1894, M. von Frey presented a publication “Contribution to the physiology of pain” and introduced
the first aesthesiometer which consisted of a piece of horse hair attached to a light wooden rod.\textsuperscript{56} His methods proved to be inadequate; however, they were refined throughout the beginning of the 20th century. In 1955, Boberg-Ans published a modification of the von Frey method by using a nylon thread on a copper head which allowed a change in the rigidity of the stimulus by lengthening or shortening the nylon thread.\textsuperscript{57} Boberg-Ans discovered the loss of corneal sensitivity in hereditary corneal dystrophies and in inflammatory conditions, including herpetic keratitis.\textsuperscript{57}

The Cochet-Bonnet aesthesiometer was developed in 1960 in France and was a modification of the Boberg-Ans aesthesiometer.\textsuperscript{58} This instrument has a 0.12mm monofilament nylon thread attached to a weight which, when applied to the cornea, can result in pressures from 11-200mg/0.0113mm squared.\textsuperscript{58} Normal human CTT values were established and confirmed the results obtained by Boberg-Ans.\textsuperscript{58} There are specific A-\(\delta\) corneal nerve fibers that respond to mechanical forces exclusively and are the
predominant nerve type stimulated by the Cochet-Bonnet aesthesiometer.\textsuperscript{31,32} On the basis of neural density counts, the Cochet-Bonnet aesthesiometer filament can stimulate as many as 100 terminal endings with a single application.\textsuperscript{28}

Drawbacks associated with the instrument include effects of humidity, unequal deflection of the nylon thread due to human error, epithelial damage, and subject apprehension.\textsuperscript{59-61} Studies have shown that the Cochet-Bonnet aesthesiometer can damage corneal epithelium even at low stimulus lengths below the CTT.\textsuperscript{54,59,60} Another design limitation is that the true sensitivity threshold can be beyond the lowest or highest stimulus length, so that the true, precise threshold is sometimes not found.\textsuperscript{60,62}

The Cochet-Bonnet aesthesiometer can be mounted on a holder which can displace it in the $x$, $y$, and $z$ axes to give greater reliability and consistency of application.\textsuperscript{63} The mounted aesthesiometer prints a tracing of eye movement as related to the stimulation and
defines the objective CTT. This is the length of the monofilament that induces a blink for 50% of the number of stimulations. This does not necessarily correlate with the patient actually feeling the stimulus, which is the subjective CTT. The objective threshold can be used to predict the subjective threshold by the following equation:

\[ X = 0.87 (Y-32.94) + 30.78. \]

Where \( X \) is the subjective threshold in mg/0.0113mm\(^2\) and \( Y \) the objective threshold in mg/0.0113mg\(^2\).

Following the advent of the Cochet-Bonnet aesthesiometer, several investigators attempted to copy the device with modifications for ease of use, which often resulted in an instrument equivocal in its advantages over the Cochet-Bonnet aesthesiometer. Investigators sought to develop a mechanical or “dynamic” aesthesiometer. Further development ultimately resulted in one that measures corneal sensitivity via a moving coil galvanometer which produces a weak electric current that in turn exerts a force onto the cornea with a
lever.\textsuperscript{14} This electronic, optical, hand-held aesthesiometer performs “dynamic” aesthesiometry and allows the stimulus force to be raised continuously without breaking corneal contact. Dynamic measurements produce a higher CTT than static instruments.\textsuperscript{14}

Noncontact pneumatic aesthesiometers stimulate corneal nerves by releasing a controlled pulse of air of a pre-determined pressure and duration, with repeatable results.\textsuperscript{60,61} The diameter of the stream of air is substantially larger than that of the nylon monofilament (0.6mm vs. 0.12mm). Therefore, differences between central and peripheral corneal sensitivity are less dramatic. \textsuperscript{61,65} There is no correlation between measured thresholds of pneumatic devices and the Cochet-Bonnet device.\textsuperscript{66} It is possible that the pneumatic stimulus contains thermal stimuli components which stimulate corneal nerve endings by a temperature change across the corneal surface, rather than by strict mechanical stimulation as with the Cochet-Bonnet aesthesiometer.\textsuperscript{61,66}
Another aesthesiometer measures corneal sensitivity to a mixture of air and CO2. The CO2 acts as a chemical stimulant and controlled pulses of air provide mechanical stimulation. Results were repeatable, but several more tests of normal values would have to be established for its widespread use.

While many aesthesiometers developed after the Cochet-Bonnet aesthesiometer boast of greater accuracy, the need for a cooperative, still patient prohibits their use in animal species. No method has been determined to be a gold standard in measuring CTT.

**Corneal sensitivity**

Corneal sensitivity is essential for the maintenance of normal corneal physiology. The blinking reflex, and normal tear secretion are essential to the well-being of the cornea and are adversely affected by corneal hypoesthesia. Corneal sensitivity influences the normal rate of involuntary blinks, and the blink rate is significantly lower following topical anesthetic administration.
In contrast to the skin, morphologic receptor differences are not found between pain, touch, pressure and thermal receptors in the cornea.\textsuperscript{14} Specific receptors in the skin transduce the various sensory modalities: touch via Meissner’s corpuscles, pressure via Vater-Pacini corpuscles, heat via Ruffini’s brushes and cold by Krause’s bulbs. It has been theorized that corneal free nerve endings can discern the difference between pain and touch by the amount of pressure applied.\textsuperscript{54,70} This difference in pressure determines which sensation is perceived and dictates the appropriate response.\textsuperscript{54,70} In the cat cornea, electrophysiological studies have separated corneal sensory nerve afferents into three separate groups with 20\% activated by mechanical factors only (mechanosensory), 70\% by chemical irritants and noxious heat (polymodal nociceptive) and 10\% by cold.\textsuperscript{67,71} However, there is no definitive study determining conscious differentiation of these stimuli, and these fields have been found to overlap within the
cornea which could confound conscious differentiation between the stimuli.\textsuperscript{67,71,72}

Corneal sensitivity has been studied in several different groups of people and parameters have been established for several normal and pathological conditions. It is well documented in humans that the cornea is more sensitive in the center as compared to the periphery.\textsuperscript{63,73} Corneal sensitivity is lowest in the morning and increases throughout the day by 28\% to be at its peak in the evening.\textsuperscript{54,74} Prolonged eyelid closure and consequent decreased oxygen availability significantly decreases corneal sensitivity.\textsuperscript{53,68,74,75} Corneal sensitivity decreases with age.\textsuperscript{14,76} Sensitivity has been found to be relatively unaffected until the fourth or fifth decade of life. At this time, the corneal fragility increases as corneal sensitivity diminishes significantly.\textsuperscript{76} Corneal fragility is defined by the value of the force needed to produce a break in the corneal epithelium, reaching a “corneal damage threshold.”\textsuperscript{76} Corneal sensitivity is higher in people
with blue eyes than brown eyes.\textsuperscript{54,77} This is not the case in albino vs. brown-eyed rabbits, suggesting a central origin to this phenomenon.\textsuperscript{54,78} Corneal sensitivity decreases from the 31st week to the end of pregnancy and has been hypothesized to correlate with corneal edema relating to water retention during pregnancy.\textsuperscript{14,54,79} Some investigators have found decreased corneal sensitivity related to the preovulatory estrogen rise.\textsuperscript{68} Decreased corneal sensitivity has also been reported as a congenital condition.\textsuperscript{80,81}

Corneal sensitivity decreases in contact lens wearers due to changes in the corneal metabolism, development of corneal edema, changes in tear flow and creation of a relative hypoxia.\textsuperscript{14,54} Long term wear produces a gradual reduction due to prolonged interference with atmospheric oxygen supply which then alters the metabolic processes in the cornea and suppresses corneal nerve function by acidosis.\textsuperscript{68,82} Wearers of hard contact lenses have a decreased sensitivity which correlates with the length of time the
lenses are worn. Recovery of sensitivity usually occurs within four months of abandoning use of lenses. The degree of fitting causes different sensitivity changes in different parts of the cornea.

