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

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Running Head: Effects of steroid and TES

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Abstract

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

Methods: Methylprednisolone (20 mg/kg) was injected through a central venous catheter twice a day for 3 days. TES was delivered with biphasic square pulses of 1 ms/phase, 100 μA of current, and 20 Hz of frequency for 60 minutes on the three hours, 1st, 4th, 7th, 14th, and 28th days after induction.

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

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

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

Introduction

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

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

TES provides neuroprotection.

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

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

Materials and Methods

rNAION induction

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

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

Treatment intervention protocol

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

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

OCT measurement

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

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

Electrophysiologic evaluation

STR Recording

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

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

The amplitudes of STR were compared in the control and the treated eye on the 1^{st}, 3^{rd}, 7^{th}, 14^{th}, and 28^{th} days after the induction.

Analysis of retinal ganglion cells

RGC staining

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

Survival RGC number measurement

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

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

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

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

Statistical Analysis

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

Results

Effect of steroids on anatomical changes and visual functions of rNAION

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

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 28th day but not on the 14th day after induction (Fig. 7). RGC survival of the TES group was significantly
155 larger than in the control group on the 14th and 28th 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

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

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

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

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

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Figure Legends

Figure 1: a. An example of the typical OCT image of the optic disc of normal rat. bar =250μm.

b. An example of the typical OCT image of the optic disc of rNAION. bar =250μm

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

b. An example of the typical STR recording of rNAION

Figure 3: An example of the microscopic fluorescence photograph. x 40 , bar =100μm.

Figure 4: The inner retinal thickness of the steroid treatment and no treatment groups on the 1, 3, 5,7,14,28, 56, and 90 days after rNAION induction. The dark gray bar is the steroid treatment group (n= 8 rats each day), and the light gray bar is the control group (n= 10 rats each day). * * $p < 0.001$. * $p < 0.005$

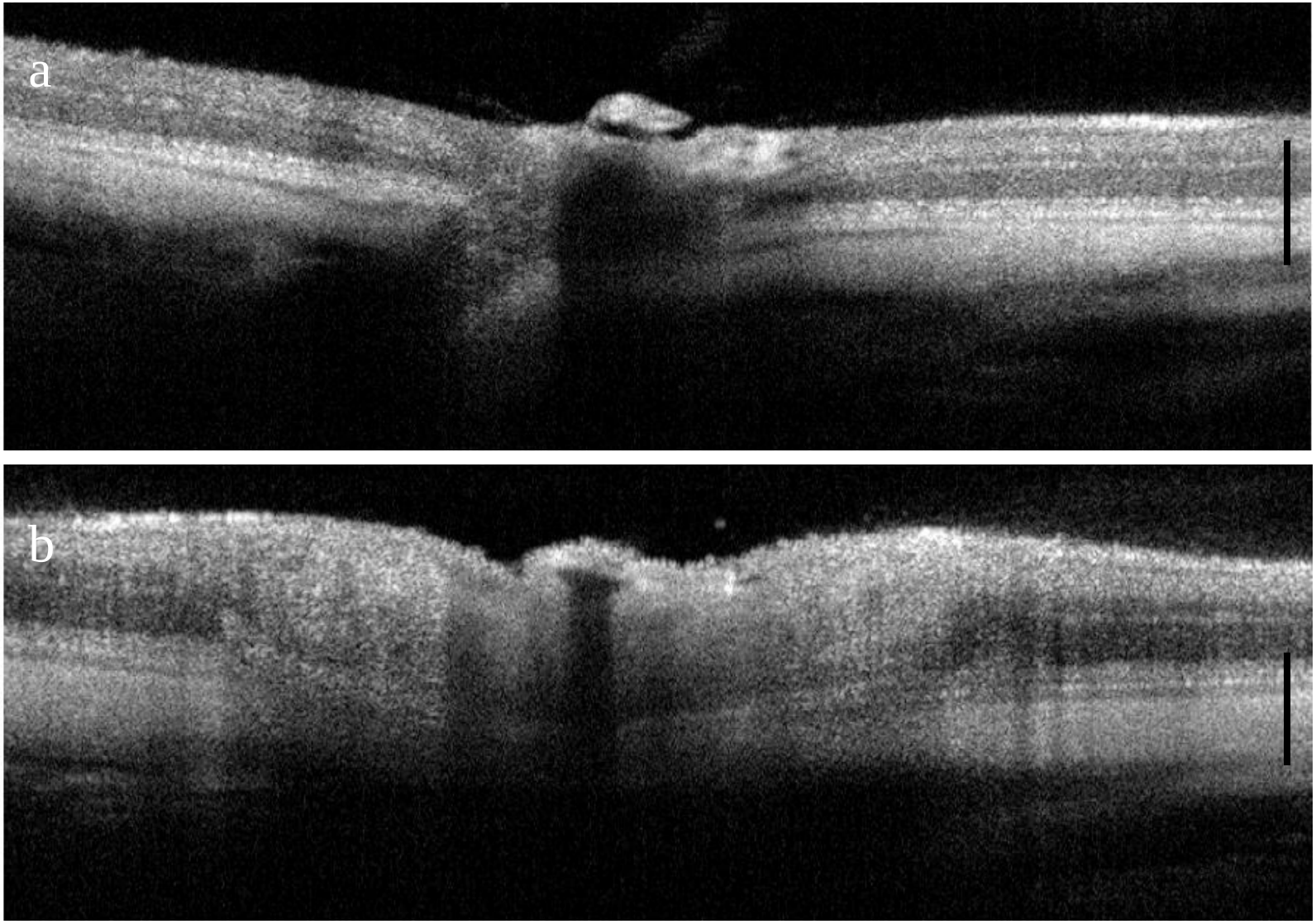
Figure 5: The amplitude of the STR in the steroid treatment and no treatment groups on the 7,14, and 28 days after rNAION induction. The dark gray bar is the steroid treatment group (n= 12 rats), and the light gray bar is the control group (n= 13 rats).

