Efficacy of Suprachoroidal–Transretinal Stimulation in a Rabbit Model of Retinal Degeneration

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PURPOSE. To develop a middle-sized animal model of outer retinal degeneration and to evaluate the effectiveness of suprachoroidal-transretinal stimulation (STS) in eliciting cortical potentials from this model.

METHODS. Twelve rabbits were intravenously injected with 0.47 mg/kg verteporfin and the retinas were irradiated with a red light for 90 minutes. Fluorescein angiography and focal electroretinography (ERG) were performed at 7 and 28 days after the irradiation. Electrically evoked potentials (EEPs) were elicited by electrical stimulation, with the STS electrode implanted over the irradiated region, 1 month and 1 year after the irradiation. EEPs were also recorded from three rabbits before and after retinotomy of the normal retina surrounding the degenerated area, to eliminate the influence of stray currents. The retina beneath the site of the STS electrode was examined histologically at 1 month (group 1) and 1 year (group 2) after the irradiation.

RESULTS. An extensive area of degeneration was detected histologically, mainly in the outer retina after the irradiation. Focal ERGs were not recorded when the stimulus was confined to the irradiated area; however, EEPs were successfully elicited by STS of the same area 1 month and 1 year after the irradiation. The 360° retinectomy did not significantly alter the amplitudes, the implicit times, or the thresholds of EEPs evoked by STS.

CONCLUSIONS. Verteporfin with light irradiation induces degeneration predominantly in the outer retinal layers in rabbits. The elicitation of EEPs by STS from the degenerated area suggests that the STS system may be useful in patients with retinitis pigmentosa. (Invest Ophthalmol Vis Sci. 2010;51:2263–2268) DOI:10.1167/iovs.09-4120

Despite extensive attempts by genetic manipulation and artificial prosthetic devices, a practical solution for the visual decrease in patients with retinitis pigmentosa (RP) has not been obtained. Because some of the inner retinal neurons are somewhat preserved in RP patients,1,2 several research groups are investigating whether an intraocular retinal prosthesis can restore vision in these patients by activating the functioning neurons.3–7

We have developed a new method of stimulating the retina called suprachoroidal–transretinal stimulation (STS),8 and experiments on normal rabbits9,10 and RCS rats11 have shown that electrically evoked potentials (EEPs) can be elicited by stimulating the retina by STS. However, a middle-sized animal model with damage predominantly in the outer retinal layer, as is observed in eyes of RP patients, is needed to evaluate the effectiveness of the STS system more completely. RCS rats, S334ter rats, and P23H rats are established animal models Steinberg RH, et al. JOVS 1996;37:ARVO Abstract 3190)12,13 of degeneration of the outer retinal layers, including the photoreceptors. Unfortunately, a rat eye is relatively small, which makes it difficult to implant an STS system that might be used in humans. A larger size eye model is necessary, because a safe and effective current level has not been determined in eyes of a size comparable to that of humans.

Several dog models of retinal degeneration have been identified,14–16 but investigating a group of dogs is difficult because of the cost and labor. Thus, the purpose of this study was to develop a middle-sized animal model with predominant degeneration of the outer retinal layer which is easily available, not expensive, and easy to handle. We selected the commonly used laboratory rabbit, and induced degeneration of the outer retinal layers including the photoreceptors by photochemical damage with verteporfin. We then evaluated the efficacy of the STS system in this model.

MATERIALS AND METHODS

Animals

Twelve eyes of 12 Dutch-belted rabbits (weighing 2.0–2.3 kg; Biotech, Saga, Japan) were used. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Every effort was made to minimize animal discomfort and to limit the number of animals to that necessary to obtain statistical significance. Nine rabbits were used for developing the retinal degeneration and the functional evaluation of the STS system; five rabbits (group 1) were used for the evaluation at 1 month, and four rabbits (group 2) were used for the evaluation at 1 year. An additional three rabbits (group 3) were used to test the validity of the model.

Light Irradiation with Verteporfin

Rabbits were anesthetized with an intramuscular injection of ketamine (35 mg/kg) and xylazine (8.5 mg/kg), and the pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride. Verteporfin (Visudyne; Novartis, Basel, Switzerland) was injected through an ear vein at a dose of 0.47 mg/kg. This dose was determined from the results of a study of photodynamic therapy (PDT) in monkeys17 and the results of our pilot study with 0.24, 0.47, and 0.96 mg/kg of verteporfin in rabbits. Verteporfin was reconstituted as recommended by the manufacturer.