Corneal sensitivity is affected by several disease processes, both systemic and ocular. Corneal sensitivity is decreased in diabetics as compared to the normal population. This is especially true in diabetics suffering from a peripheral neuropathy. Decreased sensitivity has been found to correlate with the duration of diabetes, though many patients with longstanding diabetes have normal corneal sensitivity. Morphologic degeneration and decreased bundles of subbasal corneal nerves have been detected in early stages of diabetic peripheral neuropathy and precedes the impairment of corneal sensitivity. Other systemic conditions which decrease corneal sensitivity include Hansen’s disease (leprosy) and myasthenia gravis. Hansen’s disease causes a variety of ophthalmic conditions such as prominent corneal nerves and reduced corneal
sensitivity. Increased CTTs in patients with myasthenia gravis implicate ACh in sensory transduction in the cornea although altered eyelid function could be the underlying cause.

Ocular disease processes also affect corneal sensitivity. Keratoconus, scleritis and episcleritis, lattice corneal dystrophy and glaucoma may lead to a loss of corneal sensitivity. Adie’s syndrome (parasympathetic denervation) due to lesion of the ciliary ganglion or in the short ciliary nerves causes decreased corneal sensitivity. Decreased corneal sensitivity is common in people suffering from herpes simplex keratitis. If the keratitis is superficial, sensitivity may be restored, although sensitivity can be permanently affected in patients with interstitial keratitis. Patients suffering from scleritis and episcleritis have decreased corneal sensitivity, with it being significantly more of a problem in episcleritis patients. In all patients studied, corneal sensitivity returned to normal upon resolution of the inflammation.
Corneal edema decreases corneal sensitivity by up to 55%. Hyposecretion of tears may lead to pathologic changes in corneal epithelium leading to a decline in corneal sensitivity.

Several surgical procedures result in temporary or permanent reduction in corneal sensitivity. Cataract surgery causes variable loss of sensitivity depending on the extraction method. Intracapsular extractions requiring a larger surgical incision reportedly cause a significant decrease in corneal sensitivity of more than six-fold, as compared to an unoperated eye up to two years post-operatively. The greater the size of the incision, the greater the reduction in corneal sensitivity is found when comparing an intracapsular vs. extracapsular technique. Hyposensitive areas geographically related to a scleral tunnel incision for phacoemulsification and aspiration are reported even 12 months after surgery. Photorefractive keratectomy for myopia results in a dramatic reduction of corneal sensitivity within the first few weeks that plateaus in
about a month with gradual recovery after one year.\textsuperscript{70,82} In this procedure there is an obliteration of corneal nerve architecture within the 6mm zone of ablation.\textsuperscript{82} Various other keratorefractive procedures including radial keratotomy, epikeratophakia, and LASIK, damage corneal sensory innervation and result in increased sensory thresholds.\textsuperscript{70,93} After LASIK surgery, corneal sensitivity is at its lowest 1-2 weeks postop and recovers within 6 months. Changes in geographic subbasal nerve fiber bundle morphology corresponded to decreased corneal sensitivity.\textsuperscript{93} Photocoagulation of the ocular fundus also decreases corneal sensitivity.\textsuperscript{68}

Corneal sensitivity has been studied in several domestic animal species, including the dog, the cat and the horse.\textsuperscript{18,21,94} Baseline corneal touch thresholds have been established for these species, with the horse having a more sensitive cornea than the cat or the dog.\textsuperscript{18,21,94} Corneal touch thresholds are significantly different in different breeds of dogs depending on skull type, with
brachycephalic breeds having the least sensitive corneas.\textsuperscript{18}

\textbf{Neurotrophic keratitis}

Neurotrophic keratitis is a condition that results from corneal denervation. It is characterized by degenerative corneal changes and ulcerative keratitis resulting from a loss of corneal sensitivity.\textsuperscript{13} In humans, neurotrophic keratitis has been associated with systemic diseases such as diabetes mellitus and viral infections, as well as chronic inflammatory conditions, cranial nerve V palsy, chemical burns, corneal surgery, abuse of topical anesthetics and multiple sclerosis.\textsuperscript{25,95} The precise pathogenesis of the lesions is not known but likely contributors to the keratitis include reduced blinking and reflex tear production resulting in corneal drying and repeated trauma, as well as loss of neurotrophic factors from denervation.\textsuperscript{35,37,50,95,96} This corneal condition is also associated with loss of direction of corneal epithelial migration, loss of desmosome density and intercellular edema.\textsuperscript{50}
Sensory denervation in rabbit corneas results in decreased epithelial mitosis, abnormal wound healing, decreased cell migration and altered permeability.\textsuperscript{49}

Corneal denervation of sensory nerves may occur from surgical lesions of the trigeminal ganglion, herpes virus infection, local corneal disease or surgery, and cavernous sinus disease.\textsuperscript{97} Ganglionic lesions are more likely to threaten corneal integrity than lesions between the brainstem and ganglion in the root of cranial nerve \textit{V}.\textsuperscript{98}

Denervation of the cornea results in impaired epithelial wound healing, increased epithelial permeability, decreased epithelial metabolic activity and loss of cytoskeletal structures associated with cellular adhesion.\textsuperscript{21,49} Tear concentration of substance P is decreased in patients with corneal hypoesthesia, and is more closely related to the clinical duration of the hypoesthesia than the severity.\textsuperscript{99} Substance P and CGRP have been found to modulate various aspects of corneal epithelial cell behavior, including proliferation,
adhesion and cellular migration.\textsuperscript{42,47} Topical treatment with substance P has been successful in stimulating corneal epithelialization in clinical cases of neurotrophic keratitis.\textsuperscript{100}

Following surgical injury, neural regeneration and remodeling depends on the site and depth of the incision.\textsuperscript{21,70} Recovery of sensitivity in the central cornea occurred only when incision depths were less than 53\% of the corneal thickness; being shallow enough to spare some stromal nerves.\textsuperscript{21,70} Following experimental corneal incisions in rabbits it took six weeks for neurites to extend from the wound margins 1.5-2mm.\textsuperscript{101} Following photorefractive ablation in rabbits, epithelial innervation was almost completely restored in 3 months, while stromal nerves still had abnormal features at 12 months.\textsuperscript{101}

**Corneal sensitivity and glaucoma**

Corneal sensitivity decreases with increased intraocular pressure.\textsuperscript{57,102,103} Corneal sensitivity was especially decreased at higher pressures with concurrent
optic nerve head atrophy. The decrease in corneal sensitivity seen with age is more dramatic in glaucomatous patients than the general population. Ocular hypertension causes variable degrees of mechanical deformation of the eye, tissue hypoxia and corneal edema. These pathophysiologic processes could be responsible for the decrease in corneal sensitivity in glaucomatous patients. Corneoscleral mechanoreceptors are excited with increased intraocular pressure which may explain some of the pain felt by glaucoma patients.

