F．健康危険情報

なし

G．研究発表

1．論文発表

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H. 知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし。

3．その他

なし。

I. 参考文献

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厚生科学研究費補助金（感覚器障害研究事業）

分担研究報告書

経角膜電気刺激（TES）の神経保護効果に対する刺激条件

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研究要旨

経角膜電気刺激（TES）の電気刺激の刺激条件によってどのようにRGCの神経保護効果について検討した。検討の結果　電気刺激の刺激条件によってRGCに対する神経保護効果が異なり至適条件が存在することが判明した。この結果は、TES 治療の臨床応用に用いる刺激条件を決定する上で重要であり、また人工視覚システムで用いる刺激条件を決定する上でも重要である。

A. 研究目的

外傷性視神経症、虚血性視神経症などの難治性視神経症に対する神経保護治療として我々は経角膜電気刺激（TES）治療の研究開発を行ってきた。一つの課題としてTESの刺激条件によって神経保護効果がどのように変化するのか検討する必要がある。今回ラットを用いてTES の刺激条件によって神経保護効果がどのように変化するのか検討した。

B. 実験方法

実験動物:成ウイスターラットのオスを使用した。

方法：あらかじめ網膜神経節細胞（RGC）を逆行性に標識するためにフルオゴールドを上丘に注入し、1週間後に左眼の視神経を切断した。直後にコンタクトレンズ型電極を眼球の表面にのせて角膜から網膜、視神経に対してTESを行った。

TES による神経保護効果を検討するためにTESの刺激条件を以下のように変化させて検討した。

1．パルス幅の変化による神経保護効果の検討

刺激波形は両相矩形波でパルス幅 1ms、刺激頻度 20Hz、刺激時間1時間で電流強度を50μA から 500μA まで変化させて検討した。

2．電流強度の変化による神経保護効果の検討

電流強度 100μA、刺激頻度 20Hz、刺激時間1時間としパルス幅を ms から ms まで変化させて検討した。

3．刺激周波数の変化による神経保護効果の検討

電流強度 100μA、パルス幅 1ms、　刺激時間1時間とし刺激頻度1Hz から Hz まで

変化させて検討した。

4．刺激時間による神経保護効果の検討

電流強度100μA、パルス幅　1ms、刺激頻度20Hzとし刺激時間を15分から1時間まで変化させて検討した。

5．刺激は波形による神経保護効果の検討

刺激頻度20Hz、刺激時間1時間とし、電流強度とパルス幅を図1ⅠからⅢのように変化させた。

次に同じく図1Ⅳのようにパルスとパルスの間隔を変化させて検討した。

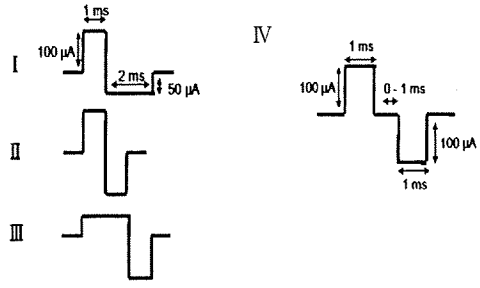


図1刺激波形　Ⅰ-Ⅲ．対称性矩形波と非対称性矩形波。Ⅳインターパルス波形

6．刺激回数による神経保護効果の検討

電流強度100μA、パルス幅　1ms、刺激頻度20Hz、刺激時間1時間として1回のみと4回刺激したものとを視神経切断2週間後に比較検討した。

視神経切断1週間後ないし2週間後に網膜伸展標本を作製して生存RGC密度を求めコントロールとしてSham刺激（コンタクトレンズ電極を装着して電気刺激を行わない）をした網膜の生存RGC密度を用い、TESの刺激条件による神経保護効果を検討した。

C．結果と考察

1．パルス幅の変化による神経保護効果の検討。図2にフルオロゴールドで標識されたRGC像を示す。図2Aの健常網膜に対し、視神経を切断して7日後には図2Bのように多数のRGCが減少したが、TESを行うと図2Cのように数多くのRGCが細胞死を免れた。また図3Aのようにパルス幅の変化によってRGCの生存細胞密度が変化することがわかった。視神経切断7日後にRGCの生存細胞密度は健常網膜の約53％に低下し、Sham刺激群も同様であるのに対し、TESを行うとパルス幅に依存し

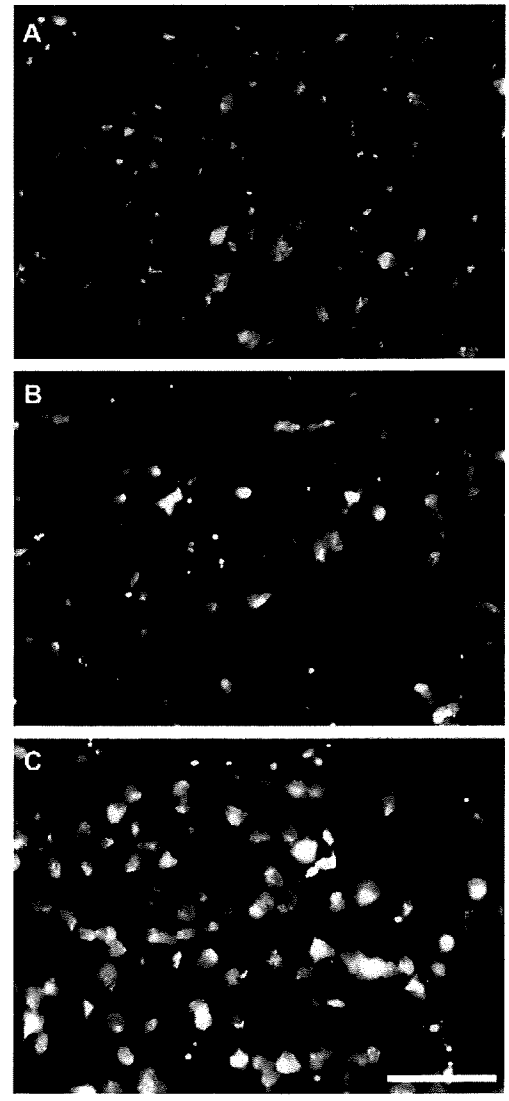


図2　網膜神経節細胞（RGC)像

A.健常網膜、B.視神経切断７日後のRGC像、C.視神経切断＋TESを行った網膜のRGC像。

て生存 RGC 密度は変化し最高で健常網膜の約８７％の RGC が生存していたが、さらにパルス幅を大きくすると RGC の生存が低下し、生存促進効果が得られるのは1から3 ms であることがわかった。

２．電流強度の変化による神経保護効果の検討。次に電流強度によってRGCの生存密度がどのように変化するか検討した。結果図３B のように電流強度に依存して RGC の生存密度が変化することがわかった。100 μA が最も効果的であることがわかった。

