Safety evaluation of subretinal injection of trypan blue in rats

Y. FANG[1], X.-Q. YAO[1], L.-L. NIU[1], J.-H. WU[2], E.F. THEE[3],
D.-F. CHEN[3,4], J.-Y. CHEN[1], X.-H. SUN[1,4,5]

[1]Department of Ophthalmology and Visual Science, Eye & ENT Hospital of Fudan University, Shanghai, China
[2]Experimental Research Center, Eye and ENT Hospital of Fudan University, Shanghai, China
[3]Schepens Eye Research Institute, Massachusetts Eye and Ear, Department of Ophthalmology, Harvard Medical School, Boston, MA, USA
[4]State Key Laboratory of Medical Neurobiology, Institutes of Brain Scienceand Collaborative Innovation Center for Brain Science, Fudan University, Shanghai, China
[5]Key Laboratory of Myopia, NHFPC (Fudan University), and Shanghai Key Laboratory of Visual Impairment and Restoration (Fudan University), Shanghai, China

Yuan Fang and *Xiaoqian Yao* contributed equally to this work

Abstract. – OBJECTIVE: To determine the appropriate concentration of trypan blue (TB) for subretinal injection in a rat model and to provide a safety profile that limits retinal toxicity while maintaining dye visibility.

MATERIALS AND METHODS: Adult rats were subretinally injected with various concentrations of either TB or phosphate-buffered saline (PBS); rats which received sham injections served as an additional control. The injected areas were visualized under a surgical microscope. Electroretinography (ERG) was performed to measure retinal function. Animals were then sacrificed, and the eyes were sectioned and examined by light microscopy. Terminal deoxynucleotidy1 transferase dUTP nick-end labeling (TUNEL) was applied to determine retinal apoptosis.

RESULTS: One day after the subretinal injection, TB stains were visible under the surgical microscope in the 0.2%, 0.08%, and 0.04% TB-injected groups, but not in the 0.02% TB-injected group. TB stain was detectable in the retina and sclera of the 0.2%, 0.08%, and 0.04% TB-injected groups for over 2 weeks after injection. However, the amplitudes of ERGa- and b-waves were affected and became significantly lower in the 0.2% TB-injected group than the amplitudes in the PBS-, or sham-injected group. Moreover, TUNEL+ cells appeared in the outer nuclear layer (ONL), ganglion cell layer (GCL), and retinal pigment epithelium (RPE) layer of the 0.2% and 0.08% TB-injected groups at 1 and 7 days after subretinal injection. In contrast, very few TUNEL+ cells were found in the 0.04% TB- or PBS-injected group. Two weeks after injection, the ONL was significantly thinner in the 0.2% TB-injected group than in the 0.04% TB-, PBS- or sham-injected group.

CONCLUSIONS: TB injection induces a dose-dependent neurotoxic effect on retinal cells. Subretinal injection of 0.04% TB is relatively safe and effective for subretinal staining.

Key Words:
    Trypan blue, Subretinal injection, Neurotoxicity, Retinal cells, Rats.

Introduction

Subretinal injections of drugs[1,2], gene constructs[3-6], or stem cells[7-10] are emerging as common procedures in experimental animals. The injected retinal area, however, is often difficult to locate, especially when it comes to preparing sections or analyzing the morphology. This is due to the quick recovery of the retinal detachment after subretinal injection. Researchers thus often use dyes to visualize the injected areas.

In clinical practice, vitreous or subretinal injection of dyes is used in vitreoretinal surgery that removes the internal limiting and epiretinal membranes[11-18] or to localize retinal breaks[19-22]. The first dye to be used in eyes was indocyanine green (ICG). However, some studies[23-26] showed that ICG exerted dose-dependent retinal toxicity in both animals and humans. Other dyes

Corresponding Author: Xinghuai Sun, MD; e-mail: xhsun@shmu.edu.cn
Junyi Chen, MD; e-mail: chenjy@fudan.edu.cn

that have been investigated include trypan blue (TB)[13,27,28], patent blue[29], triamcinolone acetonide[11], infracyanine green[30,31], and brilliant blue G[15,32,33].

