

Effects of steroid administration and transcorneal electrical stimulation on the anatomic and electrophysiologic deterioration of nonarteritic ischemic optic neuropathy in a rodent model

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1 **Effects of steroid administration and transcorneal electrical stimulation on the anatomical and**  
2 **electrophysiological deterioration of nonarteritic ischemic optic neuropathy in a rodent model**

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7 Running Head: Effects of steroid and TES

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20 **Abstract**

21 Purpose: To elucidate the effectiveness of steroid administration and transcorneal electrical stimulation (TES) on  
22 anatomical changes and visual functions in a rodent model of nonarteritic ischemic optic neuropathy (rNAION).

23 Methods: Methylprednisolone (20 mg/kg) was injected through a central venous catheter twice a day for 3 days.

24 TES was delivered with biphasic square pulses of 1 ms/phase, 100  $\mu$ A of current, and 20 Hz of frequency for 60  
25 minutes on the three hours, 1<sup>st</sup>, 4<sup>th</sup>, 7<sup>th</sup>, 14<sup>th</sup>, and 28<sup>th</sup> days after induction.

26 Results: Intravenous infusion of methylprednisolone significantly decreased the degree of acute disc edema but  
27 did not preserve inner retinal thinning, decreasing scotopic threshold responses (STR) amplitude, and decreasing  
28 RGC numbers in an rNAION. TES preserved the decreasing STR amplitude and the decreasing RGC numbers in  
29 an rNAION.

30 Conclusion: Steroids are effective for reducing disc edema in the acute stage in an rNAION. TES is effective for  
31 preserving decreasing RGC numbers and function in the chronic stage of an rNAION.

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34 **Keywords:** steroid, Transcorneal Electrical Stimulation, rodent model of nonarteritic ischemic optic neuropathy,  
35 scotopic threshold response of electroretinogram

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39 **Introduction**

40 Nonarteritic ischemic optic neuropathy (NAION) is an optic nerve dysfunction caused by an ischemia of the  
41 posterior ciliary artery (PCA) although the precise etiology is not known [1]. The incidence rate is approximately  
42 2 to 10 individuals per 100,000 [2]. Unfortunately, an effective treatment has not yet been found. Various kinds  
43 of treatment to enhance the visual function of NAION patients have been tried, including corticosteroids [3],  
44 hyperbaric oxygen therapy [4], optic nerve sheath decompression [5], transcorneal electric stimulation [6], and  
45 intravitreal bevacizumab injections [7]. Although each treatment has been reported to be effective in a short case  
46 series, they are not widely recognized as effective because a randomized, multicenter optic nerve sheath  
47 decompression treatment trial, which was thought to be effective in studies with small sample sizes [8], revealed  
48 that intervention was not effective and rather harmful [5]. Therefore, an animal model of NAION to confirm the  
49 effectiveness of a treatment and perform clinical trials [9] has been developed. We have succeeded in  
50 establishing a rodent model of NAION (rNAION) [10] and have collected reproducible objective data showing  
51 the severity of NAION, including the time course of inner retinal thickness around the optic disc using OCT [11],  
52 the scotopic threshold response of ERG [10], and an RGC count using Fluorogold (Fluoro-Gold®, Fluorochrome,  
53 Denver, USA).

54 We have found that corticosteroids have nitric oxide (NO)-independent vasodilatory effects in rabbit PCA [12].  
55 Therefore, in terms of the vasodilatory effect, corticosteroids could be effective for the treatment of NAION. In  
56 addition, NAION has two pathological steps: the acute disc edema stage and the atrophic stage. The goal of  
57 treatment must be neuroprotection due to the atrophic stage. Among the treatment options described above, only

58 TES provides neuroprotection.

59 Morimoto et al reported that TES has the protective effect for the RGC number reduction by activating  
60 endogenous retinal IGF-1 system using optic nerve transaction model of rats [13]. In the rNAION model, we  
61 showed the RGC number reduction in a chronic phase after the ischemic induction to the optic nerve. As both  
62 model showed the secondary RGC loss after the optic nerve damage, TES could show the protective effect for  
63 the RGC number reduction in the rNAION model.

64 To investigate the efficacy of, steroids and TES for anatomical changes and improving visual functions in the  
65 acute stage of an rNAION, each intervention was performed after 3 hours of rNAION induction. Subsequently,  
66 the time course of inner retinal thickness using OCT, the amplitude of STR, and the survival number of RGCs  
67 were compared to the baseline data of the controls.