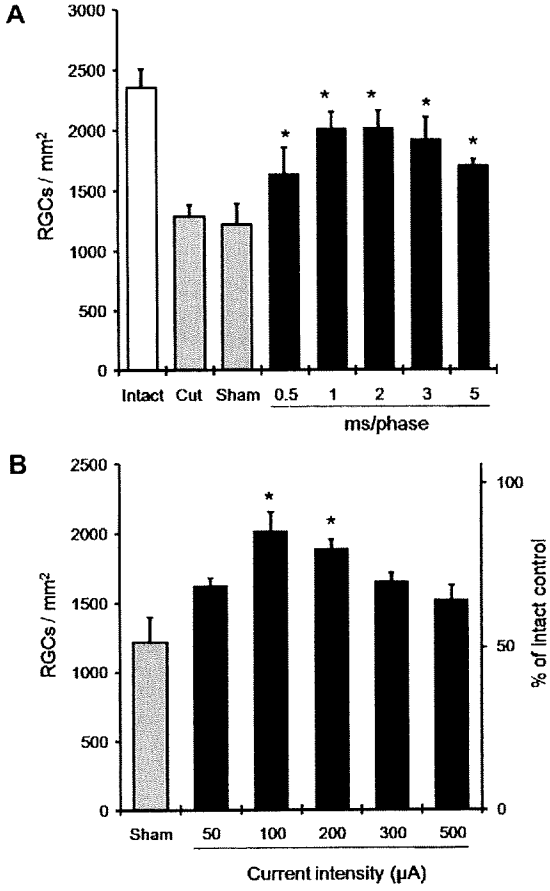


図３　刺激条件によるTESの神経保護効果の変化。A.パルス幅の変化によるTESの神経保護効果の変化。B.電流強度の変化によるRGCの神経保護効果の変化。

３．刺激周波数の変化による神経保護効果の検討。刺激周波数の変化による神経保護効果を検討したところ図４のように刺激周波数の変化によって TES の神経保護効果が変化することがわかった。1－20 Hz が最も効果的であった。

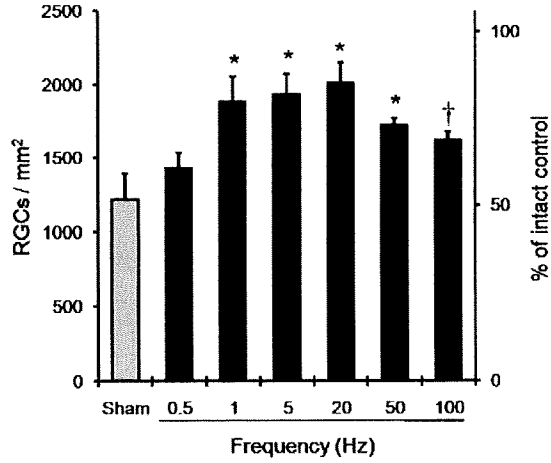


図４　刺激周波数による TES の神経保護効果の変化

４．刺激時間による神経保護効果の検討

TES の刺激時間と RGC の生存密度の変化について検討したところ図５のようにTESが神経保護効果を発揮するためには 30 分以上の時間が必要であることがわかった。

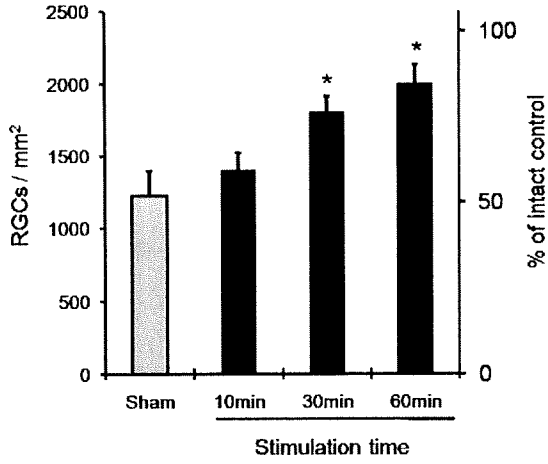
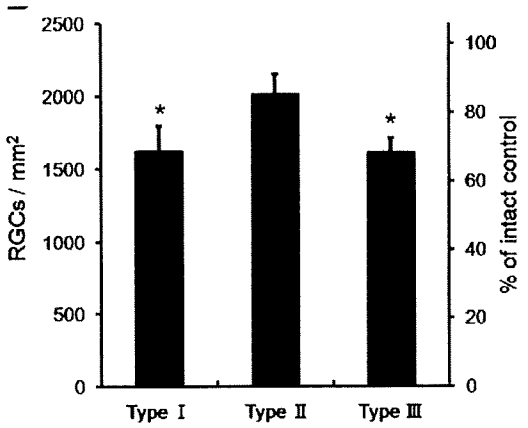


図４ 刺激時間による TES の神経保護効果の変化

5. 刺激は波形による神経保護効果の検討

図１のようにⅠからⅢの矩形波を用いて、電荷量を一定にした時、対称性の矩形波と、非対称性の矩形波で TES の神経保護効果が変化するか検討したところ図５のように対称性矩形波Ⅱを用いるほうが TES の神経保護効果が高いことがわかった。



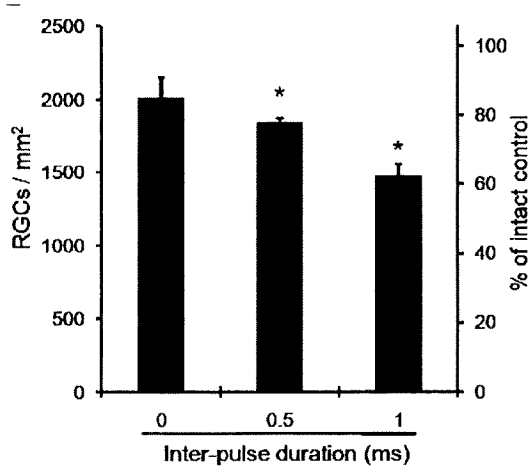


図５ 刺激波形による TES の神経保護効果の変化。A.対称性波形と非対称性波形の変化 B.インターパルスによる TES の神経保護効果の変化

次に対称矩形波で正と負の波の間にインターパルスを入れた場合の TES の神経保護効果の変化について検討したところ図５のBのようにインターパルスが長くなるほど TES の神経保護効果が低下することがわかった。

6. 刺激回数による神経保護効果の検討

TES 刺激を１回するのと４回するのでどのように変化するか検討したところ図６のように４回刺激したほうがより神経保護効果が得られることがわかった。

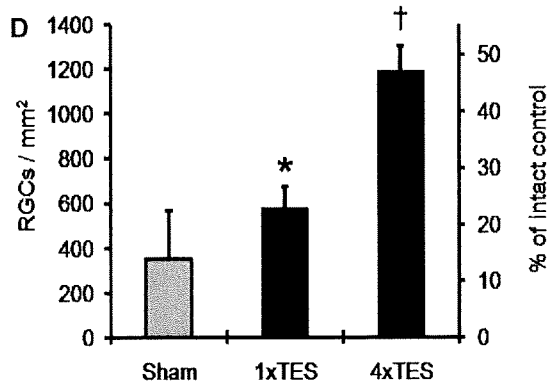


図６．TES の刺激回数と神経保護効果の変化。

以上のように今回、TES の刺激条件（パルス幅、電流強度、刺激周波数、刺激時間、刺激波形、刺激回数）について検討した。それぞれの刺激パラメータ毎に TES の神経保護効果が変化することがわかった。これらの結果は現在、我々が難治性視神経症に対して行っている TES 治療のプロトコ

ールを決定する上で重要である。また人工視覚システムで用いる刺激条件を決定する上で重要である。今後、さらに検討をすすめ TES の神経保護効果のメカニズムについて検討していく予定である。