**Medications that affect corneal sensitivity**

Medications used in the treatment of glaucoma have also been found to decrease corneal sensitivity. Local anesthetic effects of the beta-blocking agents are complex but are thought to be due to membrane stabilization effects. Timolol maleate has been shown to diminish corneal sensitivity after prolonged use. The mechanism is controversial but the drug may antagonize serotonin stimulation of epithelial chloride transport in the cornea. Different concentrations
of timolol produce anesthetic effects in older patients which last approximately 11 minutes after administration. Beta blockers have also been found to inhibit corneal re-epithelialization. Dorzolamide, a topical carbonic anhydrase inhibitor, causes a significant decrease in corneal sensitivity for up to 20 minutes following administration. This effect was more profound in patients greater than 60 years of age.

Non-steroidal, anti-inflammatory agents such as 0.5% ketorolac tromethamine (Acular®) and 0.1% diclofenac sodium (Voltaren™), can significantly decrease corneal sensitivity in normal eyes for up to 60 minutes. No change in sensitivity has been found with other topical non-steroidal anti-inflammatory agents such as flurbiprofen and indomethacin. The main anti-inflammatory effect of diclofenac lies in its ability to inhibit the cyclooxygenase pathway of the arachidonic acid cascade. However, in patients treated with diclofenac, plasma levels of beta-endorphins rise considerably, possibly contributing to analgesic
activity. Diclofenac also decreases sensory input from corneal polymodal nociceptor nerve fibers. Therefore, it is probably a combination of factors which causes a decrease in corneal sensitivity in diclofenac-treated patients.

Corneal surface anesthetic agents result in a reduction in corneal sensitivity. The topical medication diffuses into the sensory nerve endings and causes a decrease in available Na ion channels resulting in the loss of conduction by the nerve. The resultant anesthetic effect depends on the drug’s composition, lipid solubility, electrical charge and molecular weight. The duration of anesthesia increases non-linearly with increasing concentrations of 0.1%, 0.5% and 1%. Buffered solutions of bupivacaine and lidocaine have been reported to have a significantly longer anesthetic effect than nonbuffered solutions. Using a non-contact corneal aesthesiometer, the recovery of corneal sensitivity after topical anesthesia does not occur until 20-60 minutes after instillation, depending
on the agent used.\textsuperscript{109,115} Prolonged administration may have deleterious effects on corneal epithelium including the destruction of intracellular elements and extracellular connections such as desmosomes and microvilli.\textsuperscript{116} Corneal epithelial wound closure is inhibited by the application of topical anesthetics.\textsuperscript{117,118} Tetracaine has been shown to be more toxic than proparacaine in cytotoxicity tests.\textsuperscript{113} After topical anesthetic administration there is a decrease in the cytoskeletal elements actin and myosin which are important in corneal epithelial migration.\textsuperscript{117}

**Corneal sensitivity and aqueous tear production**

A lack of corneal sensitivity has been found to correlate significantly with insufficient aqueous tear production.\textsuperscript{90,119} Hyosecretion of tears may lead to pathologic changes in corneal epithelium and a disturbance in ocular surface integrity leading to a decline in corneal sensitivity.\textsuperscript{90} It has been hypothesized that damage to the corneal surface creates a destructive negative feedback on the lacrimal gland.\textsuperscript{119}
There is strong evidence that corneal stimulation drives essentially all aqueous tear flow. Damage to corneal epithelium and corneal nerves results in increased levels of epidermal growth factor and tumor necrosis factor-alpha in the lacrimal gland. This suggests a regulatory arc from the cornea to the lacrimal gland. Decreased corneal sensitivity has been found to cause lacrimal gland damage such as direct aprocrine cell damage and atrophy.

**Nd:YAG laser cyclophotocoagulation**

The Nd:YAG laser emits light in the near infrared spectrum at a wavelength of 1,064nm. The laser is produced by a photic stimulation of neodymium atoms suspended in a crystal of yttrium, aluminum and garnet. The laser mode can be pulsed or continuous, and the delivery system can be a contact or a noncontact system, both of which can influence the tissue insult and the degree of postoperative inflammation. The laser beam passes through non-pigmented tissues such as the sclera and is absorbed preferentially by melanin in
pigmented tissue. The degree of pigmentation in the sclera or conjunctiva may reduce the percentage of laser energy reaching the ciliary body. Ophthalmic applications of the Nd:YAG laser include transcleral cyclophotocoagulation for glaucoma, ablation of pigmented masses in the iris and ciliary body, as well as posterior capsulotomy. Surgical cyclodestructive methods for the treatment of glaucoma in animals include cyclocryoablation, diode laser transcleral cyclophotocoagulation and Nd:YAG laser transcleral cyclophotocoagulation. These procedures are used to damage the ciliary body to decrease production of aqueous humor. Trans-scleral cyclophotocoagulation provides improved surgical control with fewer complications than other cyclodestructive procedures. The laser beam is directed perpendicular to the sclera, 5-7mm posterior to the limbus, and passes through the sclera to be absorbed by melanin in pigmented tissue, causing direct destruction of the ciliary epithelial cells and indirect destruction by ischemia and
inflammation.\textsuperscript{2-6} The destruction of the ciliary body is maximal when the threshold between coagulation and explosive tissue disintegration is exceeded and an audible "pop" is heard meaning a maximal energy level.\textsuperscript{124,137} However, higher energies are associated with increased incidence of side effects. Cyclodestructive procedures are associated with many clinical complications, including anterior uveitis, rubeosis irides, dyscoria, lenticular opacities, corneal neovascularization and opacifications, corneal ulcerations, transient postoperative hypertension, choroidal effusions, serous retinal detachment, hyphema and phthisis bulbi.\textsuperscript{3,7,24,128,135,136}

Mechanisms for lowering intraocular pressure by cyclophotocoagulation method include 1) direct destruction of the pigmented ciliary epithelium 2) indirect ciliary epithelial cell destruction through ischemia and inflammation 3) increased uveoscleral outflow and 4) creation of alternate drainage routes such as transcleral flow.\textsuperscript{2,5,6} The optimal clinical effect is associated with approximately 100-200J of energy
delivered in short bursts,\textsuperscript{7} although fewer side effects are reported with the application of increased energy at a slower delivery to less sites.\textsuperscript{138} Therapeutic ranges on cadaver eyes report "medium" effects defined as a homogenous "bleaching" or charring of the ciliary structures without loss of tissue architecture or tissue shrinkage.\textsuperscript{137} This effect is seen at power levels of 3-5 watts for 1-2 seconds.\textsuperscript{137} Histologically, there is damage to the ciliary body due to vascular endothelial necrosis characterized by vascular congestion and a predominantly mononuclear cell infiltrate.\textsuperscript{5,6,128} This vascular necrosis causes a breakdown in the blood aqueous barrier and post-operative aqueous flare.\textsuperscript{5,6,128} Other post-operative effects are similar to other cycloablation procedures.\textsuperscript{2,3,7,128}

**Corneal nerve staining techniques**

The ability to identify nerves histologically in the cornea was initially confined to three techniques developed in the nineteenth century: methylene blue uptake, silver impregnation and gold chloride
impregnation. More recently, numerous more specific techniques have been developed.