Light irradiation was applied 5 minutes after the verteporfin infusion. A red light-emitting diode (LED; MCEP-CR8; Moritex, Tokyo, Japan) with peak emission at 630 nm was placed next to the surface of the diffuser contact lens (illuminance was 8.0 × 10^3 lux). The retina was irradiated from three directions—the center, nasal, and temporal...
to the visual streak—with an irradiation duration of 30 minutes in each direction, which resulted in a total irradiation time of 90 minutes.

**Fundus Photography and Fluorescein Angiography**

Fundus photography and FA were performed with a fundus camera (TRC-50DX; Topcon, Tokyo, Japan), with the animals under general anesthesia before and at 1 month (group 1) and 1 year (group 2) after the irradiation with verteporfin. For FA, 0.075 mL/kg of 10% sodium fluorescein was injected intravenously.

**Full-Field ERGs**

Dark-adapted, full-field ERGs were recorded in all group 1 rabbits, 1 month after the light exposure. After 20 minutes of dark adaptation and pupil dilation, the rabbits were anesthetized with an intramuscular injection of ketamine (40 mg/kg) and xylazine (4 mg/kg), and the ERGs were picked up with a corneal Burian-Allen bipolar electrode (Hansen Ophthalmic Development Laboratories, Iowa City, IA). The rabbits were placed in a Ganzfeld bowl and stimulated with stroboscopic stimuli of 1.7 log cd·s/m² (photopic units). Ten responses were averaged with a stimulus interval of 10 seconds. The a-wave amplitude was measured from the baseline to the first negative trough, the b-wave amplitude was determined by decreasing the electric current in steps. The minimum electric current that elicited the first or second positive peak of the EEP (P1 or P2) was defined as the threshold current. The EEP amplitude was measured from the baseline to the first positive trough.

**Focal ERGs**

Focal ERGs were recorded from all group 1 rabbits, 1 week and 1 month after irradiation. The techniques used for eliciting and recording focal ERGs have been described in detail. Briefly, focal ERGs were elicited by placing the stimulus spot on the visual streak. The position of the spot on the fundus was monitored during the recording with a modified infrared fundus camera. The same Burian-Allen bipolar contact lens electrode was used to record the focal ERGs. The luminances of the stimulus and the background were 30.0 and 3.0 cd/m², respectively. A 5- or 30-Hz rectangular stimulus (50% on and 50% off) was used, and a 15° stimulus spot was placed on the visual streak. A total of 512 responses were averaged by a signal processor, and the time constant was 0.05 second with a 300-Hz high-cut filter.

**Electrical Stimulation and Recording of EEPs at the Visual Cortex**

**Cortical Electrodes.** With the animal under deep general anesthesia, the top of the skull was exposed and 1-mm holes were drilled through the skull 8 mm anterior to the lambda suture and 7 mm to the right and left of the midline. Then, screw-type stainless steel recording electrodes coated with silver, were screwed into the skull to make electrical contact with the dura mater. The reference electrode was a platinum wire coated with polyurethane resin, and approximately 3 mm of the tip was exposed. The wire was inserted into the vitreous cavity and was fixed 1 mm posterior to the limbus with 80 Vicryl.

**Eliciting EEPs**

EEPs were recorded 1 month (groups 1 and 3) and 1 year (group 2) after irradiation. The electrical stimulating current was change from 50 to 1000 µA, and biphasic pulses were used for the electrical stimulation. The biphasic pulses consisted of current flowing from the vitreal electrode to the STS electrode in one phase and with current flowing from the STS electrode to the vitreal electrode. The duration of both phases was 0.5 ms. The threshold current for eliciting an EEP was determined by decreasing the electric current in steps. The minimum electric current that elicited the first or second positive peak of the EEP (P1 or P2) was defined as the threshold current. The EEP amplitude was measured from the baseline to the first positive trough.