D.結論

TES の RGC に対する神経保護効果は、刺激条件によって変化し、最適な刺激条件はパルス幅 1 －3ms、電流強度 100－200μA、刺激周波数 1-5 Hz、刺激時間 30 分以上、刺激波形　対称性両相矩形波、インターパルスなし、刺激回数 4 回であった。

E.健康危険情報

なし。

F.研究発表

1．論文発表

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2．森本　壮、下條裕史、北口善之、松下賢二、不二門尚　虚血性視神経症に対する経角膜電気刺激治療　第 227 回 OCC 2009 年 9 月　大阪

3．森本　壮、下條裕史、北口善之、松下賢二、不二門尚　非動脈炎性前部虚血性視神経症に対する経角膜電気刺激治療の治療効果の検討　第 47 回日本神経眼科学会総会、第 5 回アジア神経眼科学会総会　2009 年 11 月　東京

G.知的財産権の出願状況

なし。

厚生科学研究費補助金（感覚器障害研究事業）
分担研究報告書

高効率刺激電極の開発

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研究要旨
高精細な網膜刺激型人工視覚システム実現を目的として，IrOx などの高効率電極の試作および分散型刺激電極方式の同時多点刺激への適用について研究を行った．成膜条件を最適化した IrOx 電極を搭載した刺激デバイスを家兎眼内に埋植し網膜刺激を行い，Pt 電極との比較を行った．同時多点刺激電極デバイスは包埋方式を開発し，家兎眼内埋植を行い網膜刺激によりその有効性の確認を行った．

A. 研究目的

本研究の目的は，脈絡膜上経網膜刺激（STS: Suprachoroidal Transretinal Stimulation）や網膜下刺激に適した高密度実装を目指した分散型電極アレイの実装方式の開発である．
今年度は，高密度刺激を実現するために必要となる同時多点刺激機構の導入と電極自体の高効率化の検討を目的とした．

B. 実験方法

本研究では，これまで提案と基本実証を行った北分散型刺激デバイスの高密度刺激を目指して同時多点刺激型分散型電極アレイの設計と試作を行った．そして，ウサギ in vivo 電気刺激実験において試作したデバイスの機能実証を行った．また高効率電極材料として IrOx を取り上げ成膜条件を最適化し，ウサギ in vivo 電気刺激実験によりその有効性を検証した．

[同時多点刺激型分散型デバイス設計と試作]
本研究室において提案された分散型アーキテクチャは，フレキシブル基板上に小型の CMOS-LSI チップ（マイクロチップ）を複数配置してそれらをネットワーク接続することで少数配線での制御や刺激の高密度化およびフレキシビリティの向上を実現するものである．高密度刺激を行う場合には同時多点刺激が必要になる． 1 点で順次刺激する場合，刺激点数を多いと最初と最後の刺激点での刺激時間が大幅に変わり，その違いが知覚される懸念がある．
同時多点刺激を実現するためには，マイクロチップ内に電流源を内蔵する必要がある．昨年度このような電流源内蔵型マイクロチップを試作した．図 1は試作したマイクロチップとそのマイクロチップを 4個実装した刺激デバイスの写真である．マイクロチップの大きさは 200μm 角で，刺激電極パッドを 1個とした．このマイクロチップを分散配置した刺激デバイスを試作した．1 個のマイクロチップ上には 1 個の Pt バルク電極が搭載されている．図 1 は試作したデバイスの写真である．兎眼球の曲率にフィットできる柔軟性を有している．

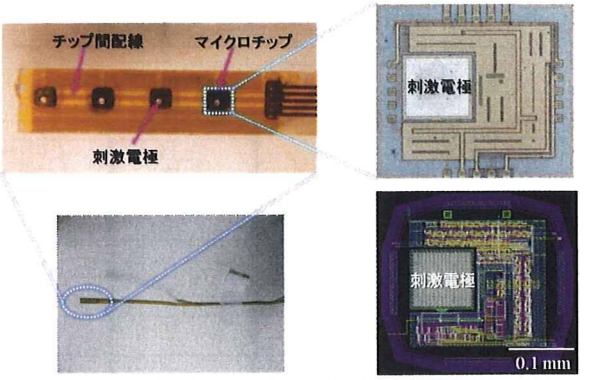

図 1：試作した分散型同時多点刺激
人工視覚デバイス用マイクロチップとその実装デバイスの写真

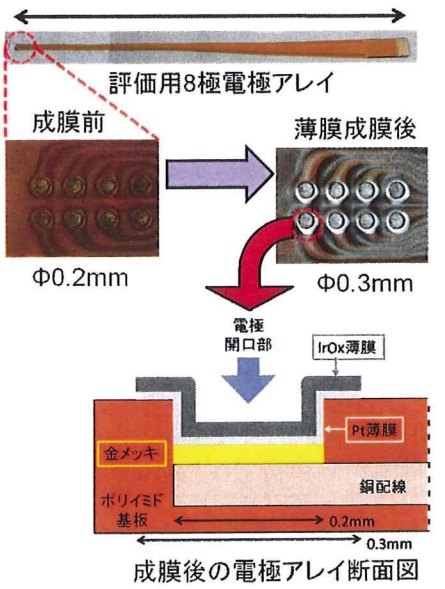

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図 2：電極材料評価用刺激電極アレイ

[IrOx 電極の作製]
人工視覚デバイス用の刺激電極としてより高性能な刺激電極の作製を目指し，一般的に用いられる電極材料である Pt に加え、IrOx, TiN をスパッタリングにより評価用電極アレイ上に直径300μmの薄膜として作製し各成膜条件別に電極性能の評価を行った．図 2 に示すようにフレキシブル基板に 2×4 個の電極を配置しており，異なる電極材料による刺激を比較できる構造としている．

表 1:Pt 成膜条件

ガス [sccm]	Ar	20.0
	O2	0
スパッタ圧力[Pa]		0.35
電力[W]	RF	200
スパッタ時間[分]		38
膜厚[nm]		1000

表 2:IrOx 成膜条件

膜タイプ		A,B	C
ガス [sccm]	Ar	8.0	0
	O2	3.0	10
スパッタ圧力[Pa]		1.0	1.0
電力[W]	RF	200	200
スパッタ時間[分]		35	60
膜厚[nm]		500~1000	1000

表 1 と 2 に Pt および IrOx のスパッタ成膜条件を示す．

C. 結果と考察
[同時多点刺激型分散型デバイス]
同時多点刺激機能を搭載した新世代の人工視覚デバイス(AR39)の試作を行った．図 3 に試作デバイスのチップ写真を示す．マイクロチップはサイズを200 μm 角に縮小し，電極数は 1 極という構成で，VDD，GND の電源入力，電極選択のためのデジタル入力である CONT1・CONT2，外部からの刺激電流を入力する STIM の計 5 本の制御配線によって動作する．この点については昨年報告した内容とほぼ同一である．相違点は実装方法を開発し，さらに信頼性の高い方式とした点である．チップ内搭載した制御回路，刺激電流生成回路により内部供給の刺激電流を 50 μA~1000 μA まで 50 μA ステップで指定可能である．昨年度はチップ単体での機能実証を行った．今年度は実際に埋込可能な刺激デバイスとしての実装を行い家兎を用いた埋植，刺激実験を行った．図1に実装したデバイスを示す．各1点の Pt バンプ電極を形成したマイクロチップを 4 個実装している．