## 68 **Materials and Methods**

### 69 **rNAION induction**

70 The animal protocols were approved by the University of Miyazaki Institutional Animal Care Committee and  
71 adhered to the guidelines recommended by the ARVO statement for the Use of Animals in Ophthalmic and  
72 Vision Research.

73 Male Sprague-Dawley rats (200-240 g; Kyudou, Kumamoto, Japan) were anesthetized with intramuscular  
74 ketamine and xylazine (80 mg/kg and 5 mg/kg, respectively). To induce rNAION, rose bengal (RB) (2.5 mM, 1  
75 ml/kg) was injected into the tail vein. After administering RB, the optic nerve (ON) was photoactivated in the  
76 optic nerve vessels using a 514-nm argon green laser with an approximately 500- $\mu$ m spot size for 12 seconds.

77 **Treatment intervention protocol**

78 For the steroid treatment, after 3 hours of rNAION induction, 20 mg/kg of methylprednisolone solution, which  
79 is similar to about 1000mg for human, with saline was injected through the central venous catheter twice a day  
80 for 3 days.

81 For the TES treatment, a monopolar contact lens electrode (Mayo, Aichi, Japan) was used as a positive  
82 stimulating electrode. The negative electrode was placed in the oral cavity using a needle. The cornea was  
83 anesthetized using 0.4% oxybuprocaine eye drops with the systemic anesthesia. A 15mg/ml of hydroxyethyl  
84 cellulose gel was used to protect the cornea. TES was delivered with biphasic square pulses from a biphasic  
85 pulse generator (BPG-1, BAK Electronics, Inc.) and a stimulus isolator (BSI-2, BAK Electronics, Inc.). The  
86 stimulus parameters were 1 ms/phase of pulse duration, 100  $\mu$ A of current, 20 Hz of frequency, and 60 minutes  
87 of stimulation. The stimulus parameter was based on the paper from Morimoto et.al, which demonstrated the  
88 protective effect of TES for the RGC number reduction using optic nerve transection model of rats [14].  
89 Stimulation of the TES started three hours after the rNAION induction and was performed on the 1<sup>st</sup>, 4<sup>th</sup>, 7<sup>th</sup>, 14<sup>th</sup>,  
90 and 28<sup>th</sup> days after the induction.

91 **OCT measurement**

92 We measured the inner retinal thickness around the disc using spectral domain OCT (iVUE-100, Optovue Inc.,  
93 CA, USA). We altered some of the measurement methods because of differences in the refractive power, axial  
94 length, and maximum dilated pupil size between rats and humans [15, 16]. The retinal image at its best focus in a  
95 rat requires a diopter compensation of approximately +20 D because of the typical strong negative spherical

96 aberration. Therefore, we fixed an achromatic +20 D doublet lens with a locking device in front of the OCT  
97 instrument. The high-power positive lens also helped reduce the required pupil diameter. After obtaining the  
98 maximum pupil dilation with eye drops of Tropicamide and phenylephrine hydrochloride (Midrin-P®), the rats  
99 under anesthesia were fixed in front of the OCT. We judged the rats to be qualified for the evaluation and  
100 counted one scan when the scan quality index (SQI) was over 45 in each scan. The examiner recorded 3 scans,  
101 and the mean value was analyzed. The diameter of the optic disc of the rat is approximately 1 mm. The  
102 measurement area was within the 3-mm diameter, the center of which was the center of the optic disc, but the  
103 area of the center circle with a 2-mm diameter was excluded. Thus, the thickness within the round, banded area  
104 of the inner retina 1 mm from the center of the optic disc was measured because in the area next to the optic disc  
105 edge, each retinal layer was obscured when the swelling developed. We evaluated the change in the thickness of  
106 the NFL as the change in the thickness of the inner retina, meaning the distance from the inner limiting  
107 membrane (ILM) to the inner plexiform layer (IPL) (Fig.1). We conducted scans before the induction and on the  
108 1<sup>st</sup>, 3<sup>rd</sup>, 5<sup>th</sup>, 7<sup>th</sup>, 14<sup>th</sup>, 28<sup>th</sup>, 56<sup>th</sup>, and 90<sup>th</sup> days after rNAION induction. We compared the change in appearance and  
109 thickness before and after the steroid treatment.