Figure 6: Survival RGC counts on the 28^{th} and 84^{th} days after induction in the steroid treatment group and control group of rNAION. The dark gray bar is the steroid treatment group (n= 8 rats), and the light gray bar is the control group (n= 5 rats).

Figure 7: The amplitude of the STR in the TES treatment and no treatment groups on the 14, and 28 days after rNAION induction. The dark gray bar is the control group (n= 8 rats), and the light gray bar is the TES treatment group (n= 12 rats). * $p < 0.005$.

Figure 8: Survival RGC counts on the 14^{th} and 28^{th} days after induction in the TES treatment group and the

control group of rNAION. The dark gray bar is the TES treatment group (n= 7 rats), and the light gray bar is the control group (n= 7 rats). ** $p < 0.001$.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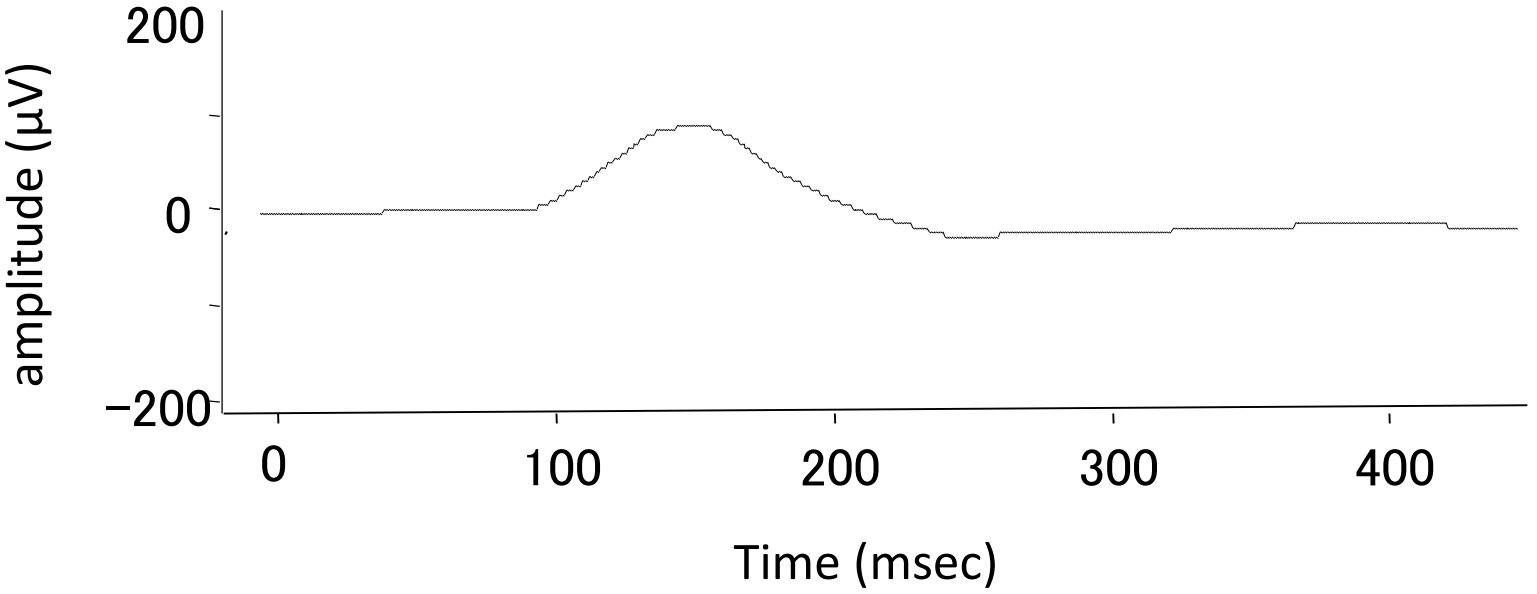
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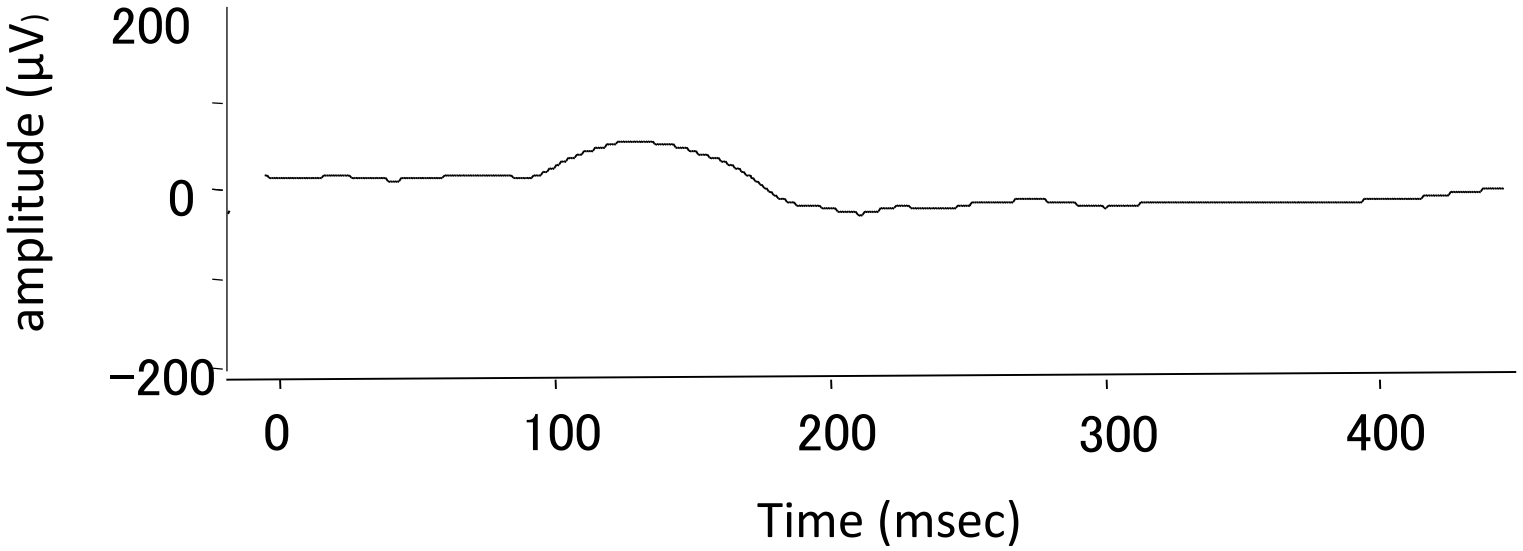
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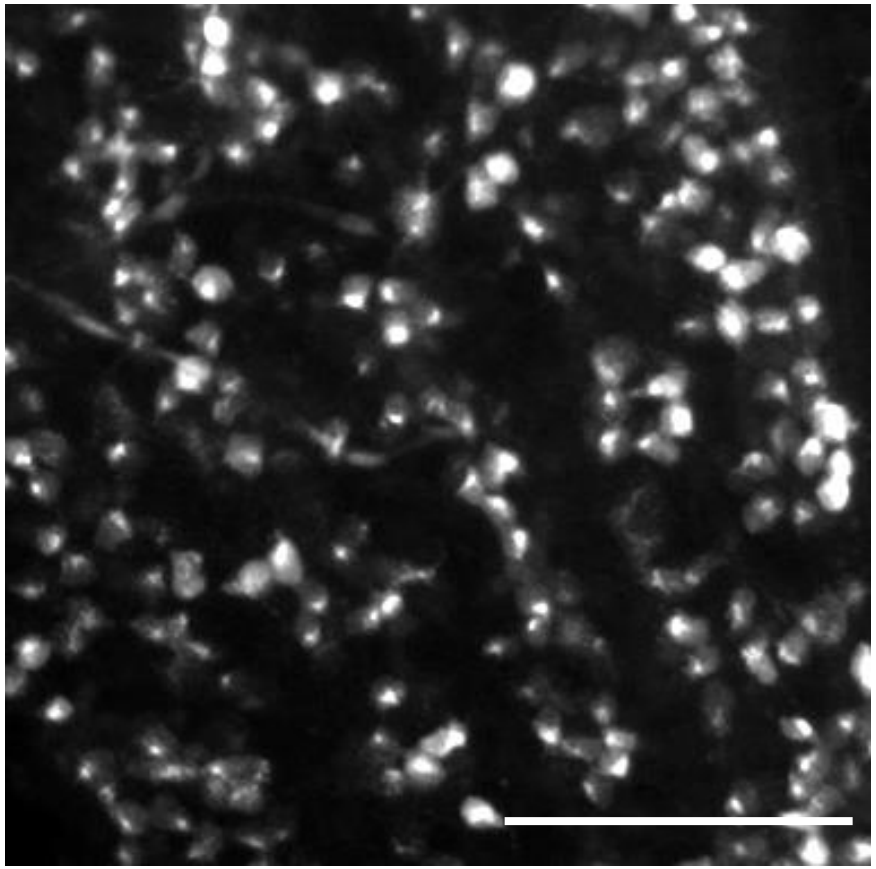

