Maintaining the cornea and the general physiological environment in visual neurophysiology experiments

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Abstract

Neurophysiologists have been investigating the responses of neurons in the visual system for the past half-century using monkeys and cats that are anesthetized and paralyzed, with the non-blinking eyelids open for prolonged periods of time. Impermeable plastic contact lenses have been used to prevent dehydration of the corneal epithelium, which would otherwise occur in minutes. Unfortunately, such lenses rapidly introduce a variety of abnormal states that lead to clouding of the cornea, degradation of the retinal image, and premature termination of the experiment. To extend the viability of such preparations, a new protocol for maintenance of corneal health has been developed. The protocol uses rigid gas permeable contact lenses designed to maximize gas transmission, rigorous sterile methods, and a variety of methods for sustaining and monitoring the overall physiology of the animal. The effectiveness of the protocol was evaluated clinically by ophthalmoscopy before, during, and after the experiments, which lasted 8–10 days. Histopathology and quantitative histology were performed on the corneas following the experiment. Our observations showed that this protocol permits continuous contact lens wear without adversely affecting the corneas. Thus, it is possible to collect data 24 h each day, for the entire duration of the experiment. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

For the past half-century, neurophysiologists have been investigating the electrophysiological properties of individual neurons at various levels in the visual system of primates, and other mammals, using what is termed the 'anesthetized and paralyzed preparation' (Kuffler, 1953; Barlow et al., 1957; De Valois et al., 1958; Hubel and Weisel, 1959). In this experimental preparation, the animal is anesthetized to eliminate any potential pain or distress. The animal is also paralyzed to eliminate natural eye movements so that the experimenter can present visual stimuli in a carefully controlled and systematic fashion. Unfortunately, the paralysis also eliminates the natural blink reflex, and because the eyelids must remain open for extended periods of time (in order to present the visual stimuli), the precorneal tear film rapidly evaporates and is not replenished. Under these conditions, the clarity of the cornea degrades within minutes, decreasing the quality of the retinal image. Although there are many different factors that determine the duration of the experiment in the anesthetized and paralyzed preparation, collection of useful data is most often limited by the amount of time that the optical quality of the open eye can be preserved.

In an attempt to preserve the optical quality, researchers have generally covered the eyes with lenses. In the very first experiments (Kuffler, 1953), the contact lenses were made of glass; in later experiments (De

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Valois et al., 1958, 1982) the contact lenses were made of plastic (polymethylmethacrylate, PMMA). Both glass and PMMA are gas impermeable. Although these lenses protect the anterior ocular media from drying-induced distortion of the corneal epithelium (thus preserving ocular clarity in the short term), corneal transparency usually deteriorates within 24 h primarily because of the lack of gas transmission through the contact lenses.

This report describes a new protocol for maintaining optical clarity, and the overall physiological environment, in the anesthetized and paralyzed preparation. Further, evidence is presented that demonstrates the effectiveness of this new protocol, in the monkey and the cat, for a minimum of 8 days.

2. Background

2.1. Maintenance of corneal clarity in the normal eye

As illustrated in Fig. 1, the mammalian cornea is comprised of three cellular layers (epithelium, stroma, and endothelium), which are separated by two acellular layers (Bowman's and Descemet's). In macaque monkeys, the epithelium accounts for $\sim 5\%$ of the corneal thickness and the stroma accounts for $\sim 95\%$. As illustrated in Fig. 2, the normal corneal epithelium is composed of three different types of cells (squamous, wing, and basal), which continually regenerate (immediately anterior to Bowman's layer) and migrate to the corneal surface, where they undergo apoptosis (Oyster, 1999) after 4–8 days (Davson, 1972), and are sloughed off. The continual turnover of epithelial cells explains the high oxygen demand of this layer. It is important to note that because the central cornea is avascular, it obtains most of its oxygen directly from the atmosphere.

The stroma has a laminated structure, each layer of which runs the full length of the cornea, parallel to the surface. These sheets are composed of a very regular lattice of collagen fibrils arranged so that scattering of light is eliminated by destructive interference from individual fibrils (Maurice, 1957); as a consequence, the cornea remains transparent. Any disruption to this regularity, for instance by excessive hydration, results in increased light scattering and decreased transparency. The barriers of the epithelium and the endothelium maintain the normal concentration of water in the stroma by means of active metabolic ionic pumps that continuously extract water (Maurice and Giradini, 1951; Klyce, 1981; for a review, see Bruce and Brennan, 1990). Immediately underlying the stroma is Descemet's membrane. Below that is the endothelium, which is composed of a single layer of non-regenerating cells.

In a normal awake animal, the excellent optical quality of the anterior surface of the cornea is maintained primarily by the action of blinking, which occurs, on average, at a rate of 12.5 blinks/min (King and Michels, 1957; McEwen, 1962). The blinking sweeps away debris and continuously distributes a 7–10 μm thick precorneal tear film evenly over the surface of the cornea, making the uneven corneal surface optically smooth (Oyster, 1999). This is essential to the optical function of the eye (Thomas, 1955). Further, the precorneal tear film is critical for proper hydration of the cornea. Finally, the tear film has direct antimicrobial action (for a general review, see McClellan, 1997) and antiviral action (Coyle and Sibony, 1988).
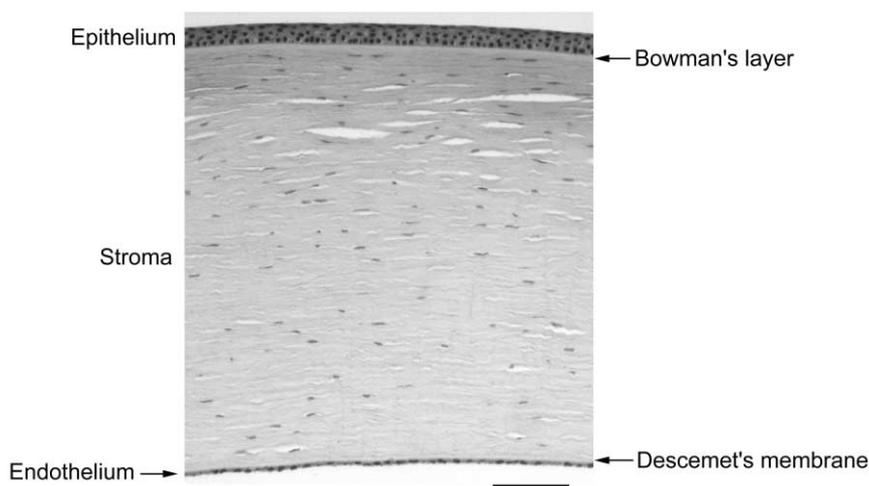