TB is an anionic hydrophilic diazo dye of a relatively small molecular weight (960 Da). It can cross the membranes of dead cells, leading to distinctive blue staining of only dead cells[34,35]. TB staining is convenient and highly stable, without any background of autofluorescence[36]. It is thus also used in clinical practice to detect retinal breaks at concentrations ranging from 0.006-0.15%, with no reported complications[19-22]. However, some clinical studies[12,14] reported that, after vitreous injection of 0.15% TB, the dye migrated from the vitreous into the subretinal space causing local chorioretinal atrophy and retinal pigment epithelium (RPE) changes. Currently, the safety profile of TB injection into the subretinal space is unknown. Therefore, the aim of this study is to determine the safe and effective concentrations of TB for subretinal injections through evaluating the histological and functional effects of TB injection in rat eyes.

Materials and Methods

Animals, Anaesthesia, and Mydriasis

All experimental procedures were performed in accordance with the Institute's Animal Care and Use Committee and adhered to the Statement for the Use of Animals in Ophthalmic and Vision Research. Male Sprague-Dawley rats (weighing 180-220 g) were maintained in a 12h light/12h dark cycle with free access to commercial rat food (Suzhou Laboratory animal feed Technology Co., Ltd. Suzhou, Jiangsu, China) and water. Rats were anesthetised by intraperitoneal injection of a mixture of 6.25 mg/kg xylazine (Sigma-Aldrich, St. Louis, MO, USA) and 10 mg/kg ketamine (Gutian Pharmaceuticals, FuJian, China). Pupils were dilated by a mixture of 5 mg/ml phenylephrine and 5 mg/mL tropicamide (Santen Pharmaceutical Co., Ltd., Osaka, Japan), and 4 mg/ml oxybuprocaine (Santen Pharmaceutical Co., Ltd) was applied as topical anaesthesia. The extent of mydriasis was checked under a surgical microscope (OM-10; Takagi Seiko Co., Ltd., Nagano, Japan). Anesthetization lasted for about 30-50 min and was sufficient for performing the subretinal injection. After the procedure, ofloxacin gel (Xing Qi Medical Company, Shenyang, China) was applied to prevent corneal opacification and reduce the risk of infection. This study was approved by the Animal Ethics Committee of Fudan University Animal Center.

Subretinal Injection and Fundus Photography

0.4% TB (Sigma-Aldrich, St. Louis, MO, USA) was diluted to final concentrations of 0.2%, 0.08%, 0.04%, and 0.02% in phosphate-buffered saline (PBS). Four μL of TB solution was slowly injected into the subretinal space of the rat eye. The contralateral eye that was injected with PBS was used as a control. Sham-injected and un-injected normal eyes served as additional control groups. Each group contained at least ten eyes. After pupil dilation, anaesthetized rats were placed under a surgical microscope in a lateral recumbent position. A 6-mm-diameter rubber ring was placed on the surface of the cornea, and one drop of carbomer eyedrops (Bausch & Lomb, Berlin, Germany) was applied within the ring to visualize the fundus. A 27-gauge needle (Meesawat Medical Industry Company, Shanghai, China) was used to puncture the sclera from a temporal direction at about 1-2 mm from the limbus (Figure 1A). The needle was inserted with the bevel up at an angle of 45° to the posterior lens face and was advanced through the sclera into the vitreous humor. At least 50% of the bevel was pushed through the sclera to produce a hole sufficient to insert a blunt 33-gauge needle (Hamilton Company, Reno, NV, USA). The tip of the blunt needle was inserted through the puncture in the sclera, advanced into the vitreous avoiding damage to the lens, and then, aimed slightly towards the retina (Figure 1B). A feeling of slight resistance to the movement of the needle indicated when it had penetrated the retina and entered the subretinal space. Four μL of fluid was then slowly injected into the subretinal space over a period of 20 s (Figure 1C). This generated a visible detachment in about 1/4 circle of the retina (the inner boundary is about 1 mm from the optic nerve head) (Figure 2A-2D). The detachment lasted no longer than 24 h (Figure 2E-2H). The needle was gently withdrawn from the eye after subretinal injection. Fundus images were then obtained with a Kodak digital camera (FZ152; Eastman Kodak Company, Rochester, NY, USA).