Methylene blue can be used as an intravenous technique due to its universal staining of nerves, but can also be used to selectively stain the cornea by simply instilling a few drops of 1% dye into the conjunctival sac. Methylene blue is a weak base and does not penetrate the cornea to stain nerves in the deeper layers. In excised corneas, incubation of the cornea in methylene blue solution results in staining which is not as specific as other techniques, however intracameral injections 30 minutes prior to harvesting the cornea provides uniform, consistent staining of nerves. Staining can be improved upon by maintaining an acidic pH.

Silver stains quickly replaced methylene blue as they were more sensitive to connections between nerve fibers and their terminals. However, background staining is heavy and it is difficult to distinguish nerves from corneal epithelium. Perfecting the
technique is difficult as results can vary from cornea to cornea.\textsuperscript{27}

The gold chloride impregnation technique was introduced in 1867 by Conheim and was based on the assumption that gold precipitated on the walls of the nerves.\textsuperscript{29} Corneas are acidified in lemon juice and impregnated with 1\% gold chloride.\textsuperscript{27} The reaction is “stopped” with glacial acetic acid, however early attempts with this method resulted in continued darkening of tissues over several days.\textsuperscript{27} Early methods also resulted in loss of detail of finer nerve fibers due to uneven precipitation of the gold onto nerve fibers and other tissues.\textsuperscript{27} Several modifications have been developed to decrease nonspecific background staining and to eliminate the progressive deterioration of stain quality with standard gold chloride techniques.\textsuperscript{139,140} Some modifications involve the use of cryoprotective agents, the removal of Descemet’s membrane prior to fixation, treatment with alpha amylase and using Kodak rapid fixer.\textsuperscript{139,140}
Intra-axonal transport of horseradish peroxidase-wheat germ agglutinin conjugate (HRP-WGA) has been used to effectively study the sympathetic innervation of the rat cornea. A 2% solution of HRP-WGA can be applied to scarified corneal surface and is taken up by corneal nerves. Subsequent inspection of the superior cervical ganglion then identifies the cell body of origin.

Catecholamines such as dopamine and noradrenaline can be specifically demonstrated in adrenergic nerves via a sodium-potassium-glyoxylic acid-induced fluorescence.

Immunohistochemical staining of nerves involves using an antibody directed toward a specific cell or tissue antigen which is labeled with a chromagen or fluorescent marker. The primary antibody reacts with the fixed or insoluble antigen in cells and tissues. High-avidity antibodies are the most useful for staining as low-avidity antibodies are often dislodged during the washing steps. The antibody can be labeled directly
with a fluorescein marker, or indirectly with subsequent layers of antibodies with other detection agents such as avidin/streptavidin with biotinylated primary antibodies.\textsuperscript{145,146} Any molecule tagged with the avidin/biotin complex can be visualized using a fluorescent dye, radioactive isotope, heavy metal or chromagen.\textsuperscript{146} The method of avidin-biotin interaction is very sensitive due to the high affinity between the avidin and biotin.\textsuperscript{146} This method is commonly used to visualize specific neuropeptides found in the cornea such as CGRP, substance P and TH.\textsuperscript{22,37,43}

The purpose of this study was to investigate the effects of Nd:YAG laser cyclophotocoagulation on canine CTT and the number and diameter of major nerve bundles in the canine cornea and development of keratitis. In addition, aqueous tear production and IOP were measured after Nd:YAG laser cyclophotocoagulation. The Cochet-Bonnet aesthesiometer was used due to its ease of use in a clinical setting with animal patients, and past studies demonstrating normal values in dogs.\textsuperscript{18} Gold
chloride staining techniques was used to visualize corneal nerves for quantitation and nerve diameter measurements.

MATERIALS AND METHODS:

Animals studied:

Fifteen conditioned adult mixed-breed dogs of varying ages, weighing between 19.7 and 27.9 kg were used for this study. One female and fourteen males were included. Physical examinations including ophthalmic and neurologic examinations were performed prior to the procedure and were found to be within normal limits. A complete blood count and serum biochemistry panel were within normal values for all dogs. Baseline measurements of CTT, IOP, and aqueous tear production were recorded, using the methods described in detail below. Dogs were acquired from the research population at the VA-MD Regional College of Veterinary Medicine and their care and use was approved by the Animal Care and Use Committee of the institution.

Nd:YAG laser cyclophotocoagulation

Dogs were premedicated with a subcutaneous administration of 0.01mg/kg acepromazine maleate(PromAce®, Fort Dodge
Laboratories Inc., Fort Dodge, IA) and 0.1 mg/kg of butorphanol tartrate (Torbugesic®, Fort Dodge Laboratories Inc., Fort Dodge, IA). Dogs were intubated, and anesthesia was maintained with a constant rate infusion of propofol to effect. The left eye was prepared with dilute 1:50 povidone iodine solution and the eyelids immobilized with an eyelid speculum. A continuous wave Nd:YAG laser (Luxus™ Series Nd:YAG Anesthesia was induced with an intravenous bolus of 1 mg/kg of propofol (Proflo®, Abbott Laboratories, North Chicago, IL) Surgical Laser System, Lasersonics® Haraeus Surgical Inc., San Jose, CA) was used with a hand-held, non-contact optical fiber. The fiber tip was positioned perpendicular to and 1-2 mm offset from the conjunctival surface. The beam was directed 5 mm posterior to the limbus over the approximate location of the pars plicata of the ciliary body. A total of 100 J of laser energy was delivered to the left eye, as was performed in an earlier study testing different energy settings and their effects. Ten laser applications of 25 watts X 0.1 seconds were delivered to each quadrant, avoiding the 3 and 9 o’clock positions. The right
eye in each dog served as an untreated control. Five dogs were treated per day on three separate days. For consistency the same investigator (AKW) performed all treatments and measurements.

**Measurement of corneal touch threshold (CTT)**

A Cochet-Bonnet hand-held aesthesiometer was used to stimulate the corneal surface until a corneal blink reflex was elicited (Figure 2). The cornea was divided into 5 testing regions: a 5mm diameter oval central region and 4 oval areas, 4-mm by 2-mm wide, 3mm axial to the limbus in the dorsal, ventral, nasal and temporal corneal regions (Figure 3). Initial stimulation was performed with the aesthesiometer nylon monofilament at 3.0 cm.

To obtain a measurement, the observer viewed the cornea from the side at close range while the filament was applied at a steady speed with perpendicular corneal contact. The cornea was touched by the tip until a slight filament bend or deflection of approximately 5% was attained. If no blink response was observed, the monofilament was shortened in 0.5cm increments until a positive blink was noted in three consecutive attempts at the same monofilament length.
Filament length was converted to milligrams of force and was recorded as the CTT. Animals were generally cooperative throughout the procedure and if not, testing would resume after a short break. Measurement of CTT was performed on both eyes prior to laser treatment, and on days 1, 3, 5, 7, 9, 11 and 13 post-laser treatment. For consistency the same investigator (AKW) performed all measurements, between 7 and 10am. The investigator was unable to be blinded to the laser treated eye due to obvious clinical signs associated with the treatment.

**Monitoring intraocular changes and corneal integrity**

All dogs were examined daily utilizing a transilluminator, slit lamp biomicroscopy and indirect ophthalmoscopy. Clinical signs such as conjunctival hyperemia, blepharospasm, aqueous flare and cells, and dyscoria were recorded daily. Conjunctival hyperemia, aqueous flare and aqueous cells were graded on a subjective scale of 0–4 with 4 being the most severe. The eyes were stained with fluorescein dye using a fluorescein impregnated paper strip and the stain retention area, indicating a defect in the corneal surface, was recorded. All eyes were treated with
chloramphenicol drops (Chloroptic®, Allergan America, Hormigueros, PR) twice daily throughout the study.