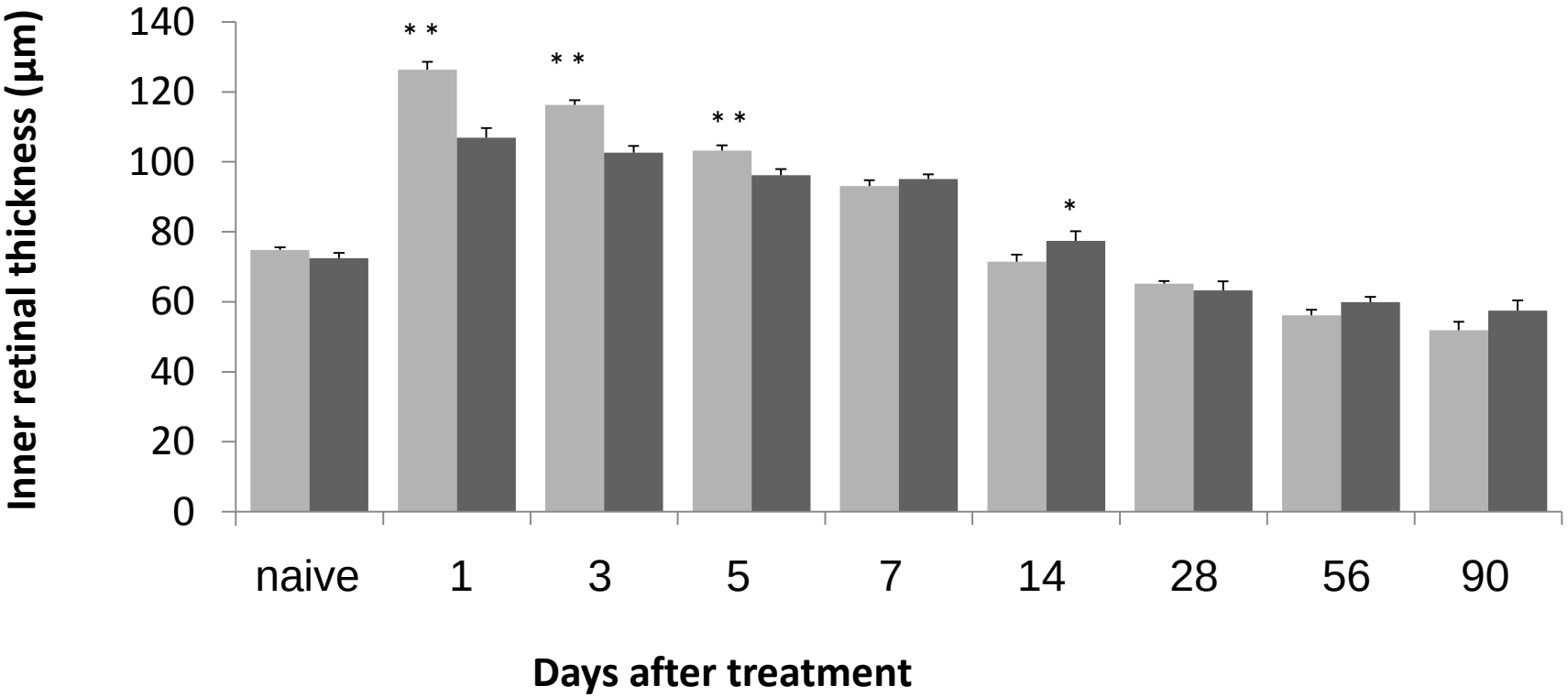
Inner retinal thickness (µm)
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