Electroretinography (ERG)

ERG was performed to assess retinal function. The rats received a subretinal injection of TB at a concentration of 0.2%, 0.08%, or 0.04% in one
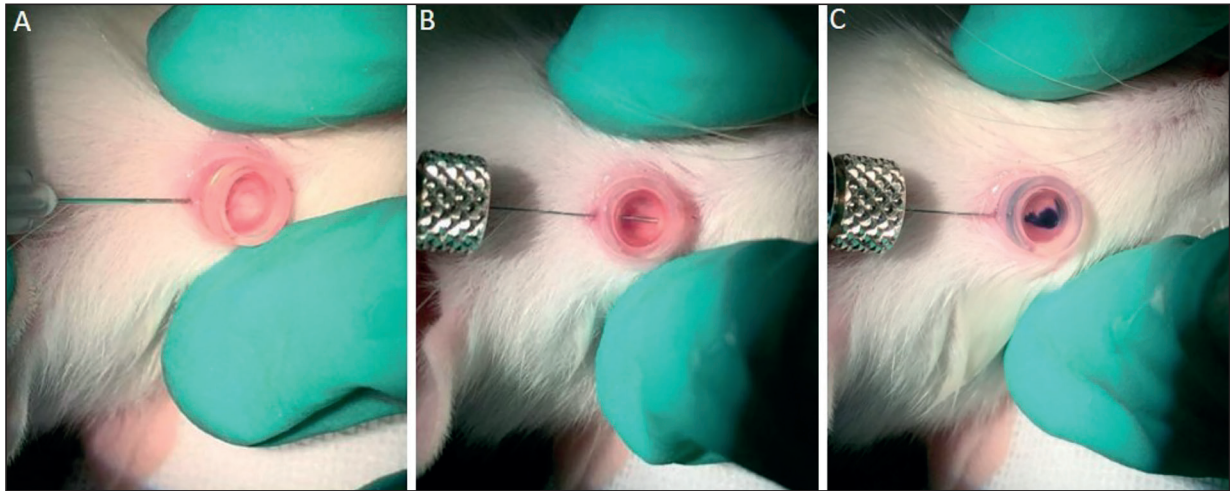
Figure 1. Procedure of subretinal injection in rat eyes. *A,* A 27-gauge needle was used to puncture the sclera from a temporal direction at about 1-2 mm from the limbus. *B,* A 33-gauge blunt needle from a microsyringe was inserted through the vitreous until it reached the retina on the opposite side of the eye. *C,* The needle was carefully inserted into the retina and the fluid was injected into the subretinal space.

eye, while the contralateral eye received PBS- or sham-injections to serve as a self-control. Full-field ERG of both eyes was recorded in all rats at 14 days after subretinal injection. Each group contained at least ten rats. Before ERG, pupils were dilated as described above, and the rats were dark adapted for 30 min. Thereafter, rats were anesthetised as described above. Under infrared, rats were placed on a warming pad to maintain the body temperature. Oxybuprocaine and carbomer gel were applied to the cornea, and corneal contact electrodes were attached. A skin electrode was placed 5 cm from the tip of the tail. The grounding electrode was affixed to the nose. The ERG recording was carried out using an Espion Visual Electrophysiology System (Espion E³; Diagnosys UK Ltd., Cambridge, UK). The procedure was designed to record retinal responses of both rods and cones. Under scotopic conditions, a single flash (10 ms) was applied at a low intensity of 1 cd-s/m² with a sweep delay of 10 s in order to record good waveforms; 10 stimuli were included in each cycle. The wave amplitude was determined for each response. The a-wave amplitude was measured from the baseline to the peak of the first negative deflection, and the b-wave amplitude was measured from the baseline to the first positive deflection.

Tissue Collection and Cryosection for Light Microscopy

Rats were euthanized at 1, 7, or 14 days after subretinal injection. Five rats were included in

each group at each time-point. The eyes (including the optic nerve) were enucleated and immersion-fixed in 4% paraformaldehyde (Weiao Bio-technology Company, Shanghai, China) overnight at 4 °C. Following fixation, the eyeballs were immersed in 20% sucrose in PBS for $\geq$ 5 h followed by 30% sucrose in PBS for 5 h. The cornea, lens, and vitreous were removed without disrupting the retina. The posterior eyecups were then embedded in Tissue-Tek OCT 4583 cryostat compound (Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen at −30°C. Cryosections (10 mm) (Leica CM3050 S; Leica Biosystems, Eisfeld, Germany) were collected onto adhesive microscope slides (Citoglas Experimental Equipment Company, Jiangsu, China) and stored at −30°C until further analysis.