[動物実験による機能評価]
大阪大学医学部の協力のもと，試作したデバイスをウサギ眼球に埋植し，網膜の同時多点電気刺激実験を行った．視覚再生に有効な刺激が行われているか確認するため，頭部に挿入した電極により脳波の測定を行い，網膜を電気刺激したことによって脳内に誘発される電位(EEP; Electrical Evoked Potential)の検出に成功した．図 3 は 4 つの波形は各々のマイクロチップにより刺激した結果である． 刺激条件は，刺激電流 1mA 設定で単極パルス(Anodic)，パルス幅 1ms である．これによりチップに搭載した刺激電流生成回路での網膜刺激に成功したことを確認した．なお刺激電流の設定値は 1mA であるが、実測で 200μA 程度となっていた．これは刺激電圧が不足しているためであり，チップの高電圧対応が今後の課題である．

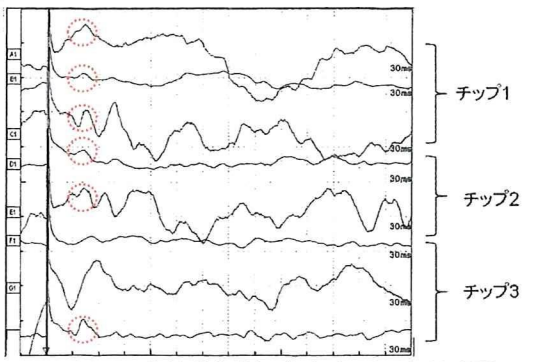

図 3:兎眼球埋植デバイスによる EEP
（1 点刺激結果）

次にこのデバイスを用いて同時多点刺激を行った．図 4 はその結果である．刺激条件は，刺激電流 1mA 設定，単極パルス(Anodic)，パルス幅 1ms で，4 チップ中の 1~3 チップを選択し同時刺激を行った．複数チップの同時動作，多点刺激に成功した．また単一チップでは弱い EEP しか得られないが，複数チップを同時動作させる事で実効刺激電流が大きくなったため，強い EEP を観測することができたと推測される．今後は同時多点刺激によるタイミング等の検討を進めていく必要がある．

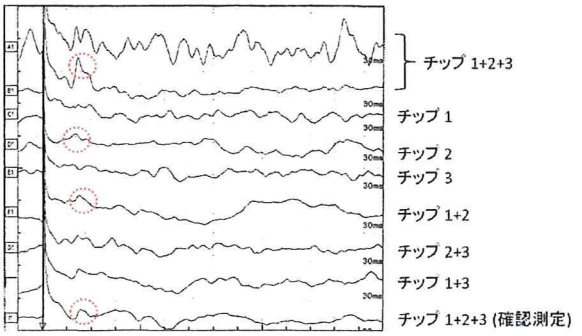

図 4：兎眼球埋植デバイスによる EEP
（同時多点刺激結果）

[IrOx 電極]

Pt, IrOx を成膜した電極の拡大写真を図 5 に示す. 電極直径は 0.3mm, 電極ピッチは 0.4mm である. IrOx は成膜条件により電極色が異なる.

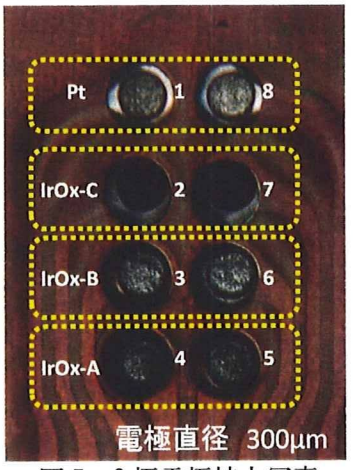

図 5：8 極電極拡大写真

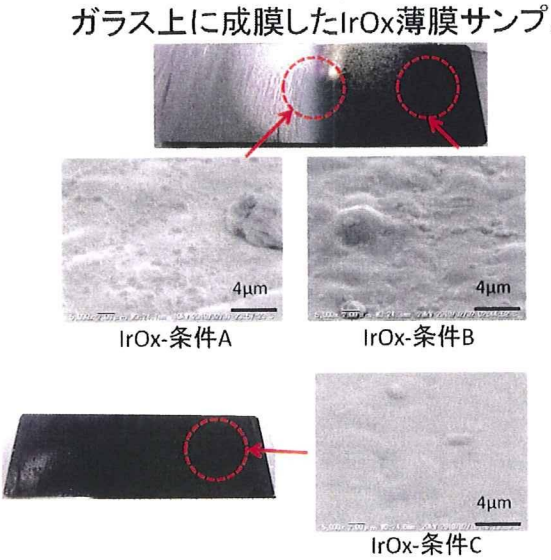

図 6：ガラス上に成膜した
IrOx 薄膜の表面写真

図 6 はガラス基板上に成膜した IrOx 薄膜の表面写真である. 成膜条件は表 2 に示したものである. 成膜条件により表面粗さが異なっている. 図 7 はサイクリックボルタンメトリ(CV)測定結果である.

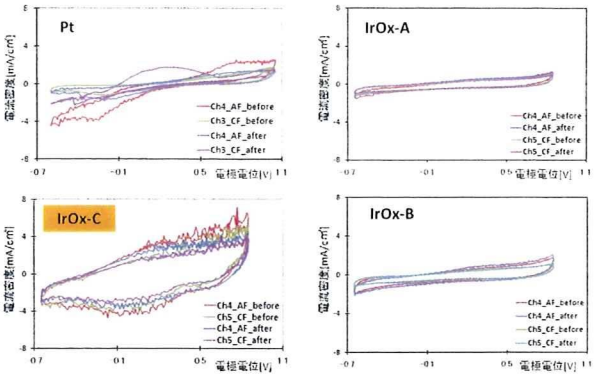

図 7：Pt と IrOx CV 測定結果

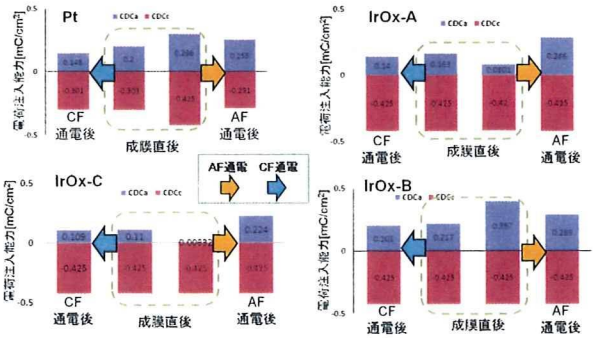

図 8：Pt と IrOx 電荷注入効率測定結果

CV 測定結果より IrOx における条件 C が最も移動電荷量が大きいことが分かった. 図 8 は電荷注入能力の評価結果である. この結果からも IrOx 条件 C が最も電荷注入効率が高いことが分かる.

Pt と IrOx 電極材料については大阪大学医学部の協力の下, 作製した電極アレイ(図 5;IrOx は条件 A, B, C の 3 種類)を家兎の眼球に埋殖し網膜電気刺激実験を行った. 刺激条件は, 1mA 定電流 双極性 Cathodic-first パルス幅 1ms パルス間隔 0.5ms である.