## 110 **Electrophysiologic evaluation**

### 111 **STR Recording**

112 For the STR recording, after the overnight (12 hours) dark adaptation, the rats were anesthetized with  
113 ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively). The body temperature of the rats was kept at  
114 37°C with a heating pad, and the pupils were fully dilated in both eyes. The retinal signals for STR were

115 recorded from the cornea using the same contact lens electrodes. The needle electrode was held stable on the  
116 skin, and the ground electrode was placed in the tail. During the STR recording, the rats were placed on the  
117 shield mat surrounded by a Ganzfeld bowl. The STR responses were obtained for flash intensities ranging from  
118  $-6.15$  to  $-3.30 \log(\text{cd s}) \text{ m}^{-2}$  in  $0.2 \log$  unit increments by averaging 15-20 responses per intensity with an  
119 interstimulus interval of 3 seconds (Fig.2).

120 The amplitudes of STR were compared in the control and the treated eye on the 1<sup>st</sup>, 3<sup>rd</sup>, 7<sup>th</sup>, 14<sup>th</sup>, and 28<sup>th</sup> days  
121 after the induction.

## 122 **Analysis of retinal ganglion cells**

### 123 **RGC staining**

124 The SD rats were anesthetized with ketamine and xylazine (80 mg/kg and 8 mg/kg, respectively). After  
125 exposing the skull, a 2-mm-diameter hole was drilled 3.5 mm bilaterally to the midline and 6 mm behind the  
126 Bregma suture. After the aspiration of the cortex, the superior colliculus was exposed. A sponge (Spongel®;  
127 Astellas Pharma, Inc., Tokyo, Japan) filled with Fluorogold (Fluoro-Gold®, Fluorochrome, Denver, USA) was  
128 placed on the superior colliculus, and the holes were plugged with ointment and closed using the overlaying  
129 skin.

### 130 **Survival RGC number measurement**

131 One week after hydroxystilbamidine placement, the rats were anesthetized with 100 mg/kg of pentobarbital  
132 (Somnopenyl, Kyoritsu Seiyaku Co., LTD, Tokyo, Japan), a perfusion fixation with 100 ml of 4% of  
133 paraformaldehyde was performed, and the eyes were enucleated. One hour after the enucleation, a cross-shaped



134 floating preparation of the retina was made, fixed on the glass slide, and mounted using PERMAFLUOR  
135 (Thermo Shandon LTD., Runcorn Cheshire, UK). The twelve 0.25 mm×0.25 mm areas (total of 3 areas in each  
136 of the 4 quadrants) were blindly selected, and the number of RGCs observed and photographed through the  
137 fluorescence microscope (Axioplan, Zeiss, Germany) with the ultraviolet filter was counted (Fig.3).

138 The RGC count was performed using CellProfiler® (The Broad Institute of MIT and Harvard). We confirmed  
139 its accuracy by comparing it with the actual count performed by two of authors (T.I. and N.K.), and we  
140 rechecked every CellProfiler® count.

141 The survival number of RGC was compared in the control and the treated eye on the 1<sup>st</sup>, 3<sup>rd</sup>, 7<sup>th</sup>, 14<sup>th</sup>, and 28<sup>th</sup>  
142 days after the induction.

### 143 **Statistical Analysis**

144 The data are summarized as the means ± SDs. To analyze the data statistically, we used a two-way ANOVA  
145 followed by post hoc Bonferroni comparisons. We considered differences significant at P <0.05.

## 146 **Results**

### 147 **Effect of steroids on anatomical changes and visual functions of rNAION**

148 The inner retinal thickness of the steroid group was significantly thinner than in the control group on the 1<sup>st</sup>, 3<sup>rd</sup>,  
149 and 5<sup>th</sup> days and significantly thicker than in the control eyes on the 14<sup>th</sup> day (Fig. 4). There was no significant  
150 difference in STR amplitude between the steroid group and control group (Fig. 5). There was no significant  
151 difference in RGC survival between the steroid and control groups (Fig. 6).

### 152 **Effect of TES on the prevention of visual functions of rNAION**

153 A decreased amplitude in the STR of the TES group was significantly better preserved than in the control group  
154 on the 28<sup>th</sup> day but not on the 14<sup>th</sup> day after induction (Fig. 7). RGC survival of the TES group was significantly  
155 larger than in the control group on the 14<sup>th</sup> and 28<sup>th</sup> days (Fig. 8). These results means that the preservation effect  
156 of STR in function could be slightly delayed compared to that in cell survival.