**Assessing Validity of This Model**

To investigate the influence of stray current beyond the degenerative area where the STS stimulating electrode was placed, we removed 360° of the normal retina surrounding the degenerative area by vitrectomy and retinectomy. EEPs were recorded from the degenerated retina immediately after the retinectomy by stimulating with the STS electrode in three irradiated eyes (group 3; Figs. 3C1, 3C2). Then, EEPs were recorded before and again immediately after retinectomy in those eyes that had only the degenerated retina and optic nerve.

**Histologic Study**

Histologic studies were performed in the areas where the electrode was placed 1 month (group 1) and 1 year (group 2) after, to ensure that the outer retina was degenerated. After the EEPs were recorded, the stimulating electrode was removed from the eye, and the rabbits were euthanatized with a 5-mL intravenous injection of pentobarbital (50 mg/mL). The eyes were enucleated, fixed with 4% paraformaldehyde, dissected, and embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetechnical Co. Ltd., Tokyo, Japan). Cryosections of 7-µm thickness were cut and stained with hematoxylin and eosin. The sections were examined under a light microscope and photographed with a CCD camera (AxioCam; Carl Zeiss Japan, Tokyo, Japan). The images were then analyzed (AxioVision 2.0 software for Windows; Carl Zeiss Japan). The numbers of nuclei in the outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL) were counted at ×40 magnification in all eyes from groups 1 and 2. Three sections from each eye were counted; at the center of the stimulating electrode, and at ≥500 µm away from the electrode. Sections were oriented along the visual streak.

**Statistical Analyses**

The Mann-Whitney test was used to calculate the significance of the differences in the full-field ERGs, EEPs, and cell counts between control and irradiated eyes. Paired t-tests were used to calculate the significance of the differences in the EEPs before and after retinectomy in group 3. P < 0.05 was considered statistically significant (all analyses: SigmaStat, ver.2.0; Systat, San Jose, CA).

**RESULTS**

**Retinal Degeneration Model**

A well-defined chorioretinal atrophy was observed in all eyes by indirect ophthalmoscopy at 1 month after the irradiation. In addition, a hypofluorescent area, that corresponded to the area of the chorioretinal atrophy was seen by FA. The lesion and the hypofluorescent area remained unchanged for 1 year, whereas the area of occluded choriocapillaris increased (Figs. 1A–F).

**Histologic Examination of the Retina beneath the Electrode**

Photoreceptors and nuclei in the ONL were almost completely absent beneath the area where the electrode array was placed. The relative number of cells (experimental eye/control eye)
was reduced to 1.5% \((P = 0.003)\) in the ONL, to 56.8% \((P = 0.006)\) in the INL, and to 84.5% \((P = 0.317)\) in the GCL (Figs. 1G–L; Table 1). At 1 year after irradiation, the cell counts in the INL were reduced to 66% \((P = 0.004)\) of the control, but those in the GCL were not significantly reduced \((P = 0.903)\).

**Full-Field and Focal ERGs**

Representative waveforms of the dark-adapted, full-field ERGs are shown in Figure 2A, and the means ± SDs of the amplitude and implicit times of the a- and b-waves are shown in Figure 2B. We found that the amplitudes of both the a- and b-waves were reduced to about one half of the control ERGs \((P < 0.05)\) at 1 month after irradiation (group 1). There was no significant difference in the implicit times of the a- and b-waves before and after the irradiation \((P = 0.548)\) and \((P = 0.095)\).

We then recorded focal ERGs to examine the retinal function in the irradiated area. We could not record any responses with the stimulus spot placed on the irradiated area from all the eyes. The amplitudes of focal ERGs were less than the noise level \((0.3 \mu V)\) for all rabbits (Fig. 2C), whereas focal ERGs with both 5- and 30-Hz stimuli from all the control eyes were recorded.