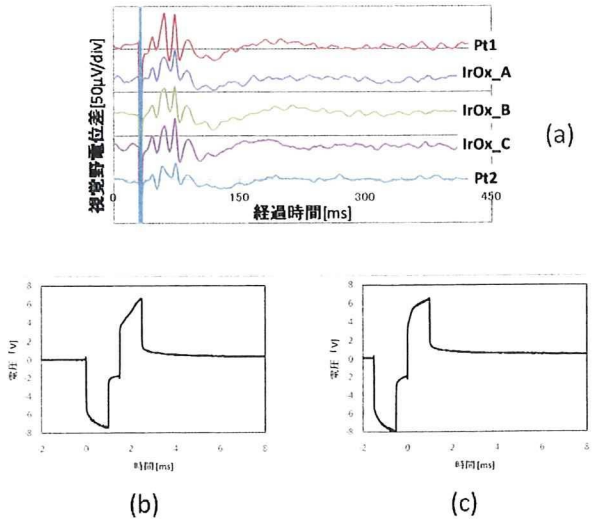

図 9：Pt と IrOx CV 測定結果(a)および電圧波形
(Pt(b), IrOx 条件 C (c))

有効な刺激が行われているか確認するため EEP を観測した. 図 9 に各電極材料からの刺激に対する EEP 波形の結果を示す. 各電極材料のいずれ

においても図 9 に示す EEP の検出に成功した．但し，刺激時の電極電位は電極材料による大きな差異を確認できなかった(図 9(b), (c))．今後詳細に検討する必要がある．

図 10：埋植通電後の IrOx 電極表面

埋植通電後取り出した電極表面を観察すると，図 10 に示すように一部剥離しているものがあった．下地膜や密着層の検討が必要である．

D. 結論

LSI による分散型人工視覚デバイスの高分解能化を目指して，同時多点機能と IrOx による高効率電極材料の検討を行った．同時多点機能搭載マイクロチップを試作し，分散型方式電極デバイスとして実装を行った．試作デバイスを家兎眼に埋植し，EEP を得ることに成功した．IrOx 電極材料について成膜条件を検討し，CV 及び電荷注入効率を評価した．成膜した IrOx 電極を用いて家兎網膜刺激を行い EEP を得ることに成功したが，Pt との顕著な違いは観測されなかった．また膜の剥離があった．今後成膜条件の最適化等を進める必要がある．

E. 健康危険情報

なし．

F. 研究発表

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2．総説・著書

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G. 知的財産権の出願・登録状況

1．特許出願

なし

研究成果の刊行に関する一覧表

1．Nishida K, Kamei M, Kondo M, Sakaguchi H, Suzuki M, Fujikado T, Tano Y. Efficacy of suprachoroidal-transretinal stimulation in a rabbit model of retinal degeneration. Invest Ophthalmol Vis Sci. 2010 51:2263-8.

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

Kentaro Nishida,[1] *Motohiro Kamei,*[1] *Mineo Kondo,*[2] *Hirokazu Sakaguchi,*[1] *Mihoko Suzuki,*[1] *Takashi Fujikado,*[1] *and Yasuo Tano*[1]

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 full-field 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 rabbits[9,10] and RCS rats[11] 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. *IOVS* 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 (33 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 monkeys[17] 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 8.0×10^4 lux). The retina was irradiated from three directions—the center, nasal, and temporal

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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-50IX; 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/m^2 (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 from the negative trough to the positive peak.

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.[18,19] 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/m^2, 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 0.03 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 lambdoid 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 then screwed into the skull at the bregma.

Stimulating Electrode. A single stimulating electrode was used. The wire (90% platinum, 10% iridium; diameter, 60 μm) was insulated with silicon and embedded in a 2-mm horizontal × 5.5-mm vertical × 0.1-mm-thick parylene plate. The tip of the wire was connected to a 500-μm-diameter single stimulating platinum electrode (see Fig. 3A). The inferior surface of the sclera was exposed by cutting the inferior rectus and the inferior oblique muscles. A scleral pocket (3 × 5 mm) was created just over the irradiated area on the visual streak. The electrode plate was then implanted into the scleral pocket and sutured with 5-0 Dacron onto the sclera just above the pocket. The insulated strand lead from the electrode was sutured at the limbus with 5-0 Dacron. The implanted electrode was confirmed to be located just under the damaged area of the visual streak by binocular ophthalmoscopy.

An electronic stimulator (SEN-7203; Nihon Kohden, Shinjyuku, Japan) was connected through a stimulus isolation unit (A-395R; World Precision Instruments, Sarasota, FL) to the STS electrode. 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 8-0 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 degenerated 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 euthanized 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)