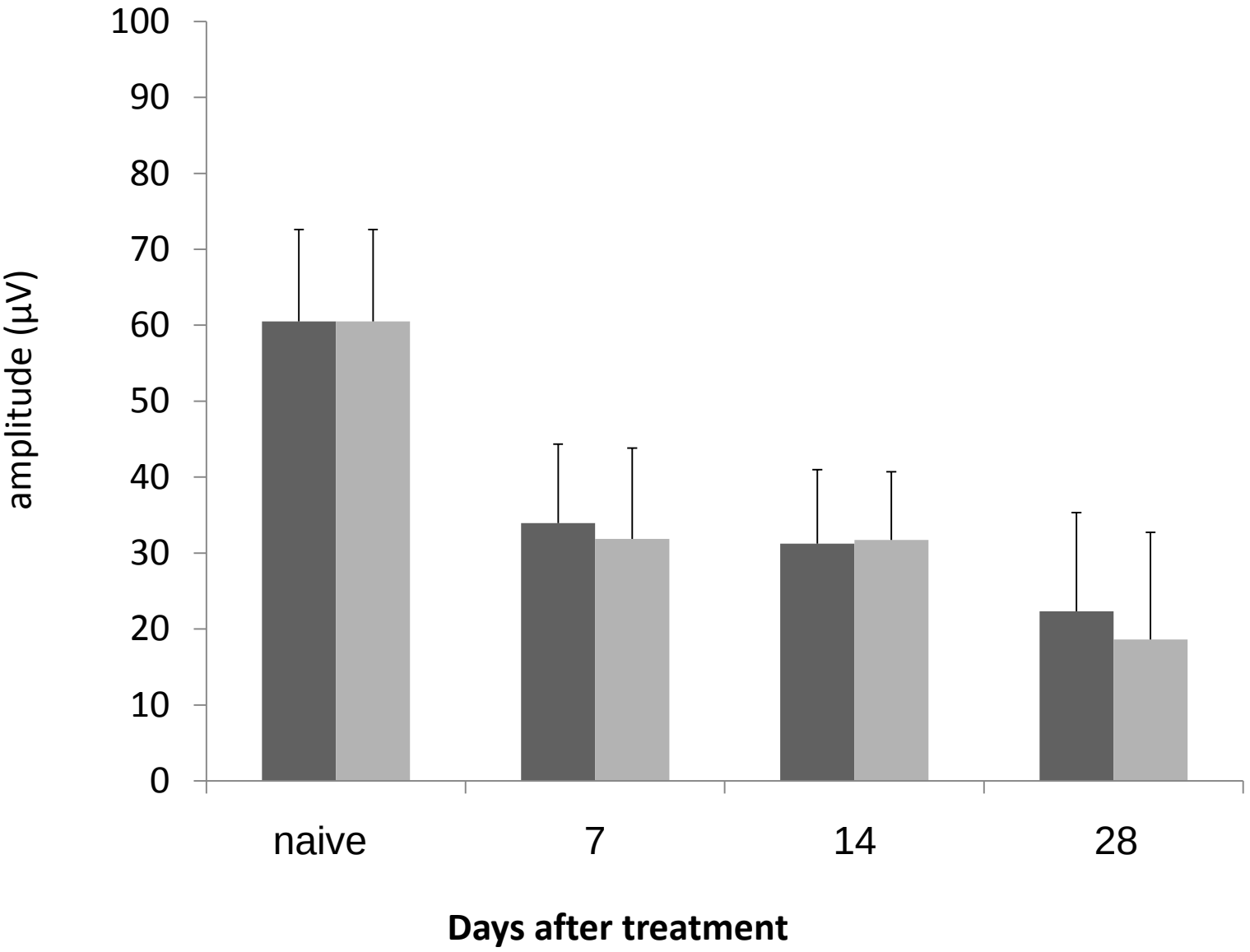
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