Terminal Deoxynucleotidy1 Transferase dUTP nick-end labeling (TUNEL)

To determine the TB toxicity on retinal cells, we examined retinal apoptosis with TUNEL at 1 and 7 days after subretinal injection. TUNEL was carried out using an In Situ Cell Death Detection Kit, TMR Red (Roche Applied Science, Mannheim, Germany). In accordance with the manufacturer's protocol, tissue sections were fixed in 4% paraformaldehyde for 30 min at room temperature and washed 4 times in PBS for 30 min. The sections were then incubated in 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) prepared in 0.1% trisodium citrate dehydrate (Sinopharm Chemical Com-
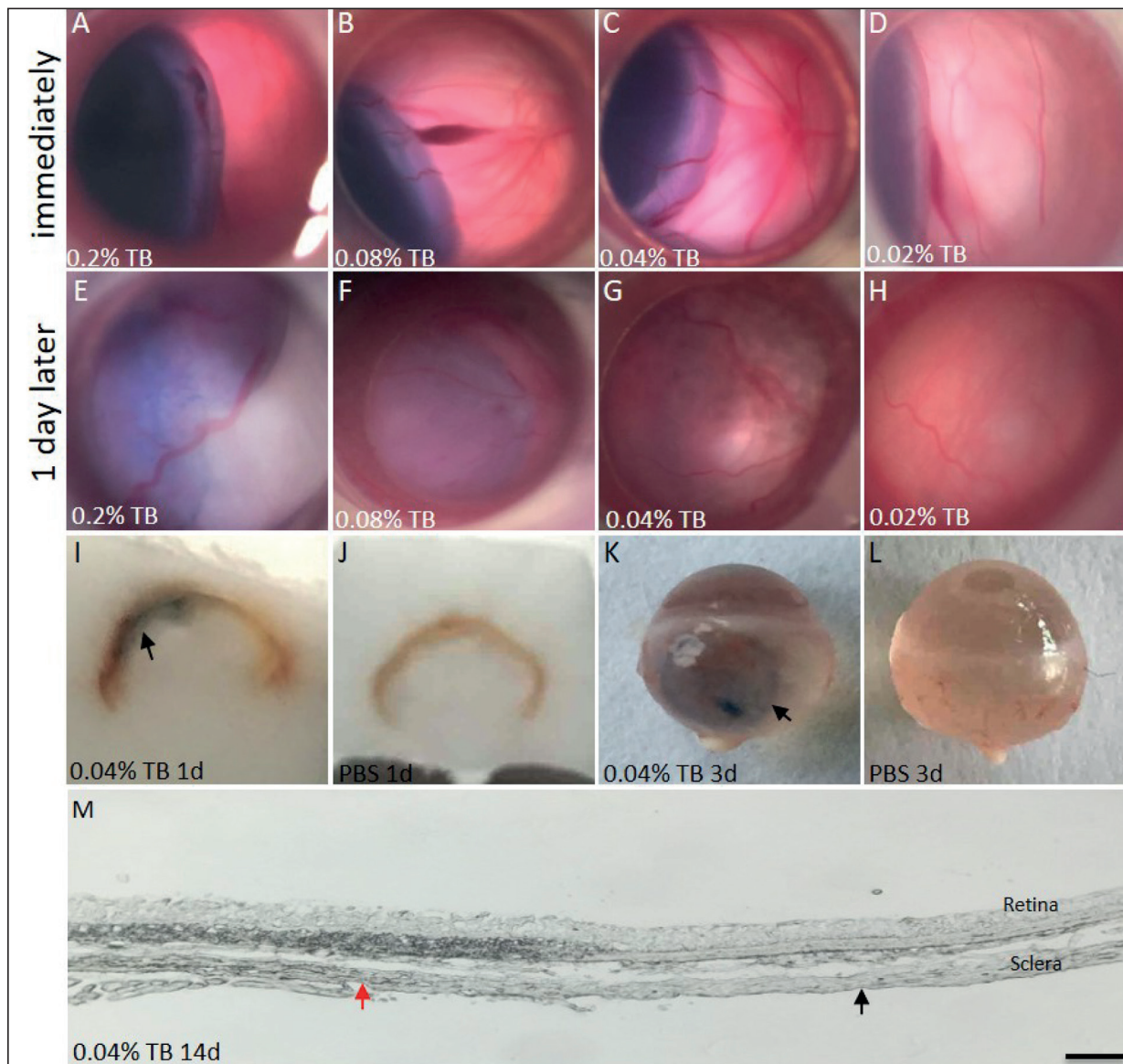
Figure 2. Subretinal injection of TB successfully stained the retina. **A-D,** Images taken by a surgical microscope immediately after a subretinal injection of TB at a concentration of 0.2% (**A**), 0.08% (**B**), 0.04% (**C**), and 0.02% (**D**). **E-H,** Images taken by a surgical microscope at 1 day after subretinal injection of 0.2% (**E**), 0.08% (**F**), 0.04% (**G**), and 0.02% (**H**) TB. (**I,J**) Images of OCT-embedded posterior eye cups taken as 1 day after 0.04% TB (**I**) or PBS (**J**) injection. Note the blue staining (black arrow) in TB-, but not PBS-injected eye cups. (**K,L**) Images of the eyeballs taken at 3 days after subretinal injection of 0.04% TB (**K**) or PBS (**L**). Note the blue stain (*black arrow*) on the sclera of TB-, but not PBS-injected eye. (**M**) Cryosection taken at 14 days after the injection of 0.04% TB. Light microscopy revealed staining on the outer layers of the retina and sclera (red arrow) that differed in appearance from the un-injected area (*black arrow*). Scale bar: 100 mm. Immediately: Immediately after injection; TB: trypan blue; PBS: phosphate-buffered saline.