**Schirmer Tear Test (STT)**

Schirmer I tear tests were conducted in each eye by placing the strip (Schirmer Tear Test sterile strips, Schering Plough Animal Health Corp, Kenilworth, NJ) in the middle to lateral third of the lower conjunctival fornix. The strip was removed after 60 seconds and the amount of strip wetting was recorded in mm per minute. Schirmer tear testing was performed in each eye prior to laser therapy, and on days 2, 4, 6, 8, 10, 12 and 14 post-laser treatment.

**Intraocular pressure (IOP) measurements**

Intraocular pressure was measured using applanation tonometry (Tonopen XL®, Biorad, San Jose, CA). The pressure was measured and recorded for both eyes of each dog prior to the laser treatment, and twice daily (7-10am, and 3-6pm) for fourteen days post-laser treatment. Only pressure readings of <5% error were accepted.

**Tissue Collection and Processing**
On day 15 following laser treatment, the dogs were sacrificed via an overdose of 150mg/kg pentobarbitone sodium (Beuthanasia®-D Special, Schering-Plough Animal Health, Kenilworth, NJ) administered intravenously. Eyes from 10 animals were harvested within 30 minutes of euthanasia and the dorsal and temporal/lateral aspect of the globes were marked with a 5-0 silk suture placed at the limbus. Whole eyes were immersion-fixed and stored in 4% paraformaledehyde-0.2% picric acid in 0.1M phosphate buffered saline, pH 7.4, for 2-3 hours. The corneas were then removed, along with 1-2mm of continuous corneoscleral limbus and stored in fresh 4% paraformaledehyde-0.2% picric acid in 0.1M phosphate buffer, pH 7.4, at 4°Celsius. Prior to sectioning, each cornea was placed in 30% sucrose in 0.1M phosphate buffered saline solution for 12-18 hours, and sectioned with a razor blade into 4 wedge-shaped segments extending from the corneal limbus to the center. The dorsolateral and ventrolateral quadrants from each animal were placed in OCT compound (Miles Labs, Elkhart, IN) for five
minutes and then sectioned with a cryostat in the anterior-posterior direction. The sections were cut tangential to the corneal surface and serial, 40um-thick sections were collected into tissue wells filled with chilled phosphate buffered saline.

**Gold chloride staining**

The dorsolateral and ventrolateral corneal sections of ten pairs of corneas were rinsed twice in 0.1M phosphate buffered saline solution, placed in 0.1M sodium citrate, pH 5.4, for 5 minutes, and then placed in 100% pure, unfiltered lemon juice (Real Lemon™) for 15 minutes, in ambient room light. The corneal sections were then placed in 1% aqueous gold chloride solution (chlorauric acid, Sigma, St. Louis, MO) for 25 minutes. The corneal sections were transferred to acidulated water (6 drops glacial acetic acid/100ml distilled water) for 4-5 hours, until nerves were visualized with the aid of a dissecting microscope, and before excessive background staining occurred. The sections were rinsed in 0.1M phosphate buffered saline solution for 10 minutes, and placed in Kodak rapid fixer with hardener (to prevent
progressive darkening of tissue) for 10 minutes, and then rinsed in 0.1 phosphate buffered saline solution for 10 minutes. The stained sections were dehydrated with graded alcohols (70%, 95%, 100%) and cleared in xylene. They were mounted in serial order on chrome alum-gelatin coated slides and coverslipped with Permount® (Fisher, Fair Lawn NJ). The optimal incubation times in gold chloride and acidulated water used in this study were determined on the basis of pilot experiments so as to maximize staining quality and minimize background staining.

**Evaluation of Corneal Nerves**

Corneal sections were critically examined with an Olympus BH2 light microscope. The major corneal stromal nerve bundles and distribution patterns of ten animals were documented by making a series of composite line drawings with a drawing tube attached to the microscope (Figure 4). The camera lucida sections were examined and the number of stromal nerve bundles entering the peripheral cornea at the corneoscleral limbus in each
corneal quadrant was quantified by two blinded observers (Table 2).

Corneal sections were also photographed at 1.5X using Ektochrome® T-64 film (Eastman Kodak Company, Rochester, NC). These photomicrographs were scanned into Adobe®Photoshop® 5.0 (Adobe Systems Inc., San Jose, CA) at 1000 dpi resolution. Using NIH image analysis software (NIH Image Research Services Branch, Bethesda, MD) the diameter of each nerve bundle was measured at its widest point as it exited the limbus (Figure 5). The examiner was blinded as to whether it was the dog’s right or left eye when counting nerve bundles and measuring diameters. Two quadrants of each cornea were examined in seven animals (8,11,21,871,883,889, 897). Only one quadrant was examined in the right eye of dogs 870 and 983, and only one quadrant was examined in the left eye of dogs 886 and 983. Nerves were measured in arbitrary units and then converted to micrometers.

Statistical analyses
Differences between the laser treated and the control eyes were calculated and the means of this difference were analyzed using repeated measures of analysis of variance (ANOVA) (SAS version 8.1, SAS Instruments Inc., Cary, NC). This was used for IOP, STT and CTT. In addition, the baseline values for IOP, STT and CTT for the right and left eye of each patient were compared to each other using a paired student t-test.

P values for IOP and STT were compared to the Bonferroni-corrected alpha level to hold the experimental wise error rate to 0.05. For the response variables where fourteen comparisons were made (IOP) the alpha for each test was lowered to 0.003938 to bring the overall alpha level back to 0.05. For the response variables where seven comparisons were made (STT) the alpha was lowered for each test to 0.0073008 to bring the overall alpha level back to 0.05. P values for CTT were compared to an alpha level of 0.05.

The number of nerve bundles and the mean nerve diameters of laser treated and control eyes were compared using a paired t-test. Because the number of nerves counted and measured varied between dogs due to the
inequality of the number of quadrants examined, weighted and non-weighted analyses were also performed.

RESULTS:

Clinical signs and corneal integrity

All laser treated eyes had varying degrees of conjunctival hyperemia, dyscoria and aqueous flare (Figure 6). Most of these clinical signs had resolved by Day 8 post-laser, with only two dogs retaining signs of conjunctival hyperemia from Day 8-12. Aqueous flare had resolved in all but 2 dogs by Day 8 post-laser treatment. Eight dogs had occasional or 1/4 aqueous cells on Day 5 of the study, seven still had occasional cells by Day 9, and one dog had aqueous cells evident throughout the entire fourteen days post laser. Ten of fifteen dogs remained mildly to moderately dyscoric throughout the study. Three dogs had a fibrin clot in the anterior chamber the day after the laser procedure which resolved by Day 4 in all three. Dog 871 developed a ventral, bulbar conjunctival cystic mass on Day 10 which grew in size for two days, before rupturing on Day 12 with the extrusion of purulent material. The mass had flattened and almost resolved by Day 14 of the study. Two dogs had evidence of
iris hemorrhage that resolved in one dog by Day 3, and in the other dog by Day 10.