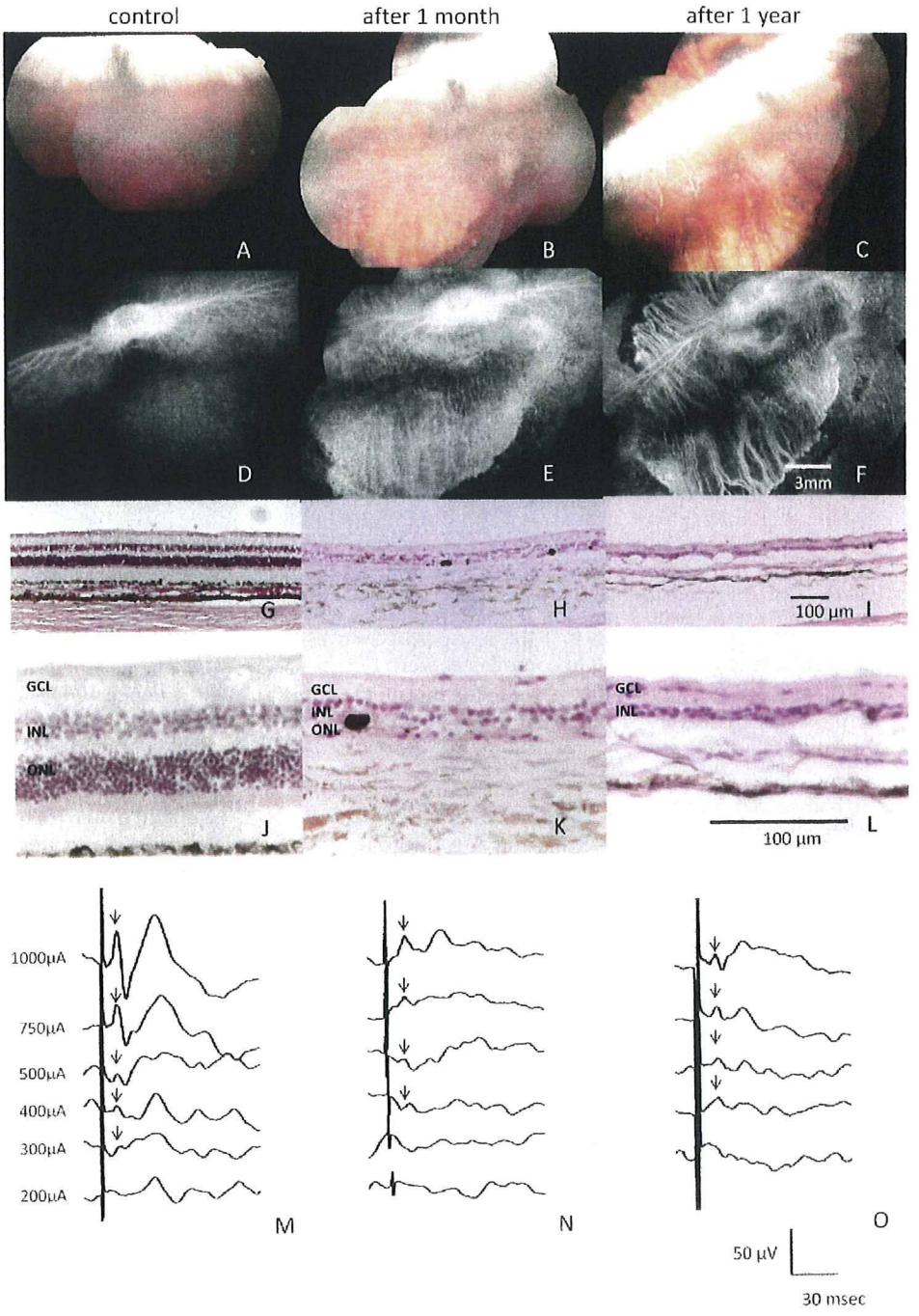


FIGURE 1. Representative fundus photographs (**A–C**), fluorescein angiograms (**D–F**), photomicrographs of the area where the electrode was placed (**G–L**), and EEPs (**M–O**) of the control and irradiated eyes at 1 month and 1 year after the irradiation with a red LED after intravenous verteporfin. (**B**, **C**, **E**, **F**) Atrophy of RPE and choriocapillaris can be seen. The occlusion of the choriocapillaris continued to be present at 1 year (**F**). Histopathology of the control eye (**G**, **J**) showed that the ONL and outer layers were fibrotic (**H**, **I**, **K**, **L**). At 1 year, the atrophy of the choroid had progressed, and the number of cells in the INL had decreased but the GCL was preserved (**I**, **L**). Scale, 100 μm. The EEPs (*arrows*) recorded after biphasic electrical pulses from the STS electrodes implanted in the control eye and degenerated eyes at 1 month and 1 year after irradiation.

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 μV) 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 ± 143.8 μA and that of the control eyes was 360.0 ± 114.0 μA. This difference was not significant ($P = 0.262$). The average current density was 20.5 and 16.4 μC/cm^2

TABLE 1. Cell Counts in the Control and Irradiated Eyes

	ONL	INL	GCL
Control	475.8 ± 84.9	186.0 ± 31.2	14.8 ± 3.9
1 Month after irradiation	7.0 ± 10.3	105.6 ± 31.1	12.5 ± 3.8
*P**	**0.003**	**0.006**	0.317
1 Year after irradiation	0	69.3 ± 32.4	14.4 ± 3.3
*P**	**0.004**	**0.004**	0.903

Data are expressed as the mean ± SD.

* Mann-Whitney Rank Sum Test with significant differences in bold.

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 $\mu C/cm^2$ 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

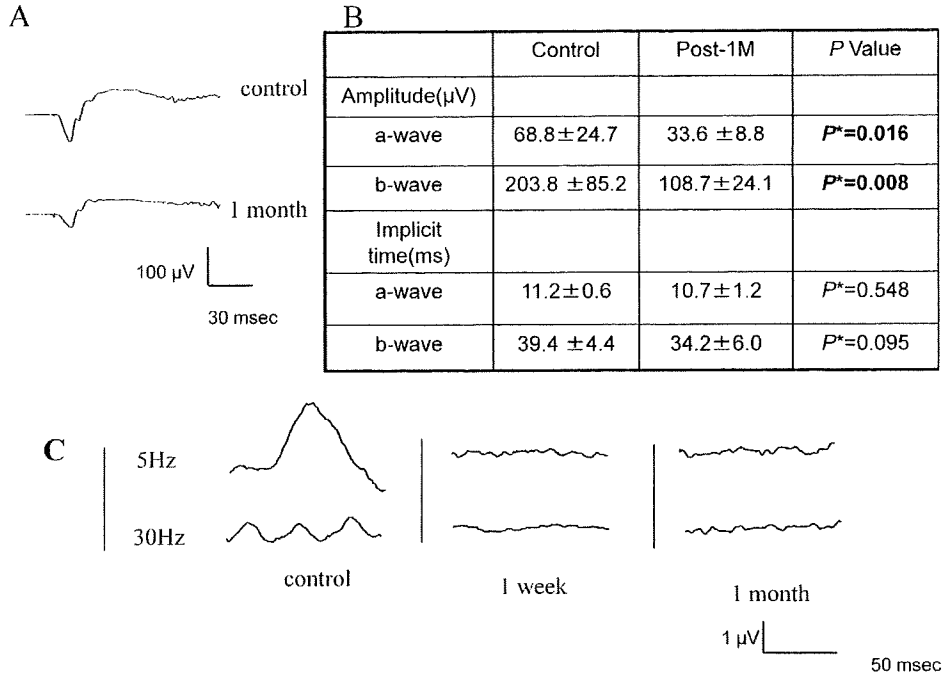
The threshold current for evoking the EEPs in group 3 was 400 ± 0 μA before the retinectomy and 400 ± 0 μA after the retinectomy. The means ± SDs of the amplitude and implicit times of EEPs elicited by 1000, 750, 500, and 400 μA are shown in Figures 3F1 and 3F2. There was no significant difference in 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]

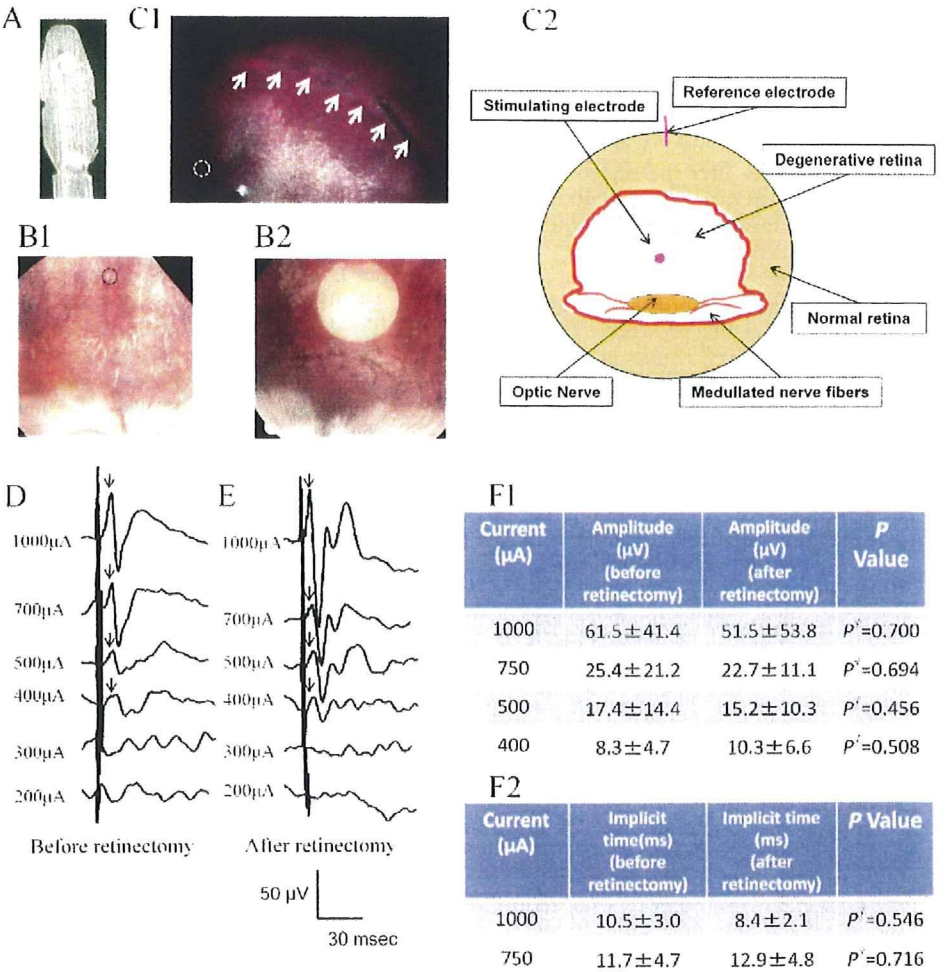
We then conducted another pilot study to develop a retinal degeneration model with verteporfin and a red LED. We changed the dose of verteporfin (0.24, 0.47, and 0.96 mg/kg), total irradiation time (45 and 90 minutes), irradiation direction (1 and 3 directions) and distance (0 and 15 mm), and finally 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 photochemical 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