pany, Shanghai, China) in PBS at 4°C for 4 min. TUNEL reaction buffer was added at a dilution of 1:9 (solution 1:solution 2), and the slides were placed at 37°C in a thermostat (Yiheng Technology Company, Shanghai, China, DHP-9052) for 1 h. Retinal sections were then counterstained with a nuclear marker, Hoechst 33258 pentahydrate (1:1000; Life Technology, Eugene, OR, USA) to reveal retinal laminar structures. Images were taken with a confocal fluorescence microscope (Leica SP8, Leica Microsystems, German). Afield size of 6.25×10^{-2} mm^2 per image was analyzed. Cells stained positive for TUNEL were counted, and a minimal 10 sections per rat, five rats per group, were analyzed.

Haematoxylin and Eosin (H&E) Staining and Immunohistochemistry for Morphological Analysis

H&E staining was performed to assess the effects of TB on retinal morphology. Enucleated eyes (including the optic nerve) were cryosectioned. The slides containing a TB stained retinal area and the optic nerve head were selected and processed for H&E staining. Briefly, retinal sections were immersed in 4% paraformaldehyde for 4 h and transferred to 70% ethanol. Individual samples were placed in processing cassettes, dehydrated through a serial alcohol gradient, and embedded in paraffin. For immunofluorescence staining, retinal sections were deparaffinized in xylene, rehydrated through a decreasing ethanol gradient, and washed in PBS. The images of H&E stained retinal sections were acquired with a light microscope (Leica DM 4000B, Wetzhan, Germany). ONL thickness was quantified by counting the layers of photoreceptor nuclei in retinal sections at ~1 mm away from the optic nerve head. A minimal 10 sections per rat, five rats per group, were analyzed.

Statistical Analysis

The mean number of TUNEL$^+$ cells and ONL thickness were compared among groups by one-way analysis of variance (ANOVA) with Tukey's post-hoc test. To compare differences of ERG data between the left and right eyes, a paired Student's *t*-test was used, and $p < 0.05$ was considered statistically significant. Error bars represent the standard deviations. All statistical analyses were conducted using GraphPad 5 Prism 5 software (GraphPad, San Diego, CA).

Results

Tissue Staining by Subretinal Injection of TB

Based on previous reports, the concentrations of TB between 0.02%-0.2% were tested. Immediately after subretinal injection of 0.2%, 0.08%, 0.04% or 0.02% TB, blue stains were observed in the injected area with a surgical microscope (Figure 2A-2D). One day after injection, the blue staining remained detectable with surgical microscopy (Figure 2E-2G) or in OCT-embedded eyecups in the 0.2%, 0.08%, and 0.04% (Figure 2I) TB-injected groups, but not in the 0.02% TB-injected group (Figure 2H) or PBS-injected group (Figure 2J). Three days after injection,

blue staining was visible in the sclera in the 0.2%, 0.08%, and 0.04% TB-injected groups (Figure 2K) and not in the PBS-injected group (Figure 2L). Moreover, one day after injection, the injected region revealed a uniform color in the outer layers of the retina and sclera (red arrow in Figure 2M) of the 0.2%, 0.08%, and 0.04% TB-injected groups compared to the un-injected region under a light microscope (black arrow in Figure 2M). A blue stained area was visible in cryosections of the 0.04% (Figure 2M) and all other (data not shown) TB-injected groups even 14 days after injection. Thus, subretinal injections of TB at a concentration $\geq 0.04\%$ effectively labeled the injected retinal areas for over 14 days. In contrast, 0.02% TB injection was inefficient in staining the retina and was thus excluded in the following experiments.