Six out of fifteen dogs (40%) developed corneal epithelial defects. On Day 1 post-laser treatment, four out of fifteen dogs (Dogs 889, 873, 865, and 897) had evidence of corneal epithelial roughening in the laser treated eye. This corresponded to multifocal, punctate areas of corneal fluorescein stain retention. Dogs 873 and 889 had such roughening on Days 2 and 8 respectively, which resolved in each within 48 hours. Dog 865 developed a rough corneal epithelial surface in the laser treated eye on Day 5 which resolved by Day 8. Dog 897 had a rough corneal epithelial surface in the laser treated eye throughout the fourteen days post-laser treatment, with new punctate areas arising and previous areas healing within that time. Three dogs (Dogs 11, 897, and 983) had corneal erosions develop post-laser treatment. A fluorescein positive corneal erosion developed in Dog 897 in the laser treated eye on Day 8 which remained static for three days, before improving on Day 12 and returning to rough on Day 13 (Figure 7). Dog 11 developed a corneal erosion in the laser treated eye on Day 4 which
healed by the next day. Dog 983 developed 1X3mm corneal erosion on Day 4 that had healed by the next day.

**Corneal sensitivity**

Mean regional CTT values in monofilament length and in grams/mm² are given in Table 3. Prior to treatment there were no CTT differences between the treatment and control eyes for baseline values of CTT for any of the corneal regions measured (P<0.05). Following laser treatment, a significant decrease in corneal sensitivity was identified in the laser treated eye as compared to the control eye in all regions of the cornea tested (P <0.05). This difference was already evident on the first day post laser treatment. The CTT difference in the central cornea ranged between 0.43–0.57mm monofilament lengths with a mean difference in CTT of 2.91gm/mm², corresponding to a 28.5% decrease in corneal sensitivity. The dorsal region had a range of differences of 0.43–0.75mm for a mean difference in CTT of 2.59gm/mm² corresponding to a 20.5% decrease in corneal sensitivity. The difference in the ventral region ranged between 0.37–0.77mm monofilament lengths with a mean difference in the CTT of 3.45gm/mm² corresponding to a 25.3% decrease in corneal sensitivity.
sensitivity. The temporal region had a range between 0.47-0.83mm monofilament lengths for a mean difference in CTT of 5.25gm/mm² corresponding to a 36.9% decrease in corneal sensitivity. The nasal region ranged between 0.53-0.77mm differences in monofilament length for a mean difference in CTT of 3.74gm/mm² corresponding to a 26% decrease in corneal sensitivity. The mean overall difference for all corneal regions in all dogs ranged from 0.55-0.64mm monofilament length, for an overall mean difference in force of 3.74gm/mm² corresponding to a 28.5% decrease overall between the laser treated and control eyes.

**Schirmer tear tests**

There were no significant differences between the eyes in the baseline measurement of STT. Over the entire observation period, the mean difference in Schirmer tear test I (STT) values between laser treated and control eyes ranged between 0.5mm/min and 2.5mm/min with a standard deviation of +/-0.95mm (Figure 8). There were no significant differences between laser treated and control eyes as the P value ranged between 0.57-0.0112.

**Intraocular pressure**

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There were no statistical significant differences between the two eyes in any of the baseline measurements for IOP. The difference in intraocular pressure between the laser treated and control eyes was significant at most measurement points. The laser treated eye had a significantly lower IOP (P<0.0039) than the control eye, in the morning on Day's 2-9, inclusive, and 14 (Figure 9). The laser treated eye had a significantly lower IOP (P<0.0039) than the control eye in the evening on Days 2-11 (Figure 10). In the morning, mean IOP in the control eye ranged from 15mm Hg to 18.2mm Hg over the entire 14 days, as compared to a range of 12.5mm Hg to 14.9mm Hg in the laser treated eye. The overall mean IOP decrease in the morning in the treated eye was 3.1mm Hg, or an overall decrease of 20.3%. In the evening, mean IOP in the control eye ranged from 16.1mm Hg to 21.1mm Hg over the entire 14 days, as compared to a range of 12.5mm Hg to 15.5mm Hg in the laser treated eye. The overall mean IOP decrease in the treated eye in the evening was 3.7mm Hg, or an overall decrease of 22.1%. Variation between subjects was modeled out of the equation.
Gold chloride staining

The number of major corneal nerve bundles was quantified in nine dogs based on composite drawings from camera lucida line drawings (Table 2). The laser treated eye had on average, 1.01 nerve bundles per quadrant less than the control eye, and this difference was statistically significant. (P<0.05) There was a greater overall sum of nerves counted in the control eye vs. the laser treated eye, 77 vs. 57. The number of nerve bundles ranged between 3-7 per right corneal quadrant and 3-4 per left corneal quadrant analyzed.

A total of 84 nerve diameters were measured from right eyes and measurements ranged from 21.24um to 322.02um, with an average of 95.77um. A total of 44 nerve diameters were measured from left eyes and measurements ranged from 37.29um - 307.74um, with an average of 100.47um. The mean corneal nerve diameters between control and laser treated eyes was not significant (P = 0.8587). Statistical analysis was performed using weighted numbers of observations (P = 0.4615).
DISCUSSION:

These findings support the hypothesis that Nd:YAG cyclophotocoagulation results in corneal nerve damage and corneal hypoesthesia. It is likely that the ulcerative keratitis seen following Nd:YAG laser cyclophotocoagulation in canine glaucoma patients is related to laser-induced ocular nerve damage.

Corneal sensitivity has been studied in several domestic animal species, including the dog, the cat and the horse. Normal CTTs have been established for these species. Corneal touch thresholds are significantly different between breeds of dogs depending on skull type, with brachycephalic breeds having the least sensitive corneas. Similar to other species, regional variation in sensation occurs in the canine cornea, with the greatest sensitivity being in the central cornea. Pre-treatment CTT values in this study were similar to those previously reported for the dog. Following cyclophotocoagulation, corneal sensitivity was significantly decreased in the laser treated eye of all
dogs for the entire two-week follow-up period. Sensitivity was diminished throughout all test regions of the cornea. This finding supports the hypothesis that Nd:YAG cyclophotocoagulation damages corneal sensory nerve fibers at a location posterior to the limbus. Avoiding the 3 and 9 o’clock positions on the globe during cyclophotocoagulation may reduce but does not negate this complication.3

Diminished corneal sensitivity has been documented in glaucoma patients.57,102,103 Sensation was especially decreased in older patients and in those with optic nerve head changes.14 Probable mechanisms include direct mechanical damage to corneal nerves by increased intraocular pressure and decreased sensitivity due to corneal edema.14,102 Damage to corneal nerves due to glaucoma could result in a partial ophthalmic neuropathy.102,103 Sensory nerve responses to ocular hypertension and subsequent decreased ocular blood flow, result in an increased firing rate at the initial onset of ocular hypertension that contributes to pain.104 It is
feasible that continued mechanical stimulation causing nerve deformation and ischemia could lead to nerve damage. By adding further insult to an already compromised corneal innervation, the effects of Nd:YAG laser cyclophotocoagulation on corneal sensitivity found in this study may be magnified in glaucomatous dogs.

Canine glaucoma patients are sometimes treated with topical diclofenac sodium and frequently treated with topical timolol maleate and dorzolamide. These drugs used singly on normal human subjects have been found to cause a decrease in corneal sensitivity.105-107,109,110,112,147 In patients treated with diclofenac, rising plasma levels of beta-endorphins and decreased sensory input from corneal polymodal nociceptive fibers contributes to analgesic activity.110 Beta-blocking agents such as timolol maleate have been credited with local anesthetic and membrane stabilization effects which contribute to decreased corneal sensitivity.105,107,110 We hypothesize that the combination of these drugs in canine glaucoma patients undergoing Nd:YAG
cyclophotocoagulation would further decrease corneal sensitivity and may contribute to corneal pathology observed following cyclophotocoagulation. Chloramphenicol was used in this study topically in both eyes and did not affect corneal sensitivity.