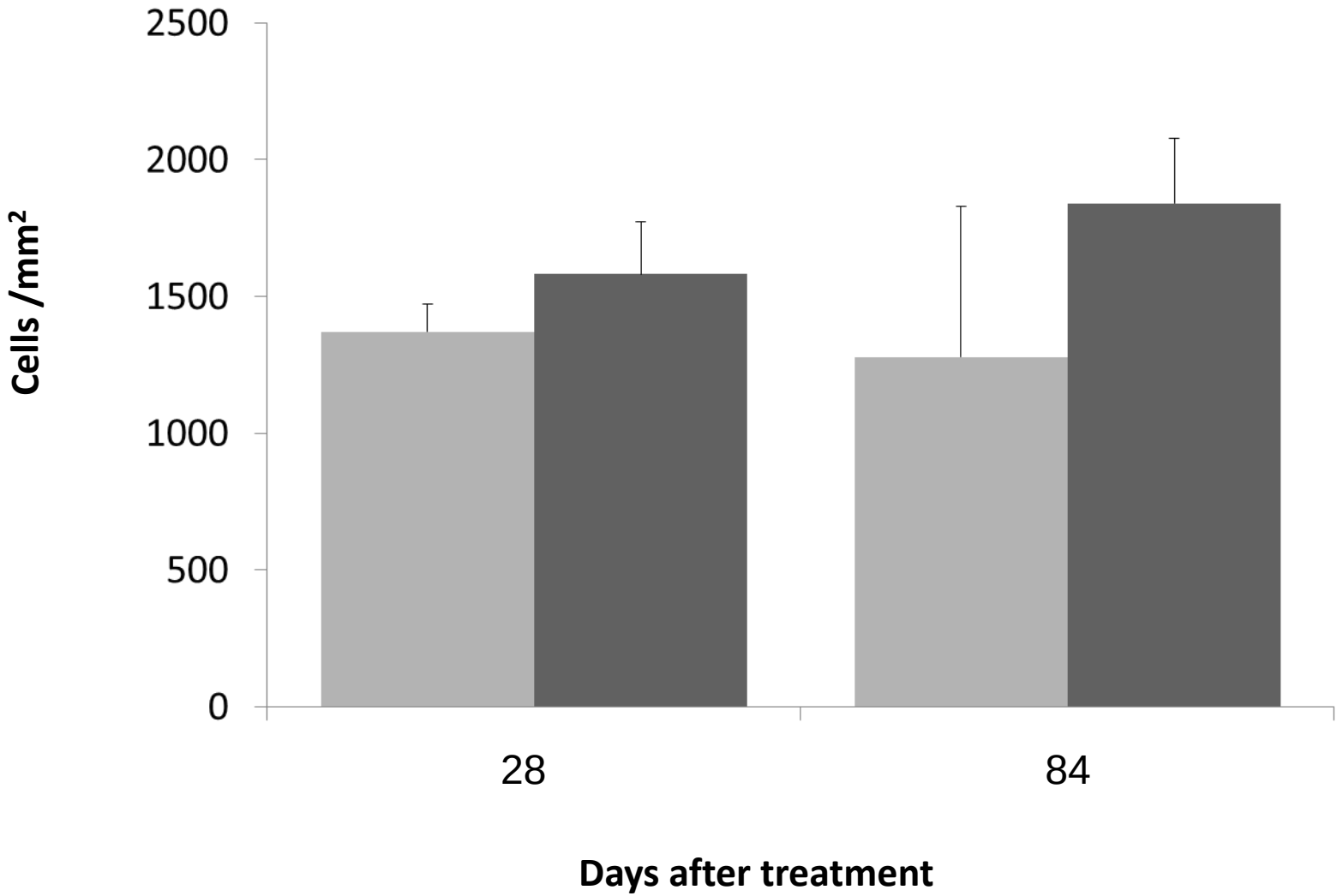
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