Fig. 1. Photomicrograph of a cross-section through a normal macaque monkey cornea (× 100). For a general review and more detailed information on the normal primate cornea, see Oyster, 1999. The three cellular layers (epithelium, stroma, and endothelium) are separated by two acellular layers (Bowman's layer and Descemet's membrane). It should be noted that the large, unstained fissure-like open spaces visible in the stroma are artifacts of the paraffin-section histology (Thomas, 1955; Walman and Hart, 1987) and are not indicative of pathology. Scale bar = 100 microns.
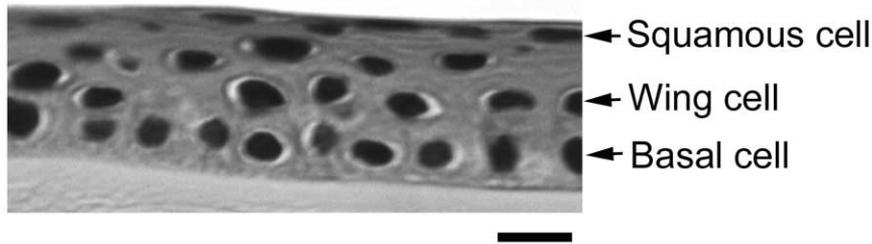
Fig. 2. Photomicrograph of a cross-section through a normal macaque monkey corneal epithelium (× 400). The labels indicate the three types of corneal epithelial cells. Scale bar = 10 microns.

2.2. PMMA contact lenses with the non-blinking eye

In the anesthetized and paralyzed preparation, there is no blinking. Further, the mechanisms for producing precorneal tear film are altered (Cross and Krupin, 1977; Ludders and Heavner, 1979; Vestre et al., 1979). Thus, both the continuous, uniform distribution of the precorneal tear film is eliminated and the protective cleansing process is eliminated. This fact, coupled with the fact that the eyelids must remain open for extended periods of time, in order to present visual stimuli, results in dehydration of the corneal epithelium and the optical quality of the eye rapidly degrades. Dehydration of the epithelium can be ameliorated by the insertion of PMMA contact lenses, because they are impermeable to water. However, while such lenses do prevent evaporation of precorneal tear film, the fact that they are also gas impermeable introduces a variety of abnormal states in all three cellular layers of the cornea, resulting in an excessive accumulation of water, ultimately leading to clouding of the cornea within 24 h.

Because PMMA contact lenses are gas impermeable, extended use results in hypoxia and hypercapnia within the cornea. This, in turn, produces a cascade of problems: a metabolic shift within the cornea towards anaerobic glycolysis, increased production and retention of lactate, compromise of the endothelial osmotic pump, and increased production and retention of water within the stroma. This edema disrupts the normal lattice arrangement of the collagen fibrils in the stroma, which increases light scattering and decreases transparency (for a review, see Bruce and Brennan, 1990).

2.3. Potential remedies for PMMA contact lens problems

Traditional palliative measures for coping with some of the deleterious consequences that result from the use of PMMA contact lenses in neurophysiological experiments are only moderately effective. To begin with, the contact lenses can be removed for some length of time to improve gas exchange; this helps reverse corneal edema. During these periods the eyelids can be closed to prevent epithelial dehydration. Although the closed eyelids diminish the amount of gas exchange, some exchange nevertheless does take place (as during sleep; Benjamin and Hill, 1986). This remediates both hypoxia and hypercapnia by allowing atmospheric oxygen to enter and carbon dioxide to be released. The condition of the cornea gradually improves to some extent (e.g. over a period of 4–6 h, stromal edema is reduced as lactate levels decrease towards baseline; O'Neal and Polse, 1985). However, the remediation is slow and incomplete, because closed eyelids cause a mildly hypoxic state even in the normal eye. In addition to removing the contact lenses, hypertonic artificial tear solutions or glycerin (Magnus, 1993) can be applied to the cornea. These solutions can help reduce stromal edema because they draw water from the stroma by osmosis. Both of these measures are somewhat effective in clearing corneal clouding due to edema. However, prolonged eyelid closure introduces its own set of problems (Baum, 1997) and repeated application of the hypertonic agents can injure the epithelium. Further, whenever the lenses are removed and replaced, there is an increased risk of introducing mechanical damage to the epithelium. Thus, these strategies for coping with corneal edema cannot be prolonged indefinitely. In any case, all of these procedures diminish the amount of time during which experimental measurements of the individual neurons can be performed.

2.4. Gas permeable contact lenses in the non-blinking eye

Advances in contact lens technology have made it possible to abandon the use of traditional gas impermeable contact lenses to protect the open unblinking eye and switch instead to rigid gas permeable, RGP, contact lenses. These lenses have been widely adopted for human use; however their use in animal experimentation has been minimal. These new lenses are essentially impermeable to liquids and thus they prevent corneal dehydration. However, they are permeable to oxygen and carbon dioxide.

3. Methods

The new protocol for maintaining corneal clarity in the anesthetized and paralyzed neurophysiological preparation: (a) utilizes a new lens material and lens design that increase gas transmissibility; (b) incorporates a more rigorous attempt to provide a sterile ocular environment; and (c) incorporates a more rigorous attempt to maintain a healthy physiological environment for the animal as a whole. All three factors help to sustain the health of the cornea.

3.1. The physiological preparation

In order to ensure a healthy physiological environment for the eyes and for the brain, and to sustain an animal that will be anesthetized and paralyzed for extended periods of time, it is necessary to take great care with the initial surgical preparation and the subsequent maintenance and monitoring of the physiological environment. This section describes in detail the procedures that help to maximize a healthy environment. Some of these procedures have been standard practice in the anesthetized and paralyzed neurophysiological preparation and they have been described in the past (for the most recent and detailed description, see Geisler and Albrecht, 1997). However, the majority of the procedures have not been standard practice in this preparation and have only recently been incorporated into the protocol. This new and complete protocol has not been described elsewhere.

All of the procedures have been approved by the University of Texas Institutional Animal Care and Use Committee, and conform to all USDA and NIH guidelines. All of the animals from which data were obtained for this paper were also used for other experiments. They were used in the electrophysiological experiments described in this section (Heeger et al., 2000; Geisler and Albrecht, 2001; Geisler et al., 2001), or in other studies covered by approved protocols. None of the animals was used for the single purpose of evaluating contact lenses.

This new protocol has been utilized on 13 young adult animals: seven domestic cats (*Felis domesticus)* and six macaque monkeys (*Macaca fascicularis* or *Macaca mulatta*). Prior to the experiment, animals are maintained in climate-controlled rooms on a light cycle of 12 h on/12 h off. Cats are housed uncaged in a social group. Monkeys are caged individually in a room with other macaques; the animals can easily view each other through the cages. Cats are fed dry cat chow, and monkeys receive monkey-biscuits, fresh fruits, and vegetables daily. All animals have continuous access to drinking water.