The percentage of study animals developing corneal erosions and ulcerations following cyclophotocoagulation in the present study was 20% vs. 60% seen in clinical canine glaucoma patients at the VA-MD Regional College of Veterinary Medicine. Fluorescein stain was used to detect these epithelial defects. Rose bengal stain, which detects devitalized corneal epithelium,148 may have detected a higher percentage of corneal epithelial lesions in both populations by demonstrating more subtle corneal epithelial irregularities. The severity of corneal epithelial defects was much less in the present study than in clinical canine patients. However, the clinical patients were diagnosed with glaucoma and had documented elevated IOP, often with optic nerve head changes, before the laser procedure. In addition, the clinical cases were commonly being treated with topical
medications that may have contributed to corneal pathology. The difference in frequency and severity of corneal epithelial defects may be related to these differences between the clinical and study populations of dogs. While the study population had documented decreased corneal sensation, these dogs did not have prior elevations in IOP, corneal edema or drug therapy that could have contributed to the corneal disease seen in the clinical canine population.

It is possible that the nylon thread of the aesthesiometer applied to the corneal surface can damage the corneal epithelium. It has been hypothesized that the invasive nature of the instrument not only damages corneal epithelium but can decrease CTT. It is unlikely that the nylon thread was responsible for the epithelial defects documented in this study, as there were no defects found in any of the control eyes at any point during the study.

The optimal clinical effect from Nd:YAG laser cyclophotocoagulation is associated with approximately
100–200J of energy delivered in short bursts,\textsuperscript{7} although there has been a report of less side effects with increased energy at a slower delivery to less sites.\textsuperscript{138} Lower energies, (<100 Joules) have been associated with only a transient reduction of intraocular pressure.\textsuperscript{7,134} Due to subject variation, variation in power levels and exposure times, and variation in the degree of scleral and uveal pigmentation it is difficult to define an “optimal” treatment protocol.\textsuperscript{7,126,134,137} In this study, as expected, there was a significant decrease in intraocular pressure in treated eyes at most time points following laser treatment up through the final days of the study. Due to the very significant decreases in corneal sensitivity across all post-treatment days, it appears that lower total energy delivery (e.g. 100 Joules) is able to compromise corneal sensitivity. However, it is likely that corneal changes associated with this loss of corneal sensitivity would be increased with higher energy delivery.
Aqueous tear production was measured every other day following cyclophotocoagulation and was found to be unchanged within that time period. Decreased corneal sensitivity has been found to correlate significantly with insufficient aqueous tear production.\textsuperscript{90,119} Hyposcretion of tears may lead to pathologic changes in corneal epithelium leading to a decline in corneal sensitivity.\textsuperscript{119} Conversely, damage to the corneal surface has been suggested to cause a destructive negative feedback on the lacrimal gland.\textsuperscript{119,121,122} It is possible that two weeks was not a sufficient time period to establish such a negative feedback on the lacrimal gland to decrease aqueous tear production. Because tear production appeared not to be significantly altered during the study period, there did appear to be a trend of lower aqueous tear production in the laser treated eyes. However, aqueous tear production was unlikely to be a contributing factor in the clinical corneal pathology noted in the study animals.
Despite the development of sophisticated and specific methods for visualizing nerve fibers, such as immunohistochemistry, techniques using the impregnation of nerve fibers with metallic salts remain useful in the study of neuroanatomy. Quantification of major stromal nerve bundles in the control dog eyes in this study was comparable to previous studies. Previous innervation studies report no anatomical differences in the number of major nerve bundles between different quadrants of the same cornea, or between the two eyes of the same animal. Therefore, it was felt that an extrapolation of total corneal innervation based on observations from representative quadrants could be performed. There was a mean 1.01 decrease in the number of major nerve bundles per corneal quadrant in laser treated eye which, when extrapolated to the whole cornea, suggests a decrease of as many as four bundles per cornea. This number was statistically significant and is likely an effect of Nd:YAG laser cyclophotocoagulation. Focal burns and histological necrosis previously reported at areas of
laser application\textsuperscript{128,135} suggest that nerve bundles present in those areas could be destroyed, resulting in nerve drop out. One study found decreased number of corneal nerve bundles in diabetics with peripheral neuropathy at 1 bundle per eye and declared it clinically relevant, even though it was not statistically significant.\textsuperscript{84} Denervation of the cornea results in impaired epithelial wound healing, increased epithelial permeability, decreased epithelial metabolic activity, and loss of cytoskeletal structures associated with cellular adhesion, which would account for the corneal epithelial defects seen in this study and in the clinical population of canine glaucoma patients.\textsuperscript{47,49-51,96} In this study, the animals which developed corneal defects had one less bundle per corneal quadrant in the laser treated eye. This was not significantly different from those animals who did not develop corneal defects.

There was no significant difference in nerve bundle diameter between laser treated and control eyes. Although the laser procedure produced no detectable change in
nerve fiber diameter, it remains possible that minor trauma to the nerves and/or transient inflammation may have compromised these nerves functionally, contributing to the decrease in corneal sensitivity and keratitic changes. In inflammatory conditions, cytokines are released and cause corneal nerve damage.\textsuperscript{119} Cytokines appear to inhibit parasympathetic neural transmission in peripheral nerves, and increased levels of cytokines are found in inflammatory conditions associated with decreased corneal sensitivity.\textsuperscript{90,119,149}

In addition to the observed decrease in corneal nerve bundles in laser treated eyes, we hypothesize that the incidence of corneal ulceration in laser treated eyes is related to diminished corneal neuropeptide expression due to laser induced corneal nerve damage. Comparison of corneal neuropeptide expression in eyes that have undergone Nd:YAG cyclophotocoagulation vs. non-treated eyes is required to validate this hypothesis. Quantitative analyses of immunohistochemically labeled corneal nerve fibers in the central corneas of normal
dogs demonstrated that SP, CGRP, TH and VIP are expressed within >99%, >99%, 29.7%, and 0% respectively, of all corneal labeled PGP-9.5-IR corneal nerves. Corneal immunohistochemical labeling was attempted on the animals used in the current study. Due to methodological difficulties associated with inadequate fixation and antigen stabilization, attempts to examine the expression of protein gene product 9.5, substance P, CGRP and TH in these corneas were largely unsuccessful. Further studies perfecting staining techniques would be required to prove this hypothesis.