**Evaluation of STS**

EEPs were successfully elicited by STS from all eyes in all groups (Figs. 1M–O, 3D, 3E). The mean threshold current evoking the EEP in the irradiated eyes at 1 month after irradiation was \(431.3 \pm 143.8 \mu A\) and that of the control eyes was \(360.0 \pm 114.0 \mu A\). This difference was not significant \((P = 0.262)\). The average current density was 20.5 and 16.4 \(\mu C/cm^2\).
for the irradiated and control eyes, respectively (P = 0.262). The implicit times of the first positive waves of the EEPs in the irradiated eyes and in the control eyes were 13.4 ± 8.7 and 17.1 ± 12.4 ms, respectively (P = 0.662). The mean threshold current of the irradiated eyes 1 year after irradiation was 300.0 ± 141.4 μA (group 2) and was 233.3 ± 115.5 μA in the control eyes. None of these differences was significant (P = 0.400). The averaged electrical density was 13.6 and 10.6 μC/cm² for the irradiated and the control eyes, respectively (P = 0.400). The implicit times of the first positive waves of the EEPs in irradiated eyes and in control eyes were 17.9 ± 4.7 and 13.9 ± 5.9 ms (P = 0.229).

There was no significant difference in the threshold current of the irradiated eyes at 1 month (431.3 ± 143.8 μA) and 1 year (300.0 ± 141.4 μA) after the irradiation (P = 0.413).

**Influence of Stray Current**

The threshold current for evoking the EEPs in group 3 was 400 ± 0 μA before the retinectomy and 400 ± 0 μA after the retinectomy. The means ± SDs of the amplitude and implicit times of EEPs elicited by 1000, 750, 500, and 400 μA are shown in Figures 3F1 and 3F2. There was no significant difference in the amplitude and the implicit times of EEPs before and after retinectomy (P = 0.058–0.716). Thus, removing the nonirradiated retina from the degenerated retina and optic nerve did not reduce the EEPs.

**DISCUSSION**

In a pilot study, we irradiated eyes with stronger light and for longer durations (24 hours) without verteporfin and failed to damage large areas of the outer retinal layer. We, therefore, used verteporfin according to a report on the predominant damage of the outer retinal layer by repeated PDT.17 We also chose a red LED for the light source because the LED light does not generate heat as easily as do other light sources, and 630 nm is the peak excitation wavelength of verteporfin.20,21 We then conducted another pilot study to develop a retinal degeneration model with verteporfin and a red LED. We changed the dose of verteporfin (0.24, 0.47, and 0.96 mg/kg), total irradiation time (45 and 90 minutes), irradiation direction (1 and 3 directions) and distance (0 and 15 mm), and finally succeeded in creating substantial damage to the outer retinal layers with a dose of 0.47 mg/kg verteporfin and irradiation from 3 directions for 30 minutes, each when the red LED was placed just in front of the diffuser contact lens. These conditions induced retinal degeneration in which the outer retinal layers were preferentially damaged and the inner retinal layers were relatively well preserved. In addition, the damage was extensive and uniform. We conclude that our technique of photocchemical damage with verteporfin and a red LED light can produce retinal degeneration resembling the histologic characteristics of eyes of patients with RP.

The amplitudes of the full-field ERGs remained about one half that of the controls (Figs. 2A, 2B) because the degenerated region did not cover the entire retina (Figs. 1A–F). In contrast, focal ERGs were not elicited when the stimulus spot was placed on the degenerated area (Fig. 2C). This result corresponded with the histologic findings that the outer retinal layers were almost completely absent in the irradiated area (Figs. 1G–L). However, the inner retinal layers were somewhat intact in the damaged region, which is known to be characteristic of the end stage of human RP.2 These results demonstrated that the damaged region of this model resembled the

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**Table 1. Cell Counts in the Control and Irradiated Eyes**

<table>
<thead>
<tr>
<th></th>
<th>ONL</th>
<th>INL</th>
<th>GCL</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>175.8 ± 84.9</td>
<td>186.0 ± 31.2</td>
<td>14.8 ± 3.9</td>
</tr>
<tr>
<td>1 Month after irradiation</td>
<td>7.0 ± 10.3</td>
<td>105.6 ± 31.1</td>
<td>12.5 ± 3.8</td>
</tr>
<tr>
<td>P</td>
<td>0.005</td>
<td>0.006</td>
<td>0.317</td>
</tr>
<tr>
<td>1 Year after irradiation</td>
<td>0</td>
<td>69.3 ± 32.4</td>
<td>14.4 ± 3.5</td>
</tr>
<tr>
<td>P</td>
<td>0.004</td>
<td>0.004</td>
<td>0.903</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD. *Mann-Whitney Rank Sum Test with significant differences in bold.