On the first day of the experiment, initial anesthesia is induced with an i.p. injection of sodium pentothal (25 mg/kg for cats; 20 mg/kg for monkeys). Anesthesia is maintained with sodium pentothal as required until gas anesthesia is introduced. Mucous secretions are minimized with an initial i.m. injection of atropine sulfate (0.025 mg/kg). To prevent dehydration of the corneal epithelium before the contact lenses are inserted (while the animal is anesthetized and the eyelids may remain partially open) the corneas are protected with a 1% sterile carboxymethylcellulose solution (CelluVisc, Allergan, Irvine, CA). This is re-applied at intervals as needed until the lenses are inserted. At the beginning of the surgical preparation, animals are given antibiotics to prevent infection: 300 000 units (i.m.) of procaine penicillin and 0.8 mg/kg (i.m.) of gentamicin. The gentamicin is repeated every 12 h, and the penicillin is repeated every 24 h. Sterile technique is used during the surgical preparation of the animals, i.e. (a) the surgical instruments and supplies are sterilized; and (b) a sterile field is created by disinfecting the skin with an iodine-based skin cleanser (Prepodyne Scrub, Amsco, Erie, PA) at each incision site and clipping a sterile fenestrated drape to the edges of the incision. Local sterility is maintained until the skin is closed.

The trachea is cannulated, and the animal is put on isoflurane anesthesia (1.5–2.5% isoflurane, 30% oxygen, 67.5–68.5% air) delivered by an Ohio Modulus gas anesthesia machine (Ohio Medical, Cincinnati, OH) and a veterinary respirator (Hallowell Engineering and Manufacturing, Pittsfield, MA). Respiration rate, inspired and expired gases, and pressure in the airway are continuously monitored via an Ohmeda respiratory gas monitor (RGM 5250, Louisville, CO). Expired carbon dioxide is maintained at $4.0 \pm 0.3\%$ by manipulating the stroke volume and respiration rate. Deep surgical anesthesia (determined by frequent pedal reflex tests) is maintained until all surgical procedures are completed.

Both femoral veins are catheterized. Physiological fluids are delivered through one catheter and pharmaceuticals are delivered through the other catheter. Using two catheters ensures that the two solutions do not form a precipitate (which could clog the catheter and change the concentration of the solutes being delivered), or interact in any other fashion.

The animal is put into a stereotaxic instrument (Baltimore Instruments, Baltimore, MD), using ear, eye, and tooth bars. Electrocardiogram (ECG) probes are placed on both sides of the chest and on one leg; these probes are connected to a veterinary cardiac monitor (Cardiac Display Corp, model CD-200, Port Washington NY), which permits continuous monitoring of the ECG. A rectal temperature probe is inserted, an oral gastric tube is inserted, and the bladder is catheterized. Throughout the experiment, body temperature is controlled automatically (Yellow Springs Instruments Model 750 temperature controller, Yellow Springs, OH) and maintained at 38.5 °C for cats and 37 °C for

monkeys with a circulating-water heat blanket (Gaymar T/pump, Gaymar Industries, Orchard Park, NY). Next, a custom-made stainless steel head holder (12 × 25 mm, curved to fit tightly on the surface of the skull) is mounted to the top of the skull with two screws such that it is positioned anterior to the Bregma and centered over the frontal poles. The head-holder is bolted to the stereotaxic frame, and the ear, eye, and tooth bars are then removed. Two screws are embedded in the skull (bilaterally) over the frontal cortex for recording of the electroencephalogram (EEG). A craniotomy is performed over the visual cortex for the insertion of microelectrodes.

Following the surgical procedures, isoflurane anesthesia is discontinued. Anesthesia and paralysis are maintained throughout the duration of the experiment through continuous intravenous infusion (via one of the indwelling femoral catheters) of the following pharmaceuticals. For cats, anesthesia is maintained with sodium pentothal (2–6 mg/kg/h) For monkeys, anesthesia is maintained with sufentanil citrate (2–8 μg/kg/h). For both species, paralysis is maintained with gallamine triethiodide (10 mg/kg/h) and pancuronium bromide (0.1 mg/kg/h) and secretions are minimized by administration of atropine sulfate (0.025 mg/kg/h). Artificial respiration is achieved using a Harvard large animal volume controlled ventilator (Model 613, Harvard Apparatus, Holliston, MA) and expired carbon dioxide is maintained at 4.0% (± 0.3%) by manipulating the stroke volume and respiration rate.

To maintain hydration and electrolyte balance, 2.5% dextrose in half-lactated Ringer's solution is administered as a continuous intravenous infusion (using the second venous catheter) at rates based on the fluid requirements of each species: 1.5 ml/kg/h for cats (Tornheim et al., 1979; Sato et al., 1996; Canton et al., 1998) and 4 ml/kg/h for monkeys (Reisback et al., 1990; Zorbas et al., 1997). The volume and the rate of the infusion of the pharmaceuticals are precisely controlled by a variable pressure volumetric pump (IVAC Model 570 San Diego, CA). Urine is collected continuously to ensure that volume and pH are within normal ranges. The normal expected pH range is 7–7.5. The normal expected urine output (for a hypokinetic monkey) is ~ 75% of fluid input (Zorbas et al., 1997). Animals are fed (via the gastric tube) a commercially available complete liquid diet (Ensure Plus, 1.6 kcal/ml) at half the rate required for weight maintenance in awake, active animals (i.e. 25 ml/kg/day for monkeys; Hansen et al., 1981; 22 ml/kg/day for cats; Kendall et al., 1983). The total caloric intake is divided into two or three feedings per day. Blood for glucose measurement is taken twice daily from one of the venous lines to ensure that the blood glucose value is within the normal range (75.2 $\pm$ 9.3 mg/dl for cats; Altman and Dittmer, 1974; 95 $\pm$ 25 mg/dl for monkeys; Hendrickson, 1984). If the value is outside of the normal range, the concentration of dextrose and the food intake are adjusted accordingly.

The EEG and the ECG complex are monitored continuously. The raw EEG signal and the Fourier-transform of the signal are quantitatively analyzed throughout the duration of the experiment to ensure that the predominant frequencies are below 4 Hz (NIH Workshop, 1988). The root mean square of the EEG amplitude is also calculated, and the trends plotted, as a measure of the general physiological condition of the animal's cerebral cortex. The average heart rate (for each individual animal) is monitored for changes in excess of 10% (Sato et al., 1996) in response to visual or tactile stimulation by the experimenter. The heart rate in sedated macaques is 120–180/min (Hendrickson, 1984); in awake cats, it is ~ 150/min (Flecknell, 1987).