TB Impairs Retinal Function as Measured by ERG

To evaluate the effects of TB of various concentrations on retinal physiology, ERGs were performed. Fourteen days after injection, the amplitudes of ERG a- and b-waves were significantly reduced in the 0.2% (Figure 3A, 3D, 3J) or 0.08% (data not shown) TB-injected group, but not 0.04% (Figure 3B, 3C, 3E, 3F, 3I) TB-injected group when compared to the PBS-injected group (Figure 3A, 3B, 3D, 3E, 3G). No significant differences in ERG a- and b-waves were noted between the 0.04% (Figure 3B, 3C, 3E, 3F, 3I) TB-injected group and sham-injected (Fig. 3C, 3F, 3H) groups. These results indicate that while injection of a high concentration of TB (e.g., 0.2%) significantly impairs ERG a- and b-waves, injection of a lower concentration of TB (e.g., < 0.04%) is as safe as PBS-and sham-injections.

Cytotoxicity of TB in Retinal Cells

The cytotoxicity of TB was further evaluated by TUNEL. One day after the injection of 0.2% and 0.08% of TB, TUNEL$^+$ cells were found primarily in the outer nuclear layer (ONL) (Figure 4A, 4B) and ganglion cell layer (GCL) (Figure 4E, 4F) of the retina. A small number of TUNEL$^+$ cells was also noted in the RPE layer (Figure 4I, 4J). By 7 days after injection, TUNEL$^+$ cells were still detectable in 0.2% and 0.08% TB-injected groups (Figure 4M, 4N). In contrast, 0.04% TB- and PBS-injected groups showed very few TUNEL$^+$ cells in the retina at 1 or 7 days after injection (Figure 4C, 4D, 4G, 4H, 4K, 4L, 4O, 4P). The numbers of TUNEL$^+$ cells were sig-
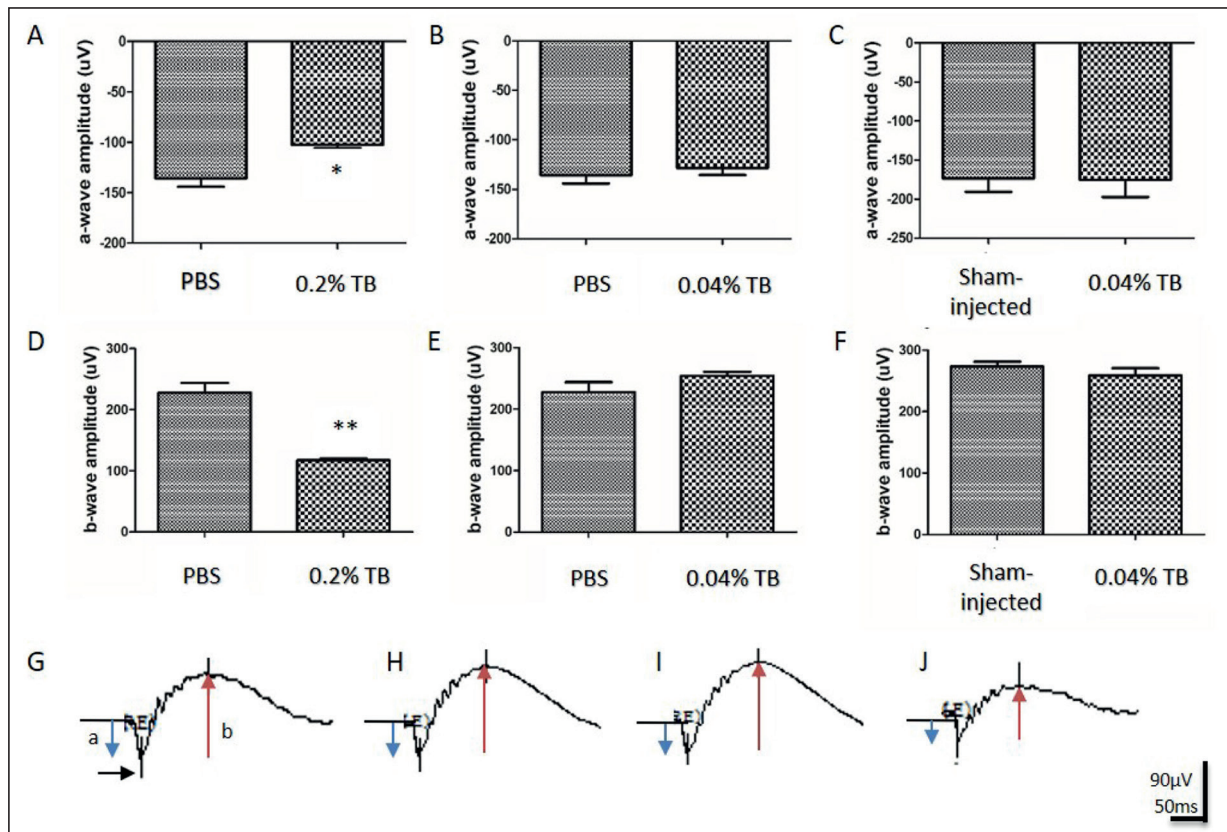
Figure 3. Retinal function measured by ERG at 14 days after subretinal injection. ***A-C,*** The amplitudes of a-waves in PBS-, 0.2% and 0.04% TB- and sham-injected groups. ***D-F,*** The amplitude of b-waves in PBS-, 0.2% and 0.04% TB- and sham-injected groups. ***G-J,*** Representative ERG waveforms taken from the PBS- (***G***), sham-(***H***), 0.04% TB- (***I***) and 0.2% TB-injected groups (***J***). Blue and red arrows indicate a-wave and b-wave, respectively. Black arrows illustrate how to measure time-to peak. *$p < 0.05$ and **$p < 0.01$ (paired Student's t test). ERG: electroretinography.