	Control	Post-1M	*P* Value
Amplitude(μV)			
a-wave	68.8±24.7	33.6 ±8.8	***P**=0.016*
b-wave	203.8 ±85.2	108.7±24.1	***P**=0.008*
Implicit time(ms)			
a-wave	11.2±0.6	10.7±1.2	*P**=0.548
b-wave	39.4 ±4.4	34.2±6.0	*P**=0.095

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.*



F1

Current (μA)	Amplitude (μV) (before retinectomy)	Amplitude (μV) (after retinectomy)	*P* Value
1000	61.5±41.4	51.5±53.8	$P^{\dagger}$=0.700
750	25.4±21.2	22.7±11.1	$P^{\dagger}$=0.694
500	17.4±14.4	15.2±10.3	$P^{\dagger}$=0.456
400	8.3±4.7	10.3±6.6	$P^{\dagger}$=0.508

F2

Current (μA)	Implicit time(ms) (before retinectomy)	Implicit time (ms) (after retinectomy)	*P* Value
1000	10.5±3.0	8.4±2.1	$P^{\dagger}$=0.546
750	11.7±4.7	12.9±4.8	$P^{\dagger}$=0.716
500	12.2±5.1	11.0±5.8	$P^{\dagger}$=0.339
400	14.0±5.8	11.4±6.0	$P^{\dagger}$=0.058

FIGURE 3. Inserted STS electrode and isolated degenerated retina and representative EEPs. (**A**) STS electrode (diameter 500 μm) inserted in the degenerated area (**B1**, *dotted black outline*) was apparently smaller than the photic stimuli for focal ERGs (**B2**). Retinectomy was performed (**C1**, *white arrows*) along the border of the degenerative retina and normal retina (**C2**, *red line*); inserted STS electrode (**C1**, *dotted white outline*) and representative EEPs (*black arrows*) before (**D**) and after retinectomy (**E**). The amplitudes (**F1**) and the implicit times (**F2**) of the EEPs after retinectomy were not significantly different from those before the retinectomy. Data are the mean ± SD. †Paired *t*-tests.

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 recorded 1 month after the irradiation.

However, the EEPs may have been evoked by stray currents that stimulated functioning neurons some distance from the irradiated area. To eliminate this possibility, we removed the normal retina surrounding the degenerated area by retinectomy. Our findings showed that EEPs of the same amplitude and thresholds could still be elicited. Thus, we conclude that the EEPs were not elicited by stray currents (Figs. 3C–F). In addition, these findings demonstrate that the degenerated area with our parameters of photocoagulation was large enough to evaluate the effect of the STS system.

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 studies[24–33] reported that an intravenous administration of either monoiodoacetic acid (IAA) or sodium iodate ($NaIO_3$) 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 reports[30–33] on the $NaIO_3$-induced retinal degeneration. Sorsby[31] injected different concentrations of $NaIO_3$ (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 study[32] an injection of $NaIO_3$ at a dose of 25

mg/kg into rabbits intravenously caused patchy RPE degeneration and photoreceptor degeneration.

Injection of $NaIO_3$ at a dose of 40 mg/kg which is the most commonly used concentration for functional evaluations of retinal prostheses[24,33] 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.[33] In another study,[24] an injection of $NaIO_3$ 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 control subjects.

In contrast to $NaIO_3$, 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[34] demonstrated that phased 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 phosphenes 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.

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Optimal parameters of transcorneal electrical stimulation (TES) to be neuroprotective of axotomized RGCs in adult rats

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ABSTRACT

We previously showed that transcorneal electrical stimulation (TES) promoted the survival of axotomized retinal ganglion cells (RGCs) of rats. However the relationship between the parameters of TES and the neuroprotective effect of TES on axotomized RGCs was unclear. In the present study, we determined whether the neuroprotective effect of TES is affected by the parameters of TES. Adult male Wistar rats received TES just after transection of the left optic nerve (ON). The pulse duration, current intensity, frequency, waveform, and numbers of sessions of the TES were changed systematically. The alterations of the retina were examined histologically seven days or fourteen days after the ON transection. The optimal neuroprotective parameters were pulse duration of 1 and 2 ms/phase ($P < 0.001$, each), current intensity of 100 and 200 μA ($P < 0.05$, each), and stimulation frequency of 1, 5, and 20 Hz ($P < 0.001$, respectively). More than 30 min of TES was necessary to have a neuroprotective effect ($P < 0.001$). Symmetric pulses without an inter-pulse interval were most effective ($P < 0.001$). Repeated TES was more neuroprotective than a single TES at 14 days after ON transection ($P < 0.001$). Our results indicate that there is a range of optimal neuroprotective parameters of TES for axotomized RGCs of rats. These values will provide a guideline for the use of TES in patients with different retinal and optic nerve diseases.

1. Introduction

Neuronal activity has a neurotrophic effect on neurons (Linden, 1994; Mennerick and Zorumski, 2000). *In vivo* and *in vitro* studies have shown that electrical stimulation (ES) or neuronal activity promotes the survival and/or neurite outgrowth of different types of neurons (Fields et al., 1990; Grumbacher-Reinert and Nicholls, 1992; Al-Majed et al., 2000a,b).

In the visual system, it has been shown *in vitro* study that ES or depolarization by KCl promotes the survival and/or neurite outgrowth of cultured retinal ganglion cells (RGCs) (Meyer-Franke et al., 1998; Shen et al., 1999; Goldberg et al., 2002). Our laboratory has shown that direct ES of a transected optic nerve (ON) in adult rats promoted the survival of the axotomized RGCs in adult rats (Morimoto et al., 2002). We also showed that transcorneal electrical stimulation (TES), which is less invasive than ES of the transected ON (ON-ES), also promoted the survival of axotomized RGCs in adult rats (Morimoto et al., 2005), and In addition, promoted the survival of photoreceptors in Royal College Surgeons rats (Morimoto et al., 2007). Miyake et al. (2007) have reported that TES immediately after crushing the ON lessened the degree of visual impairment in adult rats. In the clinic, TES has been demonstrated to improve visual function in patients with nonarteritic anterior ischemic optic neuropathy, traumatic optic neuropathy (Fujikado et al., 2006), and retinal artery occlusion (Inomata et al., 2007).