3.2. Care of the eyes

The material used in the fabrication of the lenses is 'Fluoroperm 151,' which, according to the manufacturer (Paragon Vision Sciences, Mesa, AZ) has a Dk value of 151. The design used in the fabrication of the lenses (Metro Optics, Austin, TX) is a 'heavy blended bi-curve' (see Fig. 3) with a center thickness of 0.14 mm; thus the oxygen transmissibility value, Dk/t, is 107.9. Note however that others have measured the Dk of Fluoroperm 151 and reported it to be $83 \cdot 10^{-11}$ [cm$^2 \cdot$ mlO$_2$]/[s $\cdot$ ml $\cdot$ mmHg] (see Harvitt and Bonanno, 1999); this would result in a Dk/t of $59.3 \cdot 10^{-9}$ [cm $\cdot$ mlO$_2$]/[s $\cdot$ ml $\cdot$ mmHg]. However, even this lower value is more than sufficient to ensure adequate gas transmissibility (Bennett, 1990; Harvitt and Bonanno, 1999).

The lens diameter is chosen to cover the cornea up to the limbus, and the radius of curvature varies with the diameter. For monkeys, the lenses are readily available and inexpensive because they can be made from standard material blanks that are used for humans. For cats, however, the lenses must be made from larger, non-standard blanks. It is important to fit the lenses correctly to the eyes of each individual animal. Lenses that have too high a radius of curvature trap extra fluid near the center of the lens and thereby add dioptric power; lenses that have too low a radius of curvature do just exactly the opposite. Lenses that do not fit properly can trap air bubbles. Bubbles interfere with the optics and may irritate the cornea (Bruce and Brennan, 1990). A clinical keratometer provides the most accurate method for determining the correct radius of curvature of the lenses for each animal. However, for cats, it is possible to utilize the measurements reported by Freeman (1980), which demonstrate that there is a systematic relationship between body weight and radius of curvature. Table 1 shows the parameters and the range of sizes for the lenses used in this study.

To prevent infection, all tools that are used to handle the lenses are gas sterilized before use and are subsequently stored in a lens disinfectant solution ('Renu', Bausch and Lomb, Rochester, NY). Before use they are thoroughly rinsed in sterile physiological (0.9%) saline. The contact lenses and their storage containers are washed with lens cleaner (Bausch and Lomb 'Concentrated Cleaner' for gas permeable and PMMA contact lenses, Rochester, NY), rinsed thoroughly in tap water, and then the lenses are soaked prior to use in sterile Boston Conditioning Solution (Polymer Technology, Rochester, NY). In future experiments, it would be advisable to use sterile saline as opposed to tap water for rinsing the lenses because tap water may contain pathogens.

After the surgical procedures (described above) are completed, accommodation is paralyzed (cycloplegia) and the natural pupil is dilated (mydriasis) with sterile 1% ophthalmic atropine sulfate and, for cats, the nictitating membrane is retracted with sterile 2.5% ophthalmic phenylephrine hydrochloride. For monkeys, the eyelids must be retracted away from the cornea by mechanical means because they tend to close. (This is not necessary for cats because the lids remain fully open). Retraction is accomplished with a fine (6-0 silk) sterile suture through the outer layer of each eyelid. (The eyelids must remain retracted during the experiment in order to present visual stimuli.) Prior to insertion of the lenses, the eyes are rinsed gently with a stream of sterile saline to remove the carboxymethylcellulose solution and any other foreign matter. The lenses are then inserted immediately. This is accomplished by using padded forceps to position the lens on a rubber lens-insertion tool. Once the lens is properly positioned on the cornea, two fingers (within sterile gloves) are then used to release the lens from the rubber lens-insertion tool. Air bubbles may often be removed simply by waiting for the lens to settle, instillation of an artificial tear, and/or by mechanically moving the lens. If these measures do not remove the bubble, the lenses may have to be removed and re-inserted.

Two drops of an ophthalmic antibiotic solution (sterile ophthalmic solution containing neomycin sulfate, polymyxin B sulfate, and gramicidin, Bausch and Lomb, Tampa, FL) are applied to each corner of each eye. (Based upon the instructions on the package insert, half this amount every 4 h might be sufficient.) After the outer surfaces of the lenses dry, the cotton at the tip of a sterile cotton swab is pulled out slightly (and fluffed) to form a brush. The cotton brush is moistened in sterile water and it is used to remove any salt deposits left on the lenses from the wetting solution or antibiotic drops. Application of the antibiotic drops (and the cleaning of the outer lens surface) is repeated twice daily, and there is no further unnecessary manipulation of the lenses. Unless it is absolutely necessary to clean the inner surface of the contact lenses to restore optical clarity, there is no clear benefit associated with further manipulation of the lenses. In fact, with each subsequent removal and replacement of the lenses there is the risk of producing mechanical damage to the cornea and the risk of introducing infectious microorganisms. In our experience, it is generally not necessary to remove and replace the lenses throughout the entire course of the experiment.

The eyes are examined with an ophthalmoscope to confirm that retinal landmarks can be clearly seen through the cornea. The eyes are then refracted by streak retinoscopy. Dioptric correction is applied, if needed, with appropriate external lenses. On the first day of the experiment, 56 000 units of Vitamin A is administered (i.m.) as a prophylactic measure, to help prevent a variety of corneal abnormalities (e.g. keratinization, ulceration) that have been shown in clinical studies of xerophthalmia to result from deficiencies in blinking and/or tear production (Van Horn et al., 1981;
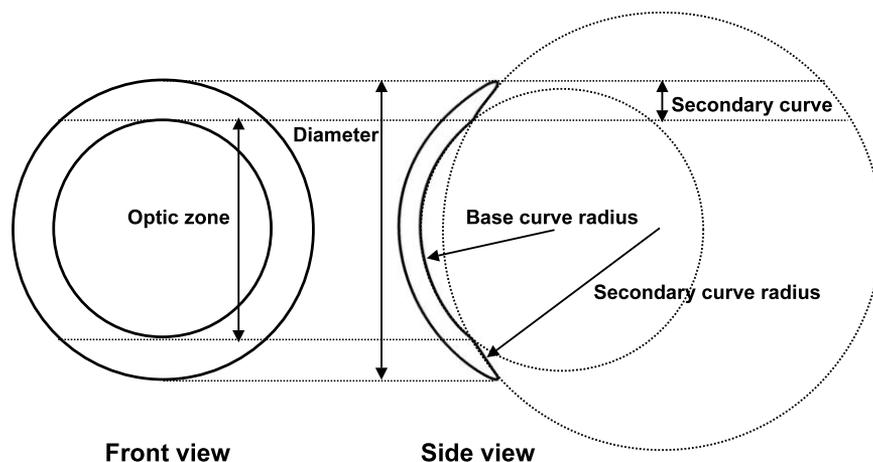

Fig. 3. Diagram of the 'heavy blended bi-curve' design for the contact lenses used in the experimental protocol. Several fundamental parameters of this lens design are illustrated. The relationship between the values of the parameters for the lenses used in this study are given in Table 1.