nificantly greater in retinal sections of 0.2% and 0.08% TB-injected groups than in those of 0.04% TB- and PBS-injected groups (Figure 4Q) ($p < 0.0001$). There were no significant differences in numbers of TUNEL+ cells between 0.2% and 0.08% TB-injected groups or between 0.04% TB- and PBS-injected groups (Figure 4Q). In conclusion, 0.04% TB injection is no more cytotoxic to the retina than PBS injection.

Histological Analysis of TB-Treated Retina

Histological analysis of TB-treated retina revealed that 14 days after TB injection, the injected area (white arrow in Figure 5A) in the 0.2% TB-injected group exhibited apparently thinner retina, particularly the ONL, than the retina in the un-injected area (black arrow in Figure 5A). It supports a direct retinal cytotoxicity by 0.2% TB. In contrast, the retinas of the 0.04% TB-injected group exhibited no differences in retinal

morphology and retinal thickness between the injected (white arrow in Figure 5B) and un-injected area (black arrow in Figure 5B). Quantification results showed no significant differences in ONL thickness among the normal (uninjected), sham-, PBS- and 0.04% TB-injected groups (Figure 5C-5F, 5H). An over 60% reduction of ONL thickness in the 0.2% TB-injected group compared to the normal retina was noted (Figure 5C, 5G, 5H) ($p < 0.0001$). The data indicate that 0.04% TB injection does not alter retinal morphology or ONL thickness.