In spite of these studies, the optimal TES parameters which will result in the best neuroprotective effect and safety of the retina in the clinical situation have not been determined. There are different parameters of the TES that need to be considered, e.g., pulse duration, current intensity, frequency, duration of stimulation, waveform, and number of sessions. Different combinations of these parameters also need to be considered. There are several studies on the effect of the electrical stimulation parameters on tissue damage (Yuen et al., 1981; McCreery et al., 1990; Harnack et al., 2004; Nakauchi et al., 2007), but reports on the relationship between the ES parameters and their neuroprotective effects on injured neurons are limited (Okazaki et al., 2008).

Therefore, the purpose of this study was to determine the optimal ES parameters for the neuroprotection of axotomized RGCs. To accomplish this, we cut the ON of adult rats and stimulated the eyes with electrical pulses of different duration, current intensity,

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frequency, duration of stimulation time, waveform, and number of TES sessions. The retinas were examined histologically to determine the effectiveness of the TES in protecting the retinal neurons.

2. Materials and methods

2.1. Experimental animals

Adult male Wistar rats (230–270 g) were obtained from SLC Japan, Inc. (Shizuoka, Japan). All experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Research Committee, Osaka University Graduate School of Medicine. The animals were anesthetized with intraperitoneal pentobarbital (50 mg/kg body weight) for all surgical procedures.

2.2. Retrograde labeling of RGCs

To identify the RGCs from other retinal cells, the RGCs were retrogradely labeled with Fluorogold (FG; Fluorochrome Inc., Englewood, CO), a fluorescent tracer, before the ON transection. The anesthetized animals were held on a surgical frame with a nose clamp. Craniotomy was performed on the posterior parietal bones to expose the occipital cortex. The occipital cortex was carefully aspirated to expose the dorsal surface of the bilateral superior colliculi (SC), avoiding damage to the superior sagittal sinus and SC. A small sponge soaked in 2% FG (in 0.9% NaCl containing 10% dimethyl sulfoxide) was placed on the surface of both superior colliculi (Morimoto et al., 2002, 2005; Okazaki et al., 2008).

2.3. ON transection

Seven days after the retrograde labeling of the RGCs, the left ON was transected as described in detail elsewhere (Morimoto et al., 2002, 2005; Okazaki et al., 2008). Briefly, a skin incision was made through the left eyelid close to the superior orbital rim, and the incision was retracted to expose the globe. The superior extraocular muscles were spread apart, the ON was exposed by a longitudinal incision of the orbital retractor muscle and perineurium. The ON was transected approximately 3 mm from the posterior pole of the eye with care taken not to damage the retinal blood vessels.

2.4. Transcorneal electrical stimulation

A bipolar contact lens electrode with an inner and outer ring (Kyoto Contact, Kyoto, Japan) was used as the stimulating electrodes. The corneal surface was anesthetized by 0.4% oxybuprocaine HCl in addition to systemic anesthesia, and the contact lens electrode was placed on the cornea of the eye with the transected ON. Hydroxyethylcellulose gel (1.3%) was used to protect the cornea and for making electrical contact with the cornea.

2.5. Stimulation parameters

TES was delivered with anodic first (cornea positive) biphasic square pulses from a constant current stimulator (SEN-7203; Nihonkoden, Tokyo, Japan; Isolator, A395R; World Precision Instruments, Sarasota, FL) (Morimoto et al., 2005, 2007). The stimulus parameters were: pulse durations of 0.5, 1, 2, 3, and 5 ms/phase with 100 μA, 20 Hz, and for 60 min; current intensities of 50, 100, 200, 300 and 500 μA with 1 ms/phase, 20 Hz and for 60 min; frequencies of 0.5, 1, 5, 20, 50, and 100 Hz at 100 μA, 1 ms/phase, and for 60 min; stimulation duration of 15, 30, and 60 min at 100 μA, 1 ms/phase, and 20 Hz (Table 1).

The waveform of the TES was also changed from symmetrical, asymmetrical, and symmetrical with an inter-pulse interval of 0.5 ms or 1 ms at 100 μA, 1 ms/phase, 20 Hz, and for 60 min. In addition the effect of repeated sessions of TES of 100 μA, 1 ms/phase, 20 Hz, for 60 min on days 0, 4, 7, and 10 after the ON transection was compared with a single session of TES with the same parameters of stimulation was investigated 14 days after ON transection.

Table 1
Stimulation parameters tested in this study.

Experiment	Current intensity (μA)	Pulse duration (ms/phase)	Frequency (Hz)	Stimulation duration [min]
Pulse duration	100	0.5–5	20	60
Current intensity	50–500	1	20	60
Frequency	100	1	1–100	60
Stimulation duration	100	1	20	15–60

2.6. Quantification of RGC density

Seven or 14 days after the ON transection and TES, the animals were deeply anesthetized and perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). Both eyes were enucleated and small incisions were made at the dorsal pole of the eyes for retinal orientation. The eyes were stored in 4% PFA in 0.1 M PB overnight at 4 °C. The retinas were dissected from the eyes, and four radial cuts were made to flatten the retinas on a glass microscope slide.

To calculate the density of the surviving RGCs, the number of FG-labeled RGCs was counted using a fluorescence microscope (Axioskop, Carl Zeiss, Oberkochen, Germany) with UV excitation at 365 nm. The RGCs in three areas of 0.5 × 0.5 mm^2 along the naso-temporal and dorso-ventral axes (nasal, temporal, dorsal and ventral) at 1, 2 and 3 mm from the optic disc were counted using a microscanner (Sapporo Beer, Saitama, Japan). The mean density of RGCs was calculated from the number of surviving RGCs counted in the 12 areas of every retina (Morimoto et al., 2002, 2005; Okazaki et al., 2008).

2.7. Statistical analyses

Data are presented as the mean ± standard deviations (SDs) and the data were statistically analyzed using a commercial software (SigmaStat, version 3.11; Systat Software, Inc). Comparisons among the groups were made by one-way analysis of variance (ANOVA), when the equal variance test passed, and was followed by the Tukey test or by Kruskal–Wallis one-way ANOVA on ranks, when the equal variance test failed, followed by the Dunn's method. Comparisons between two groups were made by Student's t tests. The level of statistical significance was set as $P < 0.05$.

3. Results

3.1. Effect of pulse duration on neuroprotection of axotomized RGCs

The somas of the RGCs in the intact control retinas were round with the fine dots of FG in the perinuclear cytoplasm and proximal dendrites (Fig. 1A). The mean RGC density in the intact control retinas was 2357 ± 150 cells/mm^2 (mean ± SD; $n = 12$; Fig. 2A). Seven days after the ON transection and without TES, the number of FG-labeled RGCs were markedly reduced to 1290 ± 96 cells/mm^2 ($n = 8$) which is 54.7% of that of intact retinas. The RGCs were irregularly shaped and debris of dead RGCs were present (Fig. 1B). The mean RGC density in the retinas with sham electrical stimulation 7 days after ON transection, was 1221 ± 176 cells/mm^2 ($n = 6$) which was 51.8% of intact retinas. This density was not significantly different from that in the retinas with ON transection without TES, and was used as the control value to evaluate the neuroprotective effect of TES.