Ocular Pigmentation Protects the Rabbit Retina From Gentamicin-Induced Toxicity

Esther Zemel,* Anat Loewenstein,† Bo Lei,* Moshe Lazar,† and Ido Perlman*

Purpose. This study was designed to investigate the possibility that gentamicin-induced retinal toxicity is dependent on ocular pigmentation by comparing the effects of the drug on the functional and morphologic integrity of the retina in albino and pigmented rabbits.

Methods. In each rabbit, a solution of gentamicin sulfate was injected into the vitreous of one eye, and saline was injected into the other eye. Retinal function was assessed by electroretinogram (ERG) at different time intervals after injection. Retinal structure was examined at the light microscopic level.

Results. In albino and pigmented rabbits, functional retinal damage developed to a maximal level within the first week after gentamicin injection. Thereafter, gradual recovery could be seen in eyes that suffered less than 80% maximal reduction in the ERG b-wave. For each dose >0.1 mg studied, retinal damage was more severe in the albino rabbits than in the pigmented ones. The degree of damage was not affected by the level of ambient illumination, nor was it reduced by the administration of N-acetylcystein, a free radical scavenger, together with gentamicin.

Conclusions. Ocular pigmentation partially protects the rabbit retina from the toxic action of gentamicin. This protection probably reflects binding of the drug by the melanin, which thereby reduces the concentration of the free gentamicin. When the initial gentamicin-induced retinal damage is expressed in <80% reduction in the ERG, substantial recovery may occur in both strains of rabbits. Invest Ophthalmol Vis Sci. 1995;36:1875–1884.

Because of their effectiveness against gram-negative and gram-positive bacteria,1 aminoglycosides are the preferred antibiotics for the treatment and prophylaxis of ocular infections. However, the use of these antibiotics has been limited by their well-documented toxicity to the retina.

Numerous studies have been conducted on different animal species to determine the safe, nontoxic dosage of gentamicin for intravitreal injection. A wide range of "safe" dose has been reported that partially could be accounted for by species differences. However, even in the same species, different results were obtained. In one study on albino rabbits, retinal damage was detected when gentamicin in a dose as low as 0.03 mg was injected with the bevel of the needle pointing to the retina, whereas a dose of 0.2 mg was not harmful when the bevel was pointed toward the anterior chamber.2 A second study on the same animal species reported that a dose of 0.08 mg gentamicin produced functional retinal damage as evidenced by electrophysiological recordings.3 In pigmented rabbits, a dose of 0.2 mg of gentamicin was found to be toxic based on electrophysiology and light microscopy.4 In another study, a dose of 0.5 mg was found to cause structural damage,5 but it was reported to be safe in a third study.6 Others found damage only when a dose of at least 0.8 mg was used.7,8 Research conducted in monkeys found 0.4 mg gentamicin to be safe to the retina, as judged by ophthalmoscopy, electrophysiological testing, and light and electron microscopic criteria,9 whereas others found doses of gentamicin from 1.0 to 10.0 mg to be toxic.10–12

The wide diversity of results in these animal studies did not allow accurate determination of a safe dose for intravitreal injection of gentamicin in human patients. Moreover, species differences may cause a certain dose to be safe in one animal but toxic in another.
In fact, many incidences of retinal toxicity in humans after the injection of supposedly safe doses are still being reported.\textsuperscript{18-20} It is now obvious that although most patients can tolerate an intra vitreous dose of 0.1 mg of gentamicin sulfate, there may be specific, yet unidentified, characteristics of the eyes of some patients that make them susceptible to injury at this low dose level.\textsuperscript{21}

The cause of varying thresholds of gentamicin toxicity emerging from animal studies is most probably multifactorial. The facile explanation of species differences will not suffice because wide ranges of allegedly safe doses have been reported in studies of the same animal species.\textsuperscript{3-8} Thus, other variables must be considered. The clinical factors involve the surgical state of the eye,\textsuperscript{17} the presence and severity of infection,\textsuperscript{8} and the position of the needle bevel.\textsuperscript{2} In the animal studies, most of these factors are well controlled so that only those physiological variables that may either augment or reduce gentamicin-induced toxicity need to be considered. The physiological factors include the degree of ocular pigmentation, the level of ambient illumination, room temperature, and stress. Exposure to light,\textsuperscript{18,19} high temperature,\textsuperscript{20} and stress\textsuperscript{31} are known to induce retinal damage both by themselves and by augmenting the effects of other retinopathic agents. They, therefore, also may play a role in gentamicin-induced retinal damage. Melanin in the pigment epithelium may play a dual protective role in drug-induced retinopathy. First, it reduces the exposure of the retina to light by absorbing the photons that bypass the photoreceptors. Therefore, albino animals are rendered more susceptible to light-induced retinal damage than are pigmented animals.\textsuperscript{29} Additionally, melanin is known as a scavenger of free radicals.\textsuperscript{30} If gentamicin-induced toxicity is mediated by free radicals, the melanin of the pigment epithelium may provide some protection.