Table 1
Lens design

Base curve radius (mm)	Diameter (mm)	Optic zone (mm)	Secondary curve radius (mm)
A. Cat lenses			
6.5	11.0	9.0	7.5
7.0	12.0	10.0	8.0
7.5	13.0	11.0	8.5
8.0	14.0	12.0	9.0
8.5	15.0	13.0	9.5
9.0	16.0	14.0	10.0
B. Monkey lenses			
5.6	9.0	7.5	6.8
5.8	9.5	8.0	7.0
6.0	10.0	8.0	7.2
6.2	10.0	8.0	7.4
6.4	10.0	8.0	7.6
6.6	10.0	8.0	7.8
6.8	10.0	8.0	8.0

Values of the parameters for cats (A) and for monkeys (B) of the 'heavy blended bi-curve' contact lenses used in this study.

Sommer and Tarwotjo, 1982; Sommer, 1983). Vitamin B Complex is also given (0.75 ml, i.m.) once a day to supplement the diet and prevent the development of visual defects (Damodaran et al., 1979).

During the experiment, the eyes are examined ophthalmoscopically (see Section 3.3) at least twice daily to re-assess the clarity of the optics and the potential need to remove, clean, and replace the lenses. Indications that lenses need to be removed and cleaned are lack of a clear view of the retina, or obvious accumulations of exudate under, and around, the lenses. Deposits on the surface of the lenses can be carefully removed with a sterile fluffed cotton swab moistened in sterile water. It is not necessary to remove material that collects at the margins of the eyes unless it encroaches on the area under the lenses. If the lenses must be removed, and if there appears to be dried exudate (i.e. dried discharge) at the margins of the lenses, then the eyes should be thoroughly irrigated with sterile physiological saline prior to removal. The reason for this is that such dried material may cause the lens to adhere to the cornea, and if it is not softened prior to lens removal, the adhesion may cause tearing of the epithelium. If the lenses are removed for cleaning, carboxymethylcellulose drops are used to protect the eyes for the few minutes the lenses are absent; this is rinsed off prior to lens re-insertion, as before.

3.3. Evaluation of the cornea

To evaluate the potential effects of extended contact lens wear in the anesthetized and paralyzed neurophysiological preparation, ophthalmoscopic examinations were performed on all of the 13 animals (seven cats and six monkeys). In addition, for two of the monkeys from this group, the corneas were excised and histopatholog-

ical examinations were performed. Finally, quantitative histological measurements were performed. For comparison, histopathological examinations and quantitative histological measurements were performed on two corneas excised from two lens-naive monkeys (one from each animal), which were not used in any vision research protocol. These lens-naïve corneas served as normal controls (i.e. they underwent no experimental manipulations).

3.3.1. Ophthalmoscopic examination

The degree of corneal clarity was monitored over the course of the experiment by inspecting the cornea and the retinal landmarks (through the contact lenses) with an ophthalmoscope. Direct ophthalmoscopy (Jean, 1915; Gordon, 1971; Paton et al., 1976) was performed at the beginning of the experiment, as soon as the eyes had dilated. First, the corneas were examined grossly for any signs of opacities, injuries, or scarring. Then, all four quadrants of the fundus were carefully examined for abnormal pigmentation, scarring, masses, hemorrhage, and retinal detachment. The optic disc was observed with respect to color, clarity of border, and configuration of the physiological cup. The blood vessels were inspected for arterio-venous diameter ratio, configuration at the optic disc, color, abnormal dilation, occlusion, or beading. The macula was observed for color. The configuration and condition of the retinal landmarks observed at each successive 12 h of the experimental period were noted and compared with those observed on the first day.

3.3.2. Histopathological examination

For two of the monkeys that had worn contact lenses continuously for 8 days (61M and 62M), a histopathological examination of the condition of the corneas was

performed at the end of the experiments by a patholo-gist (SLT) and an ophthalmologist (HGR). The only difference in the circumstances of lens wear between the two monkeys was that 62M did not have the lenses removed for cleaning during the 8 days, while 61M did have the lenses removed for cleaning on day 3 and then on day 7. The eyes of these two monkeys (the contact lens group) were removed ~ 1 h postmortem. After removal, the eyes were immersed immediately in a fixative (10% phosphate-buffered formalin with a pH of 7.4) and stored at room temperature.

The corneas of the contact lens group were compared to the corneas of two other monkeys (T57 and V49) that had never worn contact lenses, and that had not been used in any vision research experiment. The eyes of these two animals were removed ~ 15 min post-mortem. After removal, one eye from each of these two animals was immersed immediately in the fixative solu-tion (the normal group) and stored at room tempera-ture. (Because the corneas of the contact lens group and the normal group were both stored at room tempera-ture, any effect of temperature on the corneas would have been similar for both groups.)

The corneas from both groups were dissected from the eyes, embedded in paraffin, sectioned (at room temperature) serially at 3–4 μm, mounted on slides, and stained with hematoxylin and eosin. All of the tissue was processed at the same pathology laboratory using the same standard protocol. Sections were taken across the full extent of the cornea (including the limbus) in and around the optic axis. The corneas were examined for any evidence of pathology using both conventional and polarized light microscopy.

3.3.3. Quantitative histological examination

To determine if there were any significant anatomical changes in the corneas of the contact lens group, in comparison to the normal group several quantitative histological measurements were made from representa-tive sections.

First, total corneal thickness and epithelial thickness were measured directly, under the microscope, using a calibrated graticule.

Second, photomicrographs of the entire thickness of the central cornea were taken at a magnification of $100 \times$ on Fujichrome Provia 100F color slide film (with a 35 mm Olympus camera mounted on an Olympus light microscope). Similar photomicrographs of the ep-ithelium alone were taken, at the same central position, using a magnification of $400 \times$. The photomicro-graphic slides were scanned into a computer by means of a Polaroid SprintScan 35 Plus. All of the quantita-tive histological measurements performed on a cathode ray tube display (e.g. measurements of cell area and cell aspect ratio) are subject to distortion and error if the pixels are not square. Therefore, to assess whether the

pixels were square, a stage micrometer (a finely cali-brated set of markings that denote microns) was pho-tographed horizontally and vertically and then processed in exactly the same fashion as the images of the cornea. The fact that the measurements between the micrometer divisions in the vertical and horizontal pho-tographs were the same indicates that the pixels were square.