In conclusion, corneal sensitivity was significantly decreased in the laser treated eye of all dogs, in all regions of the cornea, for the entire two week period following Nd:YAG laser cyclophotocoagulation. Three out of fifteen dogs (20%) developed corneal erosions in the treated eye, one of which was refractory to healing. Six out of fifteen dogs (40%) had some loss of corneal epithelial integrity. Intraocular pressure was significantly decreased in the laser treated eye as compared to the control eye in both the morning and

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evening on most post-treatment days. There was no significant decrease in aqueous tear production as measured by STT in the laser treated eye as compared to the control eye. The number of major nerve bundles entering the cornea at the corneoscleral limbus was significantly decreased by approximately one nerve per quadrant in the laser treated eye, an average of a 34% decrease per quadrant. There was no apparent difference in maximum stromal nerve bundle diameters between the control and laser treated eyes.
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<table>
<thead>
<tr>
<th>Case</th>
<th>Breed</th>
<th>Date</th>
<th>Joules delivered</th>
<th>Complications</th>
<th>Healing time</th>
</tr>
</thead>
<tbody>
<tr>
<td>4500</td>
<td>1990 M Sharpei</td>
<td>08/26/1997</td>
<td>98J OS 7</td>
<td>corneal erosion</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>02/12/1998</td>
<td>110J OD 8</td>
<td>corneal erosion</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>11/12/1998</td>
<td>273 J OU 8</td>
<td>corneal ulcers OU</td>
<td>2 months</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>blind and phthisical OU</td>
<td></td>
</tr>
<tr>
<td>4952</td>
<td>1988 M Cocker</td>
<td>05/26/1998</td>
<td>157 J OD 8</td>
<td>corneal ulcer</td>
<td>2 weeks</td>
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<tr>
<td></td>
<td>spaniel mix</td>
<td>06/04/1998</td>
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<td></td>
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<tr>
<td>2550</td>
<td>1989 Min Poodle</td>
<td>07/18/1998</td>
<td>147 J OD 7</td>
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<td></td>
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<td>3584</td>
<td>1989 JRT</td>
<td>07/14/1998</td>
<td>174 J OD 8</td>
<td>ulcer OD</td>
<td>3 weeks</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>113J OS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5100</td>
<td>1992 bloodhound</td>
<td>04/15/1998</td>
<td>216J 8</td>
<td>increased IOP - BLIND</td>
<td></td>
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<tr>
<td>3761</td>
<td>1991 Sharpei</td>
<td>02/08/1999</td>
<td>cryo OD 5</td>
<td>corneal ulcer</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>01/07/1999</td>
<td>126 J 8</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>05/13/1999</td>
<td>244J</td>
<td>corneal edema</td>
<td></td>
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<td>ID</td>
<td>Year</td>
<td>Breed</td>
<td>Date</td>
<td>Date</td>
<td>Condition</td>
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<td>--------</td>
<td>------------------------</td>
<td>------------</td>
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<tr>
<td>3566</td>
<td>1990</td>
<td>Bassett Hound</td>
<td>08/15/1995</td>
<td>5</td>
<td>255J OD Hyphema, blind phthisical</td>
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<tr>
<td>4194</td>
<td>1990</td>
<td>Dandy Dinmont</td>
<td>03/29/1996</td>
<td>6</td>
<td>103J OD corneal erosion</td>
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<tr>
<td>1702</td>
<td>1986</td>
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<td>7</td>
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<td>03/13/1997</td>
<td>7</td>
<td>79 J OD</td>
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<tr>
<td>4253</td>
<td>1992</td>
<td>Shih Tsu</td>
<td>04/11/1996</td>
<td>6</td>
<td>83J OS</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td>267.5 J</td>
<td>OS</td>
</tr>
<tr>
<td>4010</td>
<td>1985</td>
<td>Basset Hound</td>
<td>08/16/1996</td>
<td>5</td>
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<td></td>
<td></td>
<td></td>
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<td>267.5 J</td>
<td>OS</td>
</tr>
<tr>
<td>4475</td>
<td>1991</td>
<td>Chihuahua</td>
<td>06/13/1997</td>
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<td>132J OS</td>
<td>corneal erosion</td>
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<tr>
<td>4813</td>
<td>1989</td>
<td>Cocker Spaniel</td>
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<td>1988</td>
<td>Bull terrier</td>
<td>01/21/1996</td>
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Table 2: Number of major nerve bundles found per quadrant in study dogs.

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<tr>
<th>DOG</th>
<th>Control eye</th>
<th>Laser treated eye</th>
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<tbody>
<tr>
<td>883</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>889</td>
<td>4</td>
<td>3</td>
</tr>
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<td>886</td>
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<td>983</td>
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<td>11</td>
<td>5</td>
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<td>11</td>
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<td>2</td>
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<td>21</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>897</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>TOTAL</td>
<td>77</td>
<td>57</td>
</tr>
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</table>
Table 3: CTT values for all dogs. A significant decrease in corneal sensitivity was identified in the lasered eye as compared to the control eye in all regions of the cornea tested (P<0.05).

**MEAN REGIONAL CORNEAL TOUCH THRESHOLD**

<table>
<thead>
<tr>
<th></th>
<th>Central</th>
<th>Nasal</th>
<th>Temporal</th>
<th>Dorsal</th>
<th>Ventral</th>
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<tbody>
<tr>
<td><strong>Pairs of eyes</strong></td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
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<tr>
<td><strong>Average gram/mm²</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control eye</td>
<td>7.31</td>
<td>10.66</td>
<td>8.3</td>
<td>10.07</td>
<td>10.19</td>
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<tr>
<td>Laser treated eye</td>
<td>10.22</td>
<td>14.4</td>
<td>13.15</td>
<td>12.66</td>
<td>13.64</td>
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<tr>
<td><strong>Average monofilament length (cm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control eye</td>
<td>1.85</td>
<td>1.31</td>
<td>1.57</td>
<td>1.4</td>
<td>1.4</td>
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<tr>
<td>Laser treated eye</td>
<td>1.34</td>
<td>0.83</td>
<td>0.97</td>
<td>0.88</td>
<td>0.93</td>
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<tr>
<td><strong>Mean difference gm/mm²</strong></td>
<td>2.91</td>
<td>3.74</td>
<td>5.25</td>
<td>2.59</td>
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<tr>
<td><strong>Percentage decrease in corneal sensitivity</strong></td>
<td>28.5</td>
<td>26</td>
<td>36.9</td>
<td>20.5</td>
<td>25.3</td>
</tr>
</tbody>
</table>
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**Intraocular pressure - Morning**

![Graph showing mean intraocular pressures across 14 days.](image-url)
Figure 10: Mean intraocular pressure between the control eyes and laser treated eyes, measured across 14 days post laser treatment in the evening. Asterisks indicate statistically significant values. ($P < 0.0039$)

$a = $ baseline measurement
Vita:

Anne Christine Kelley Weigt was raised in Saginaw, Michigan. She graduated from Douglas MacArthur High School in 1983 and attended Miami University in Oxford, Ohio. She graduated in 1988 with B.A. degrees in Zoology and English/Technical Writing. Anne worked with the Kendall Healthcare Products Company as a sales associate and then with the University of Cincinnati School of Medicine as a research assistant in a neuroscience laboratory. Anne moved to the Republic of Panama in 1991 and worked as a research assistant for the Smithsonian Tropical Research Institute from 1991-1993. During that time, she was also a biology and english instructor at the Panama Canal College and a music teacher at the Curundu Pacific Theater Arts Center. In 1993, Anne attended Ross University School of Veterinary Medicine in St. Kitts for one year and then transferred to the University of Tennessee where she obtained her doctorate in veterinary medicine in 1997. After completing a small animal medicine and surgery internship at the VCA Franklin Park /Berwyn Animal Hospitals in Chicago, Illinois, she began a residency program at the VMRCVM in ophthalmology. Anne will complete her Master’s and residency in July, 2001. She is married to Mr. Lee A. Weigt who is a faculty member at the Virginia Bioinformatics Institute, has a son Eric, who is 2 and 1/2 years old, and a new baby due in November. In Anne’s spare time, she enjoys music, horsebackriding, and painting. Ophthalmology interests include surgery and neuroophthalmology.