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**Figure 2.** Representative full-field (A) and focal (C) ERGs at 1 month after irradiation. The amplitudes of the a- and b-waves at 1 month were reduced to one half of the control value (B). Data are shown as the means ± SD. *Mann-Whitney rank sum test with significant differences in bold.
histologic and physiological characteristics of eyes of patients with RP.

Despite the absence of focal ERGs when the stimulus was placed on the damaged area (Fig. 2C), EEPs could still be elicited by the STS electrode placed beneath the irradiated area (Figs. 1N, 3B1, 3B2). These results indicate that the STS electrode can stimulate the inner retina in the area that has been damaged by the irradiation to evoke EEPs. One year after irradiation, the ONL had entirely disappeared, and even the INL was significantly decreased but partially remained as shown in the histologic sections (Fig. 1L, Table 1).

Because the EEPs are evoked from ganglion cells and partly from bipolar cells, even though the ONL was completely absent, the EEPs at 1 year after the irradiation were not significantly different from those before the retinectomy. Data are the mean ± SD. †Paired t-tests.

The shapes of the EEPs in our rabbits were different from those of RCD1,22,23 which may be because of the differences in retinal prosthesis, the number of stimulating electrodes, and the current densities.

Earlier studies24–33 reported that an intravenous administration of either moniodoacetic acid (IAA) or sodium iodate (NaIO3) can damage the retina. IAA is well known to be retinotoxic and to damage the photoreceptors selectively,25–27 and the damage of the inner retinal layer is much less severe than that of the outer retinal layers.24,28 However, the effect of this toxin is uneven among individuals and occasionally even between the eyes of the same animal. 28 Liang et al.28 injected IAA intravenously at a dose of 20 mg/kg into 23 rabbits and found a uniformly decreased ONL in only 3 eyes, partial damage of the ONL in 9 eyes, and no change in 11 (48%) eyes. Because a dose of 20 mg/kg IAA is relatively high and results in a high mortality (20%),29 increasing the dose of this drug to damage the ONL more uniformly is not practical. Our method has the advantages that a predominant outer retinal degeneration can be created with almost 100% certainty.

There are many reports30–33 on the NaIO3-induced retinal degeneration. Sorsby31 injected different concentrations of NaIO3 (10–60 mg/kg) into rabbits intravenously and concluded that an incidence of 100% of retinal lesions was attained at a dose of 25 mg/kg. But he did not evaluate the size of the lesions. In another study32 an injection of NaIO3 at a dose of 25
mg/kg into rabbits intravenously caused patchy RPE degeneration and photoreceptor degeneration.

Injection of NaO₃ at a dose of 40 mg/kg which is the most commonly used concentration for functional evaluations of retinal prostheses caused apoptosis in the photoreceptor layer and in the INL at 1 week after the injection and apoptosis in the GCL at 3 weeks. A severe apoptosis of the GCL was noted 4 months after the injection. In another study, an injection of NaO₃ at a dose of 40 mg/kg led to a reduction of 76% in the a-wave and of 67% in the b-wave amplitudes of the neural retina in degenerative retinal models.

In contrast to NaO₃, the retinal degeneration of our model was limited and uniform and large enough for a functional evaluation of a retinal prosthesis. Our model at 1 year (group 2) showed that the cell counts in the GCL were not significantly different (P = 0.903; Table 1). More important, we were able to elicit EEPs by STS electrode at 1 year after the irradiation (Fig. 1O). Thus, our model can be used as a retinal degenerative model for testing retinal prostheses for at least 1 year after irradiation.

A recent study demonstrated that phase tissue remodeling and functional reprogramming of the neural retina may occur in degenerative diseases such as retinitis pigmentosa. However, most studies on developing a degenerative model including our model did not investigate the possibility of neural reprogramming, and more investigations are needed to confirm tissue remodeling and functional reprogramming of the neural retina in degenerative retinal models.

In conclusion, we succeeded in developing a middle-sized animal model of photoreceptor degeneration. Our model will help to determine the optimal stimulus parameter to elicit EEPs in degenerated retinas by STS electrode. In addition, these parameters may be helpful to elicit phosphens from patients with RP. This middle-sized animal model is easy to handle and to be created, and should be helpful to evaluate not only the STS system but also other types of retinal prostheses including subretinal and epiretinal stimulations.

References