Third, every distinguishable epithelial cell in the pho-tographic field of each section was labeled and counted. Cell density was estimated from these counts. The height and width of each labeled cell was measured in Adobe Photoshop. The aspect ratio of each cell was calculated by dividing the cell's height by its width. The characteristic shape of epithelial cells systematically varies with depth in the epithelium (see Fig. 2). In the upper zone, the squamous cells tend to be flatter; in the middle zone, the wing cells tend to be ovoid or ellipti-cal; and in the lower zone, the basal cells tend to be roundish. Therefore, to avoid inappropriate compari-sons of aspect ratio, the epithelium was divided into three zones prior to any analysis, based upon the average area occupied by the three different shapes of cells: (1) Upper zone (the upper 23% of the depth of the epithelium, containing the squamous cells); (2) Middle zone (the middle 43% of the depth, containing the wing cells); and (3) Lower zone (the lower 34% of the depth, containing the basal cells). The same criteria were used for all four of the corneas. The aspect ratios of the cells in each zone were analyzed separately.

Fourth, intercellular distances were measured (from cell boundary to cell boundary) for the epithelium and the endothelium. For the epithelium, the distance be-tween each cell and the three nearest cells was mea-sured. For the endothelium, the distance between each cell and the two nearest cells was measured.

Fifth, intercellular distance measurements (from cell boundary to cell boundary) were made in the stroma for a sample of 60 adjacent cells. The distance between each corneal cell and the four nearest cells was measured.

Sixth, the area of each epithelial cell was measured using NIH Image: Each cell was outlined and the area was calculated. The resulting area measurements (in square pixels) were converted to square microns by comparing distances in pixels to actual measurements between the same pairs of anatomical landmarks made with the microscope and calibrated graticule.

Finally, endothelial cell density was measured.

3.3.4. Postmortem examination

As described above (Section 3.1), in an effort to maintain the physiological state of the experimental animals within the normal range, the following indices were continuously monitored: body temperature, EEG, ECG, inspired and expired O_2 and CO_2, fluid intake,

urine output, urine pH, caloric input, and blood glucose level. In order to help evaluate the effectiveness of these procedures, monkey 61M underwent a thorough postmortem examination by a pathologist (SLT).

4. Results

4.1. Ophthalmoscopic examination

Prior to the introduction of the methodological protocol described above, our protocol included the use of traditional PMMA contact lenses to prevent corneal dehydration. Further, although systemic antibiotics were administered, the regimen for maintaining ophthalmic sterility was less rigorous. Finally, because the experiments were necessarily of shorter duration (as a consequence of early corneal deterioration), the physiological support measures, along with the physiological monitoring techniques, were less sophisticated and less exhaustive (Albrecht and Hamilton, 1982; De Valois et al., 1982; Albrecht et al., 1984; Albrecht and Geisler, 1991; Albrecht, 1995).

Using the older procedures, after ~ 24 h, there were signs of corneal abnormalities that could easily be seen through examination with an ophthalmoscope: Localized areas of cloudiness could be observed, and if no palliative measures were introduced (see Section 2.3), the cloudiness spread, and ultimately, the entire cornea became opaque. Even with palliative measures, the ability to maintain corneal clarity was very limited, and as might be expected from stromal edema (Bruce and Brennan, 1990), significant changes in the dioptric correction were observed from day to day. All of these observations are consistent with what has been reported in the clinical literature in humans, and in the experimental literature in animals, concerning the detrimental effects on the cornea that result from the use of PMMA contact lenses, even in a blinking eye (e.g. Bergmanson and Chu, 1982; Dada et al., 1989; Hideji et al., 1989; Ichijima et al., 1992; MacRae et al., 1994; Ren et al., 1999; Wiffen et al., 2000).

In contrast, using the new protocol (described in Sections 3.1 and 3.2), no signs of corneal deterioration could be observed in preparations that lasted from 8 to 10 days. The central corneas looked normal in all respects in all of the cats and all of the monkeys throughout the entire course of the experiment. Specifically, there were no signs of opacities, ulcerations, injuries, keratinization, infections, or general cloudiness and all of the retinal landmarks, even the smallest observable blood vessels, could be seen in sharp focus at the initial and all subsequent examinations.

Further, under ophthalmoscopic examination of the fundus, no evidence of any abnormality was seen within the eyes of any of the thirteen animals before, during,

or after the recording experiments took place. In every case, at each observation, the quality of the view did not change from that of the first day.

Finally, one sign of central corneal edema is a significant increase in focal power (Bruce and Brennan, 1990) compared to the original correction. Using the new protocol, daily refraction of the eyes revealed no such changes.

### 4.2. Histopathological examination

Bowman's layer, the stroma, Descemet's layer, and the endothelium were similar in the central corneas of the contact lens group and the normal group: There was no evidence of either stromal or endothelial pathology as a result of contact lens wear. However, significant differences were revealed in the epithelia. Specifically, in the contact lens group, the epithelial cells were somewhat flattened, or compressed, in the area of the corneas covered by the contact lenses. Such flattening of the epithelial cells (which is described quantitatively below) has been reported with contact lens wear in normal human patients (Bergmanson et al., 1985; Bruce and Brennan, 1990; Iskeleli et al., 1996). However, such changes in cell shape have not been implicated in reduced quality of vision (Ruiz-Montenegro et al., 1993).

The corneas of the contact lens group were examined using optical coherence tomography (Ducros et al., 1999, 2001) before they were excised. Stromal thickness, epithelial thickness and corneal birefringence were measured and compared to the same measures in normal monkeys (that had never worn contact lenses). There were no discernible differences.