## 157 **Discussion**

158 This study showed that a methylprednisolone intravenous infusion significantly decreases the degree of the  
159 acute stage of disc edema but did not preserve the thinning of the inner retina in an rNAION (Fig. 4). In addition,  
160 the methylprednisolone intravenous infusion did not provide protection from the reduced RGC function and  
161 number observed in the chronic stage of rNAION. Hayreh et al. treated 613 patients with NAION using  
162 corticosteroids or observation and compared the visual recovery [3]. The authors observed a significant and more  
163 rapid reduction of disc edema as well as a higher probability of improvement in visual acuity and the visual field  
164 in the steroid treatment group than in the observation group. Our experimental results support the Hayreh's  
165 findings that showed that steroid treatment significantly decreased the degree of disc edema in the acute stage.  
166 However, our experimental results did not support Hayreh's results because steroid treatment did not provide  
167 protection from the reduced RGC function and number in the chronic stage of rNAION. Hayreh et al. speculated  
168 about the mechanism behind this effect and considered that corticosteroid therapy reduces optic disc edema by  
169 reducing capillary permeability [3]. However, we have found that there was no inflammation in the optic nerve  
170 of rNAION using an HE stain and immunohistochemistry [10]. We also found that steroids have nitric  
171 oxide-independent vasodilatory effects on the rabbit PCA [12]. From these results, we speculate that the

172 vasodilatory effect of corticosteroids could be the cause of the rapid reduction of disc edema rather than the  
173 reduced capillary permeability. The disc edema of NAION could be attributed to the slowed axonal flow at the  
174 lamina cribrosa of the optic nerve due to the insufficiency of the blood flow to the optic nerve [17]. The  
175 vasodilatory effect evoked by the corticosteroid could increase the blood flow to the optic nerve and lead to an  
176 increase of the axonal flow, thereby reducing disc edema rapidly and successfully. However, steroids have no  
177 effect on the visual recovery, according to this study. There has been no report that steroids have a  
178 neuroprotective effect. For better visual recovery, some neuroprotective options are still needed following the  
179 steroid treatment.

180 This study showed that TES protects against the reducing RGC function and number in the chronic stage of  
181 rNAION.

182 Fujikado et al. performed TES on three patients with NAION, and two of three patients had improved visual  
183 acuity by at least 0.3 log (minimum angle of resolution) [6]. They also reported that TES could rescue the  
184 axotomized RGC by increasing the level of IGF-1 production by Muller cells [13]. In this experiment, although  
185 an anatomical change could not be observed using OCT because of potential corneal damage, the  
186 neuroprotective effect of TES was significant.

187 In conclusion, a methylprednisolone intravenous infusion ameliorated the acute stage of disc edema but could  
188 not preserve the inner retinal thickness and RGC function in the chronic stage of rNAION. A TES protected  
189 against deteriorating RGC function and decreasing RGC counts of rNAION. As NAION as well as rNAION  
190 have two different phases of processes, we might consider the different strategy for the treatment for NAION.

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248 **Figure Legends**

249 Figure 1: a. An example of the typical OCT image of the optic disc of normal rat. bar =250 $\mu$ m.

250 b. An example of the typical OCT image of the optic disc of rNAION. bar =250 $\mu$ m

251 Figure 2: a. An example of the typical STR recording of normal rat.

252 b. An example of the typical STR recording of rNAION

253 Figure 3: An example of the microscopic fluorescence photograph. x 40 , bar =100 $\mu$ m.

254 Figure 4: The inner retinal thickness of the steroid treatment and no treatment groups on the 1, 3, 5,7,14,28, 56,

255 and 90 days after rNAION induction. The dark gray bar is the steroid treatment group (n= 8 rats each day), and

256 the light gray bar is the control group (n= 10 rats each day). \*\* p < 0.001. \* p < 0.005

257 Figure 5: The amplitude of the STR in the steroid treatment and no treatment groups on the 7,14, and 28 days

258 after rNAION induction. The dark gray bar is the steroid treatment group (n= 12 rats), and the light gray bar is

259 the control group (n= 13 rats).

260 Figure 6: Survival RGC counts on the 28<sup>th</sup> and 84<sup>th</sup> days after induction in the steroid treatment group and

261 control group of rNAION. The dark gray bar is the steroid treatment group (n= 8 rats), and the light gray bar is

262 the control group (n= 5 rats).

263 Figure 7: The amplitude of the STR in the TES treatment and no treatment groups on the 14, and 28 days after

264 rNAION induction. The dark gray bar is the control group (n= 8 rats), and the light gray bar is the TES treatment

265 group (n= 12 rats). \* p < 0.005.

266 Figure 8: Survival RGC counts on the 14<sup>th</sup> and 28<sup>th</sup> days after induction in the TES treatment group and the

267 control group of rNAION. The dark gray bar is the TES treatment group (n= 7 rats), and the light gray bar is the

268 control group (n= 7 rats). \*\* p < 0.001.

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