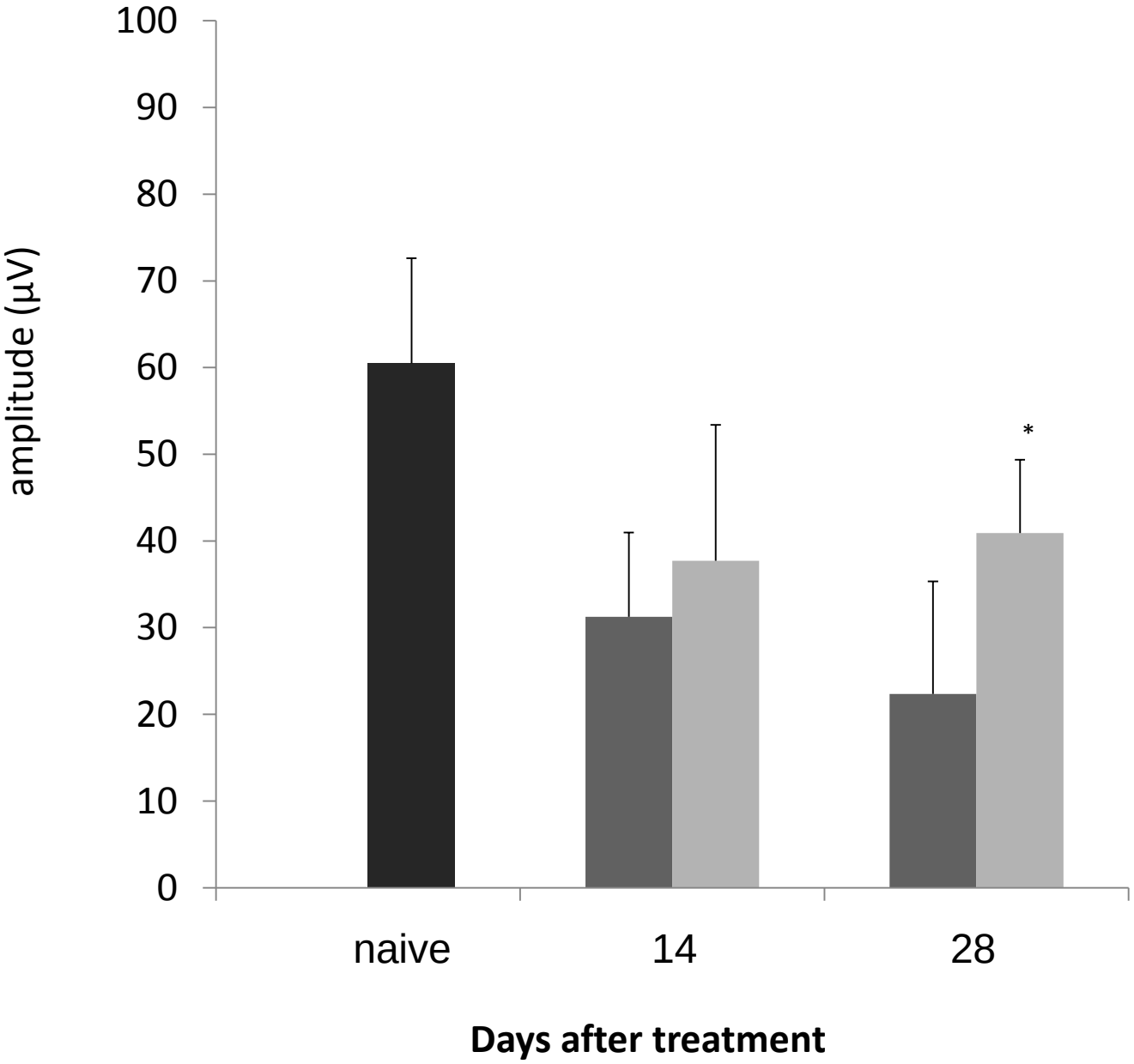
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