4.3. Histological measurements of thickness

Fig. 4 shows a low magnification ($100 \times$) photomicrograph of one cornea from each of the three groups. The overall thickness of each cornea, as well as the thickness of the corneal epithelium, was measured directly from the histological slides using a calibrated graticule in the microscope. (It should be noted that these results are limited by the fact that corneal thickness measurements were not made before the insertion of the contact lenses.) Table 2 shows the results of these measurements. The average value for corneal thickness was 516 μm (SE = 92) for the normal group and 370 μm (SE = 48) for the contact lens group; the average value across those four corneas was 443 μm. The average difference among these four corneas was 16.5%. The magnitude of this variation was well within what might be expected, given that the variation in the total thickness of the central cornea can be quite large among individuals of the same species (Prince et al., 1960). For example, in humans the variation can be as
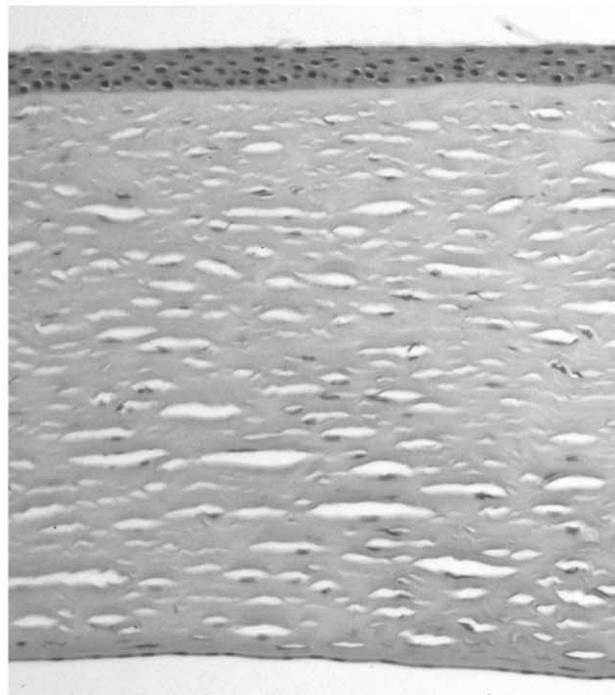
T57 (normal)
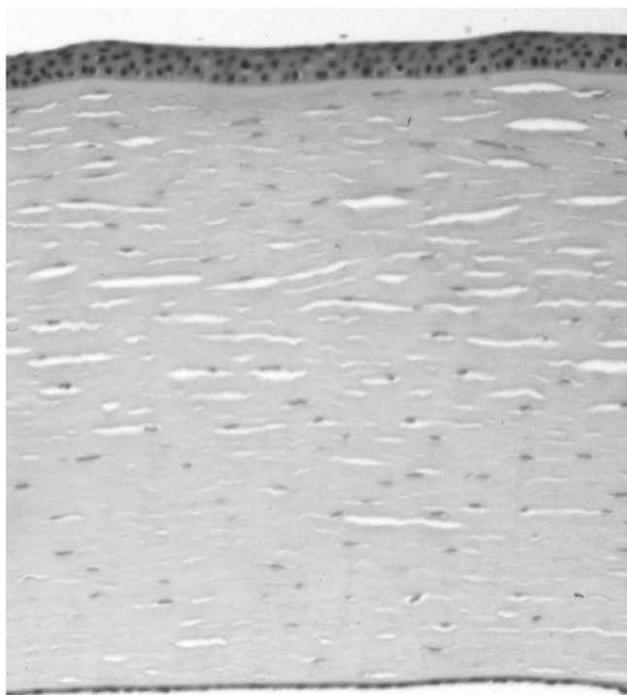
61M (contact lens)

Fig. 4. Photomicrograph of a cross-section (× 100) through a cornea from each group of animals: the normal group, and the contact lens group. These sections (and similar sections) were utilized for quantitative analysis of the cellular morphology (see text). As can be seen, there are no significant differences between the corneas of the normal group and the contact lens group. Scale bar = 100 microns.

large as 25% (Maurice, 1984). Nonetheless, the fact that the corneal thickness for the contact lens group did not exceed the thickness for the normal group supports the ophthalmoscopic observations that the contact lenses did not induce stromal edema.

The thickness of the epithelium is also given in Table 2. As can be seen, there was some variation in the epithelial thickness among the animals and the groups. However, these differences were quite small: the average difference was 10.5%. It is worth noting that there is a great deal of variation in the thickness of the epithelium among individuals of the same species. For example, in humans, the variation can be as large as 100% (Davson, 1972; Thomas, 1955). Thus, it is likely that these small epithelial thickness differences represent normal intraspecies variation.

4.4. Histological measurements of cell morphology

Cell density for the epithelium was estimated by counting all of the cells in a given photographic field and dividing that count by the area represented in the field. The density for the normal group was 1.58 cells/100 μm^2 (SE = 0.11) and the density for the contact lens group was 1.40 cells/100 μm^2 (SE = 0.18). (The samples for these measurements consisted of all the epithelial

cells in representative 188 μm lengths of the epithelium of each central cornea: a total of 128 cells for the normal group, and 127 cells for the contact lens group.) In a study on rhesus monkeys that were awake and blinking, Bergmanson and Chu (1982) observed mild corneal edema and early degenerative cell changes as early as 2 h after insertion of PMMA lenses; after 24 h,

Table 2
Corneal and epithelial thickness

Group	Animal		Corneal thickness (μm)	Epithelial thickness (μm)
Contact lens				
	61M		418	24.0
	62M		322	24.0
		Mean	370	24.0
		SE	48	0
Normal				
	T57		424	22.4
	V49		608	20.8
		Mean	516	21.6
		SE	92	0.77
		Grand mean	443	22.8

Neither corneal thickness nor epithelial thickness was significantly different (at the 0.05 confidence level; two-tailed *t*-test) for the contact lens group and the normal group.

Table 3
Epithelial cell aspect ratio

Epithelial zone		Contact lens	Normal
Upper	Mean	0.30	0.44
	SE	0.09	0.05
Middle	Mean	0.51	0.79
	SE	0.02	0.04
Lower	Mean	0.75	1.19
	SE	0.03	0.04

A two-tailed t-test revealed that all three comparisons were statistically significant at the 0.001 level of confidence.

there was premature epithelial cell loss. The similarity in epithelial cell density found in this study agrees with work by MacRae et al. (1994), who found that the mean epithelial cell density of humans that wore RGP lenses was not different from the density of non-lens wearers.

The area of each epithelial cell was measured for 128 cells in the normal group and 127 cells in the contact lens group (see Section 3). The average cell area for the normal group was 16.06 μm^2 (SE = 0.59) and the average cell area for the contact lens group was 15.07 μm^2 (SE = 0.46). This small difference is not statistically significant. Thus, the cell area measurements show that the average epithelial cell size was the same for both

groups. This result agrees with a recent human clinical study (Tsubota et al., 1994), which showed that epithelial cell area did not change throughout the course of 1 year for patients that wore RGP lenses.

The distance between each epithelial cell and the three closest cells was measured for 128 cells in the normal group and 127 cells in the contact lens group. The average intercellular distance for the contact lens group was 3.42 μm (SE = 0.11) and the average for the normal group was 3.27 μm (SE = 0.09). (The samples for these measurements consisted of all of the epithelial cells in representative 188 μm lengths of epithelium of each central cornea.) The lack of any difference in the intercellular distances is noteworthy because increased distance between epithelial cells indicates epithelial edema (Bergmanson and Chu, 1982; Bergmanson, 1987; Bruce and Brennan, 1990; Ren et al., 1999). Thus, there is no evidence of epithelial edema in the contact lens group.