The aim of this study was to compare quantitatively the development of gentamicin-induced retinal toxicity between albino and pigmented rabbits to determine the role of ocular pigmentation in this pathologic process.

METHODS

Animals

Fifty-two albino and 30 pigmented rabbits weighing 1.5 to 3.2 kg each were used in this study. Each experiment was performed in parallel on a similar number of albino and pigmented rabbits of similar weights to minimize the variability in gentamicin toxicity unrelated to ocular pigmentation.

The animals were housed in separate cages under 12-hour light–12-hour dark cycles in the Animal Facility of the Faculty of Medicine, Technion–Israel Institute of Technology (Haifa, Israel). After the intravitreal injections, most of the animals were returned to the same room on a 12-hour light–12-hour dark cycle. One experimental group of eight albino rabbits was placed in total darkness for 1 week after gentamicin injection to test for a possible interaction between ambient illumination and gentamicin toxicity. All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Procedure

The animals were anesthetized by an intramuscular injection (0.5 ml/kg body weight) of a "cocktail" prepared in the following proportions: 1 ml Ketalar (Parke–Davis, Gwent, UK), 0.2 ml acepromazine maleate, and 0.3 ml xylazine. The pupils were fully dilated with cyclopentolate hydrochloride 1%. Topical anesthesia (benoxinate HCL 0.4%) was administered before carrying out electroretinogram (ERG) recordings and before intravitreal injection. The ERG was used to estimate the functional integrity of the retina. The test was performed at different time intervals after intravitreal injection of the drug. After the last electrophysiological recording session, eyes were enucleated and prepared for histology, and the rabbits were killed by an overdose of sodium pentobarbital.

Drugs

Gentamicin sulphate (Teva, Jerusalem, Israel) at a concentration of 40 mg/ml was used. It was diluted as necessary with physiological saline to produce the desired concentrations. The most concentrated gentamicin solution used in this study (10 mg/ml) had an osmolarity of 255 mOsm and a pH of 4.3. N-acetylcystein (Sigma, St. Louis, MO) was used as an antioxidant to test for possible involvement of free radicals in gentamicin toxicity.\textsuperscript{24,25}

Intravitreal Injection

All intravitreal injections were administered as described\textsuperscript{20} with a 25-gauge needle using a 1.0-ml tuberculin syringe. The needle was introduced 3 mm posterior to the limbus under visual control by means of an indirect ophthalmoscope and was advanced toward the region of the optic disk as close to the retina as possible. In every rabbit, a volume of 0.1 ml of drug solution was injected into the experimental eye while a similar volume of saline was injected into the fellow, control eye. The injection was carried out with the bevel of the needle pointing away from the retina to prevent any damage to the retina.

Electroretinogram

The ERG responses were recorded simultaneously from the experimental and control eyes with conoal
electrodes (Medical Workshop, Groningen, Holland) as described. The reference and ground electrodes were inserted into the ears. The ERG signals were amplified by a factor of 20,000 and filtered (0.3 to 300 Hz) by differential amplifiers (P511; Grass, Quincy, MA). The output of each amplifier was digitized at a rate of 2 kHz (Lab-Master; Scientific Solutions, Encino, CA) and stored in a 386 computer for off-line analysis. Light stimuli were obtained from one of two sources. A xenon photostimulator (PS22; Grass) was used to elicit the ERG responses in the light-adapted state (background illumination of 11 foot lamberts). The intensity of this light source was controlled by the instrument settings (II to I16). The dark-adapted ERG responses were evoked by an electronic camera flash that could be attenuated by a set of neutral-density filters covering a range of 5 log units.

Electroretinographic analysis was based on amplitude measurements of the b-waves. It was measured from the trough of the a-wave to the peak of the b-wave. For each rabbit in each ERG recording session, the b-waves of the experimental eye were divided by the corresponding values recorded from the control eyes. The mean b-wave ratio (experimental eye/control eye) was used as an index for retinal function in the experimental eye.