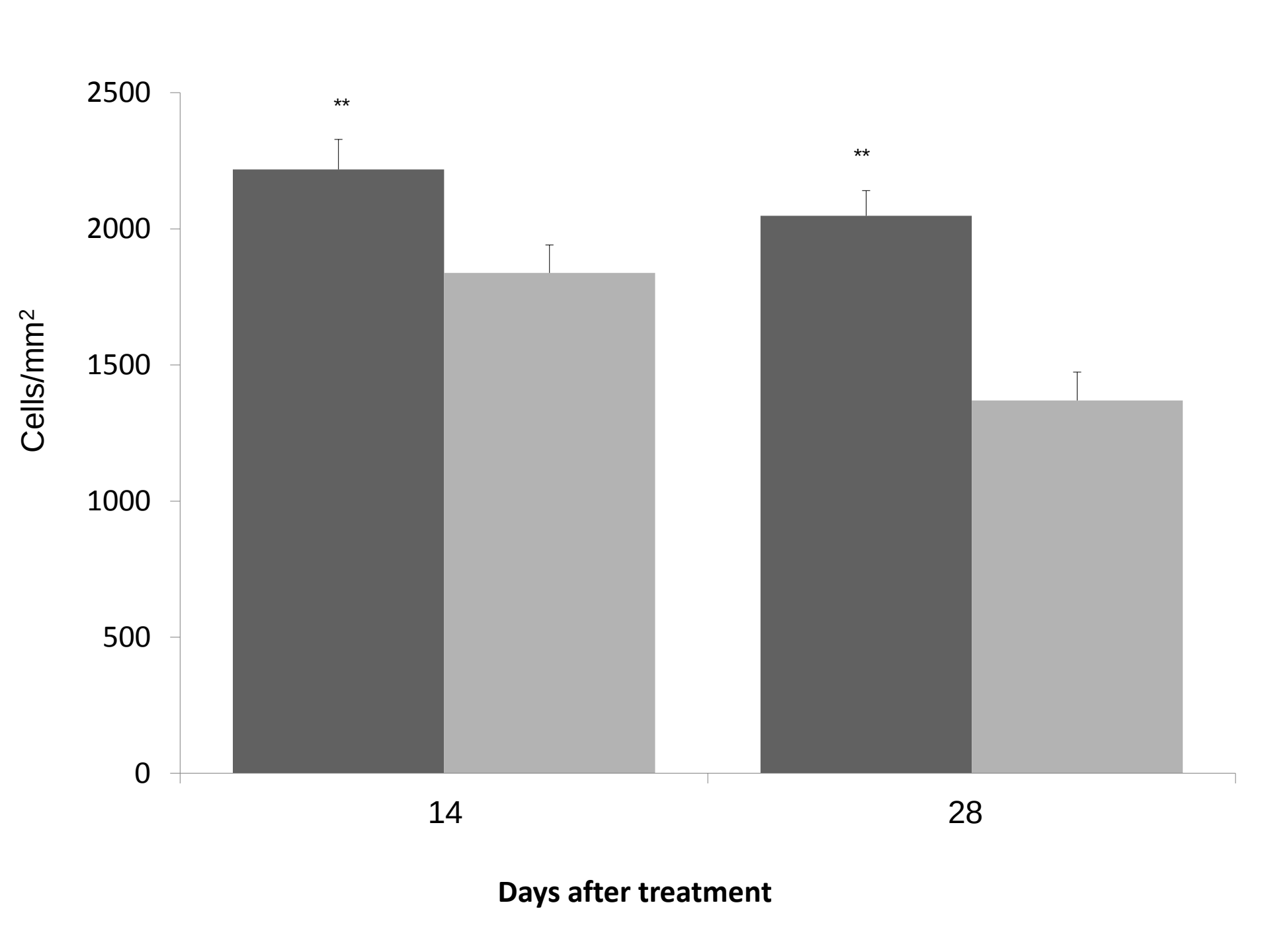
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