The height and width of the 128 epithelial cells were measured for the normal group and the 127 cells in the contact lens group. The cell height was divided by the cell width to get the aspect ratio. This was done separately for each of the three zones (which approximately correspond to the zones containing the squamous, wing, and basal cells; see Section 3). The results of these measurements are shown in Table 3 and are illustrated
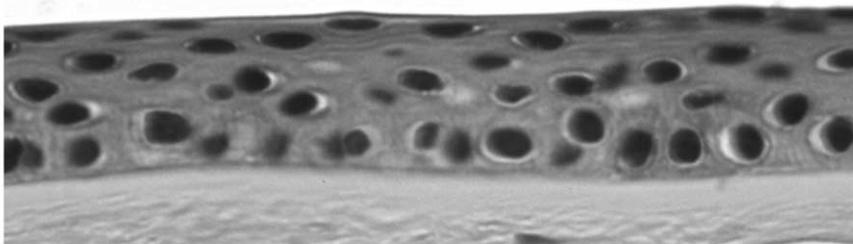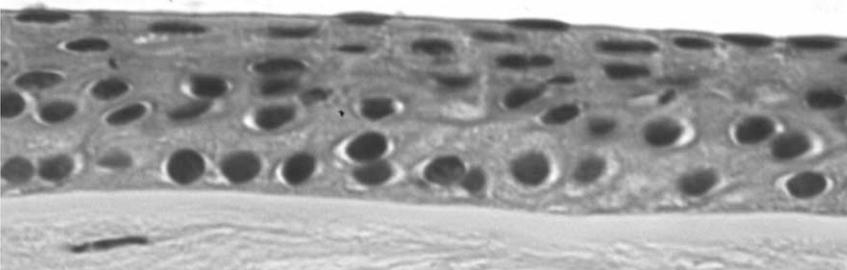

Fig. 5. Photomicrograph of a cross-section (× 400) through the corneal epithelium of a monkey that had worn contact lenses for 8 days and the corneal epithelium of a monkey that had never worn contact lenses. These sections (and similar sections) were utilized for quantitative analysis of cellular morphology (see text). Although the corneal epithelium is similar in both cases, there is significant flattening of the cells in all three epithelial zones for the cornea that had worn contact lenses. This is a natural consequence of extended contact lens wear. Scale bar = 20 microns.

Table 4
Stromal intercellular distances

Group	Animal		Intercellular distance (µm)
Contact lens	61M		6.9
	62M		7.0
		Mean	7.0
		SE	0.05
Normal	T57		6.8
	V49		6.3
		Mean	6.6
		SE	0.25

Stromal intercellular distances were not significantly different (at the 0.05 confidence level; two-tailed t-test) for the contact lens group and the normal group.

in Fig. 5. As can be seen, the aspect ratios of the epithelial cells in the contact lens group were smaller in comparison to the normal group. That is, the epithelial cells in the corneas that had worn contact lenses were flatter in comparison to the epithelial cells in the corneas that had not worn contact lenses. This difference in the aspect ratios was statistically significant for all three epithelial zones (at the 0.05 level). This suggests that some flattening of the corneas took place as a result of lens wear. As mentioned above, such flattening of the cornea with contact lens wear has been reported (Bergmanson et al., 1985; Bruce and Brennan, 1990; Iskeleli et al., 1996), but the causes remain uncertain (Bergmanson et al., 1985), and there is no indication that such changes in epithelial cell morphology in contact lens wearers have any effect on the quality of vision, or visual acuity, in these patients (Ruiz-Montenegro et al., 1993).

In the stroma, intercellular distances were measured. (The samples for these measurements consisted of 60 contiguous stromal cells in representative sections of the stroma of each central cornea.) Specifically, the distance between each corneal cell and the four nearest cells was measured. The results of these measurements are shown in Table 4. As can be seen, there is no significant difference in the mean intercellular distances

for the contact lens group in comparison to the normal group. Thus, it seems reasonable to conclude that stromal edema was not present in the contact lens group.

Finally, in the endothelium, the mean intercellular distance and the mean cell density were measured for the normal group (103 cells) and the contact lens group (101 cells). (The samples for these measurements consisted of all contiguous endothelial cells contained in representative 188 µm lengths of each central cornea, from a total of twelve different histological sections.) The results of these measurements are shown in Table 5. As can be seen, there were no significant differences in these measurements. If damage to the endothelium had occurred as a result of contact lens wear, it would be expected that there would be disturbances to the integrity of the endothelium, cell loss, and/or increased intercellular distances (Dada et al., 1989; Madigan and Holden, 1992; Erickson et al., 1998; Setala et al., 1998). Given the results of these measurements, it seems reasonable to conclude that there was no obvious endothelial damage in the contact lens group.

4.5. Postmortem examination

Gross observation revealed that monkey 61M (see Section 3) had retained a normal proportion of body fat, was properly hydrated, and had no signs of systemic or local infection. Further, gross observation, as well as microscopic examination, of the vital organs revealed that there were no pathological abnormalities.

4.6. Summary

In summary, using the rigorous procedures described in Section 3 for maintaining the physiological environment of the animal, along with rigid gas permeable high Dk/t contact lenses, the central corneas of monkeys utilized for 8–10 days in the paralyzed anesthetized preparation for visual neurophysiology showed no signs of pathology that would compromise the function of the corneas. Overall, the results of this study agree with

Table 5
Endothelial cell density and intercellular distances

Group	Animal		Endothelial cells/100 µm	Intercellular distance (µm)
Contact lens	61M		6.3	47.8
	62M		8.6	27.5
		Mean	7.5	36.7
		SE	1.1	10.2
Normal	T57		6.7	43.0
	V49		8.3	28.6
		Mean	7.5	35.8
		SE	0.8	7.2

Neither endothelial cell density nor endothelial intercellular distance was significantly different (at the 0.05 confidence level; two-tailed t-test) for the contact lens group and the normal group.

clinical work that has shown no difference in corneal clarity between human subjects who wore rigid, gas permeable contact lenses, and human subjects who wore no contact lenses (Carlson et al., 1988). These human clinical studies conclude that vision is stable and that the corneas are free of pathology (Iskeleli et al., 1996) during extended wear of high rigid Dk/t gas permeable contact lenses. Future studies on corneal thickness in the anesthetized and paralyzed preparation might include the use of pachymetric measurements of corneal thickness before and after contact lens wear.

5. Conclusion

In this report a new protocol is described for extending the viability of the cornea and the overall physiology in the anesthetized and paralyzed preparation for visual neurophysiology. The protocol utilizes rigid gas permeable contact lenses designed to optimize gas transmission. The protocol also incorporates rigorous procedures for sterility, along with rigorous procedures for maintenance of the overall physiological environment of the animal. Using two different clinical assessment procedures (ophthalmoscopy and histopathology), along with a quantitative histological analysis of corneal cell morphology, no substantive differences were observed between the corneas of the monkeys that wore no contact lenses and the corneas of the monkeys that wore contact lenses continuously for 8 days. Using the procedures described in this new protocol, corneal clarity can be preserved for extended periods, thus enabling uninterrupted data collection throughout the entire duration of the experimental preparation.

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