Histology

The eye was soaked for 10 minutes in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). It was slit 2 mm posterior to the limbus to facilitate fixation. After 72 hours, the anterior segment of the eye was removed by a circumferential incision 2 mm posterior to the limbus. After removal of the vitreous, the posterior eyecup was bisected at the level of the optic disk. One half of each eyecup was rinsed in water and then dehydrated twice in 70% alcohol for 3 hours and twice in 96% alcohol for another 3 hours. Embedding was performed in a JB-4 resin (Bio-Rad, Watford, England). Tissue sections were cut by a Reichert Jung microtome at a thickness of 2 μm and mounted onto slides. For light microscopy, the sections were stained with Richardson's stain.

In a previous study, we showed that small volumes of drugs injected into the vitreous might produce only localized structural effects. Therefore, two loci were always inspected for each rabbit—one close to the site of injection and the other approximately 15 mm more peripherally.

RESULTS

The effects of 1.0 mg gentamicin on representative ERG responses of one albino and one pigmented rab-

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933411/1877/1877Gentamicin-Induced Retinal Toxicity-1877Gentamicin-Induced Retinal Toxicity-1877Gentamicin-Induced Retinal Toxicity-1877Gentamicin-Induced Retinal Toxicity-1877Gentamicin-Induced Retinal Toxicity)
FIGURE 2. Gentamicin-induced structural damage to the albino rabbit retina. The eyes were prepared for histology 2 weeks after injection of 1.0 mg gentamicin. Light micrographs from the experimental retina (C,D) are compared to corresponding areas from the control retina injected with saline solution (A,B). The micrographs were taken from the site of injection (A,C) and from a remote (15 mm from the optic disk) site (B,D). Calibration bar: 50 μm.

For each stimulus intensity, the amplitude of the b-wave recorded from the experimental eye was divided by the corresponding value recorded from the control eye. These ratios were averaged to give the light- and dark-adapted b-wave ratios. For the two rabbits whose ERG responses are shown in Figure 1, the mean (± SD) of the light- and dark-adapted b-wave ratios were 0.16 ± 0.09 and 0.12 ± 0.08 for the albino rabbit and 0.33 ± 0.12 and 0.35 ± 0.15 for the pigmented rabbit. Thus, the effect of 1.0 mg gentamicin was more pronounced in the albino rabbit retina.

The functional loss induced by a dose of 1.0 mg gentamicin was supported by histologic observations as demonstrated for an albino rabbit in Figure 2. The eyes of this rabbit were enucleated 2 weeks after the injection of gentamicin, and both retinas were prepared for light microscopy. Micrographs of the experimental and control retinas, obtained from similar loci relative to the site of injection, were compared. The retina of the control eye injected with saline solution appeared unharmed in both loci; one was adjacent to the site of injection (Fig. 2A) and the other was more peripheral (15 mm from the optic disk) (Fig. 2B). The micrographs that had been obtained from the experimental retina exhibited severe damage at the site of injection (Fig. 2C) and more peripherally (15 mm away from the optic disk) (Fig. 2D). According to these micrographs, gentamicin-induced structural damage seemed to be restricted to the outer retina. It was manifested by an almost complete loss of inner and outer segments of the photoreceptors, whereas the outer nuclear layer and the inner layers of the retina appeared normal. The mean (± SD) light- and dark-adapted b-wave ratios of this rabbit were 0.20 ± 0.05 and 0.10 ± 0.05, respectively.

To estimate the safe dose of gentamicin, specifically the maximal dose that would not produce functional or structural retinal damage, or both, to the albino and pigmented rabbits, solutions containing different concentrations of the drug were injected intravitreally. Their toxic action was assessed from the b-wave ratios of the ERG responses 1 week after injec-
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The data in Figure 3 indicate that the b-wave ratio was higher in the pigmented rabbits than in the albino rabbits for every dose of gentamicin studied. The differences were tested for statistical significance, and a significant \( P < 0.05 \) difference between the b-wave ratios of albino versus pigmented rabbits was obtained for two doses, 0.3 mg and 1.0 mg (denoted by the asterisks above the bars, Fig. 3). The dose of 0.1 mg gentamicin did not cause any apparent ERG deficit in either albino or pigmented rabbits, whereas the damage caused by 0.6 mg gentamicin was more severe in albino than in pigmented rabbits. This difference, however, was not statistically significant. These data indicate that the safe dose of gentamicin is higher for pigmented rabbits than for albino rabbits as determined electroretinographically 1 week after injection.

It was demonstrated that synthetic melanin markedly reduced the activity of aminoglycosides by binding of the antibiotic. This findings are significant: injected gentamicin may remain longer in the pigmented eye than in the albino eye. If so, gentamicin-induced toxicity to the pigmented rabbit retina, even though lower initially, may develop slowly over a longer period of time to reach a level similar or even greater than that seen in the albino eye. To investigate this possibility, we performed eight consecutive ERG recordings at different time intervals after the injection of 0.6 mg gentamicin in four albino and four pigmented rabbits. The first ERG recording was performed 1 hour after injection, and the last was performed 84 days later. The changes in the mean \( \pm SD \) light- and dark-adapted b-wave ratios with time are shown in Figure 4 (open circles, albino rabbits; filled circles, pigmented rabbits). Maximal reduction in retinal function, assessed from the ERG b-wave ratio, was seen 1 to 2 weeks after injection in both groups of rabbits. Thereafter, the b-wave ratios in the pigmented rabbits gradually recovered and almost reached the preinjection values within 84 days. The albino rabbits exhibited little recovery of the dark-adapted b-wave ratios and virtually none of the light-adapted b-wave ratios within the follow-up period (84 days). The mean b-wave ratios representing the pigmented rabbits (filled circles) were significantly \( P < 0.05 \) larger than the corresponding values for the albino rabbits (open circles) for every time interval longer than 7 days after injection (Fig. 4, asterisks).

The data in Figure 4 indicate that the rabbit retina may recover from the deleterious effects of gentamicin even if severe damage initially is seen. To assess poten-
FIGURE 4. The time course of gentamicin (0.6 mg)-induced retinal damage in four albino (open circles) and four pigmented rabbits (filled circles). The mean (± SD) b-wave ratios for light-adapted (upper part) and dark-adapted (lower part) states are compared between the two groups. Significant ($P < 0.05$) differences are denoted by asterisks.

FIGURE 5. The dependency of functional recovery (maximal b-wave ratio) measured 84 days after injection on the initial, maximal damage (minimal b-wave ratio) determined 1 week after injection. Data for 10 albino (open circles) and 10 pigmented rabbits (filled circles) injected intravitreally with different doses of gentamicin are compared. Error bars denote the standard deviation of the b-wave ratio for each rabbit at each electroretinogram (ERG) recording session. The continuous line is the identity line. The dashed vertical line indicates an initial damage of 80% reduction in the ERG responses.
FlCURE6.

Structural retinal damage in two albino rabbits that underwent permanent electroretinogram deficit after the injection of 0.6 mg gentamicin. The retina of one rabbit was prepared for light microscopy at 3 weeks (A,B), and the retina of the other rabbit was prepared at 12 weeks (C,D) after injection. Light micrographs were obtained from the site of injection (A,C) and a more peripheral (15 mm from the optic disk) site (C,D). Calibration bar: 50 μm.

that the melanin in the pigment epithelium provides the rabbit retina with some degree of protection against the toxic effects of gentamicin. Melanin may influence gentamicin-induced retinopathy by at least three mechanisms. It absorbs stray light bypassing the photoreceptors thereby reducing the degree of light exposure; it is a scavenger of free radicals and may act as an antioxidant; and it binds the drug and reduces the concentration of free gentamicin. These alternatives were tested by comparing the effects of 0.6 mg gentamicin on the ERG in 25 albino rabbits that were divided into three groups, as shown in Figure 7. One group was maintained in a 12-hour light–12-hour dark cycle. The second group was kept in total darkness after injection, and the animals in the third group were injected with a mixture of gentamicin and the antioxidant N-acetylcystein. The mean (± SD) light- and dark-adapted b-wave ratios of these groups did not differ statistically. The data shown in Figure 7 suggest that neither the lighting conditions nor the addition of an antioxidant provided any protection to the albino rabbit retina against gentamicin-induced damage. It should be mentioned that in preliminary experiments, we found that neither 1 week of darkness nor N-acetylcystein produced any effect on the b-wave ratios (right eye:left eye) of albino rabbits.

DISCUSSION

These findings support those of previous reports on the toxic action of gentamicin on the rabbit retina. Functional retinal damage, assessed from the ERG responses, could be detected as early as 3 hours after intravitreal injection of the drug. The degree of functional damage slowly developed with time, reaching a maximal level within 1 to 2 weeks of injection. Thereafter, it either stabilized or exhibited slow but progressive recovery. In some of the rabbits, the ERG responses completely recovered to the normal preinjection level within 12 weeks (Fig. 4). There seems to be a clear threshold that separates retinas that can recover from those that suffer from permanent damage (Fig.
were injected with gentamicin alone and were kept in complete darkness. The number of animals in each experimental group is denoted in the figure.

5). Eyes that were characterized by more than 80% reduction in the ERG responses (b-wave ratios < 0.2) exhibited little recovery, whereas those suffering from less initial damage (b-wave ratio > 0.2) exhibited substantial recovery and could attain the preinjection b-wave ratio. The retinas that recovered from the toxic action of gentamicin appeared unharmed at the light microscopic level (data not shown). The observation that the rabbit retina may recover from gentamicin-induced damage raises the possibility that some of the variability in the safe dose of gentamicin, determined in different studies, can be accounted for by differences in the experimental protocols used. Thus, a dose of gentamicin that leads to a 50% ERG reduction when measured 1 week after injection and that is therefore reported as highly toxic may appear safe if the ERG is measured several weeks later.

Morphologic observations indicated that the initial site of gentamicin toxic action was the outer retina. Two weeks after injection, when the physiologic effect of gentamicin had reached its peak, the inner retina and the outer nuclear layer seemed intact, whereas the inner and outer segments of the photoreceptors were missing (Fig. 2). This type of structural damage is reminiscent of the one induced by hemicholinium-3, a drug that is a competitive inhibitor of choline uptake and that interferes with the outer segment renewal process. Gentamicin also may exert its toxic action by interfering with the synthesis of new outer segment disks. Two cellular processes needed for the production of new outer segment disks, protein synthesis and lipid metabolism, may be the site of action of gentamicin. The aminoglycoside antibiotics are known to act on the bacterial ribosomes, leading to the production of faulty proteins and eventually to death of the microorganism. Alternatively, aminoglycoside-induced ototoxicity has been attributed to the interactions of the drug with polyphosphoinositols. Phosphatidylinositol is abundant in the rod outer segment and is needed for synthesis of outer segment disks. Therefore, interaction of gentamicin with this phospholipid may interfere with the outer segment renewal process.

At prolonged periods of time after gentamicin injection, the rabbit retina may exhibit either substantial recovery or permanent functional and structural damage, depending on the degree of initial damage (Fig. 5). When the initial damage is severe (b-wave ratio < 0.2), the synthesis of new outer segment disks fails to restore the outer segments, which leads to permanent damage. In these retinas, structural damage, first seen in the outer segments, spread with time to the entire retina (Fig. 6). In retinas experiencing only mild to moderate initial damage (b-wave ratio > 0.2), the rate of synthesis of new outer segment disks is probably sufficient to restore the integrity of the photoreceptors. In that case, the retina appears structurally and functionally normal at longer time intervals after injection.

We found that the degree of gentamicin-induced functional damage strongly depended on ocular pigmentation. A dose of 0.1 mg did not cause any noticeable functional (Fig. 3) and structural damage (not shown here) either in albino or in pigmented eyes and could, therefore, be considered safe for both. This dose can be converted to a vitreous concentration of 68 µg/ml, assuming a vitreous volume of 1.47 ml for rabbits weighing 1.8 to 2.7 kg and complete mixing of the drug in the vitreous. This concentration is similar to the levels used clinically.

However, for every higher dose tested (0.5, 0.6, and 1.0 mg), the maximal degree of damage, assessed 1 week after injection from the b-wave ratio, was larger in albino than in pigmented rabbits (Fig. 3). Therefore, permanent damage was considerably more apparent in albino than in pigmented rabbits. Approximately 3 months after injection of gentamicin, 7 out of 10 albino rabbits exhibited severe ERG deficit whereas only 3 out of 10 pigmented rabbits exhibited similar degrees of permanent functional damage (Fig. 5). Thus, the safe dose of gentamicin is expected to be higher in pigmented eyes than in albino eyes. Similar differential susceptibility to gentamicin of albino and pigmented animals has been demonstrated in the cochlea of guinea pigs.

The protection that melanin provided the rabbit retina from gentamicin toxicity could arise from the absorption of stray light, from the absorption of free...
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... our data suggested against the first two alternatives. Reducing the level of ambient illumination by keeping the rabbits in darkness or mixing gentamicin with a scavenger of free radicals did not protect the retina of albino rabbits from gentamicin-induced damage (Fig. 7). Therefore, binding of free gentamicin by melanin was probably the mechanism by which ocular pigmentation partially protected the retina from gentamicin toxicity. These observations indicate that gentamicin toxicity to the retina is mediated by the free, unbound drug only and that bound gentamicin does not affect the retina.

Key Words
electroretinography, gentamicin, melanin, rabbit, retina

References