Discussion

This study presents a series of *in vivo* experiments that analyzed the safety and tissue-staining efficacy of TB following a subretinal injection in the rat. Our data show that subretinal injection of TB at a concentration as low as 0.02% was
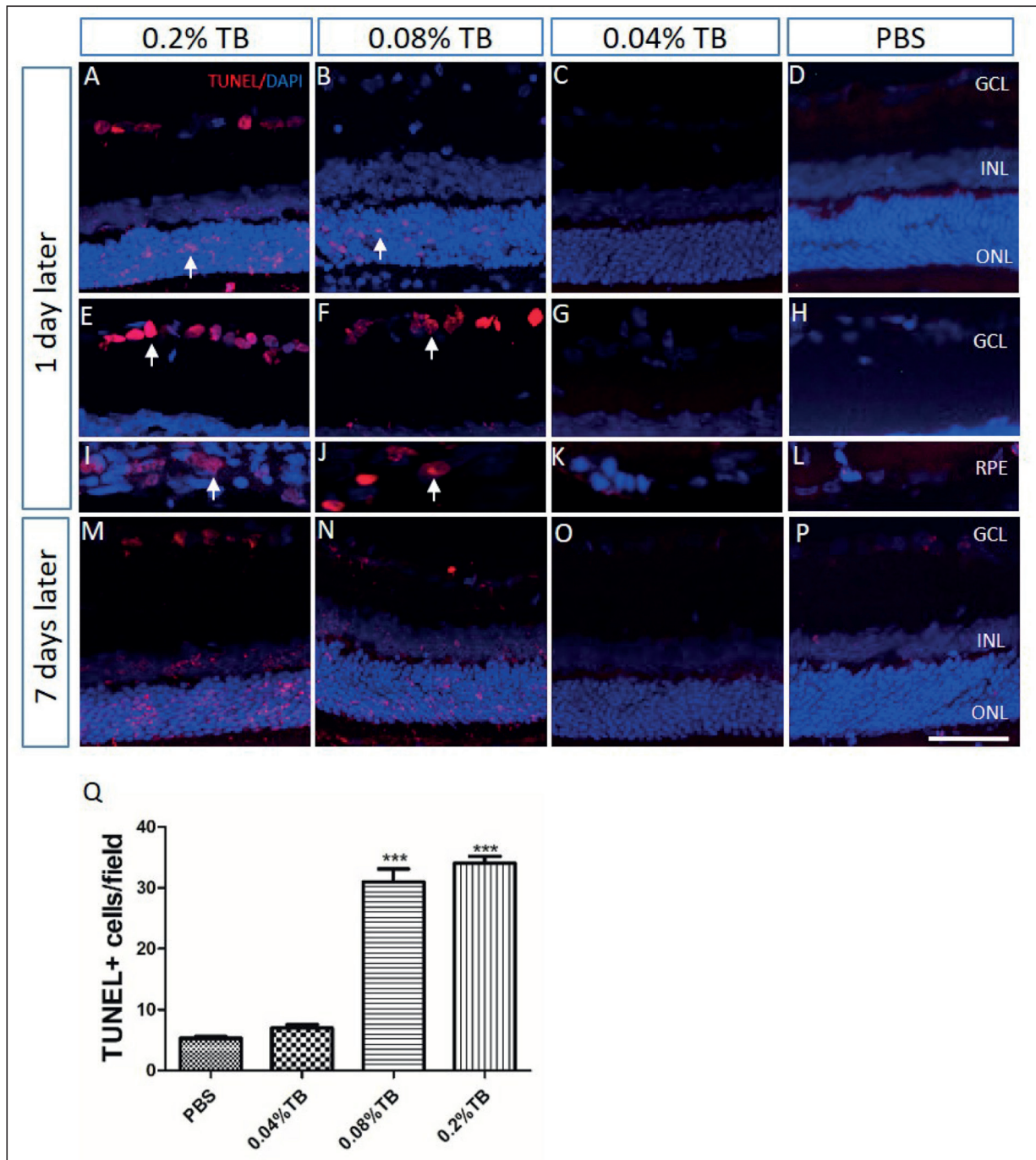
Figure 4. Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) in the retina. ***A–D,*** Representative photomicrographs of TUNEL-labelled retinal sections taken from rats at 1 day after subretinal injection of 0.2% (***A***), 0.08% (***B***), 0.04% (***C***) TB, or PBS (***D***). Note TUNEL$^+$ cells (arrows) in the ONL of 0.2% (***A***) and 0.08% TB-injected groups (***B***). ***E–H,*** Representative photomicrographs of the GCL taken from rats at 1 day after subretinal injection of 0.2% (***E***), 0.08% (***F***), 0.04% (***G***) TB, or PBS (***H***). Note TUNEL$^+$ cells (arrows) in the GCL of 0.2% (***E***) and 0.08% TB-injected groups (***F***). ***I–L***, Representative photomicrographs of the TUNEL-labelled RPE layer from rats at 1 day after subretinal injection of 0.2% (***I***), 0.08% (***J***), 0.04% (***K***) TB, or PBS (***L***). Note TUNEL$^+$ cells (arrows) in the RPE layer of 0.2% (***I***) and 0.08% TB-injected groups (***J***). ***M-P,*** Representative photomicrographs of TUNEL-labelled retinal sections taken from rats at 7 day after subretinal injection of 0.2% (***M***), 0.08% (***N***), 0.04% (***O***) TB, or PBS (***P***). Scale bars: 50 mm. GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer; RPE: retinal pigment epithelium. (***Q***) Numbers of TUNEL$^+$ cells (ANOVA with Tukey's post-hoc test). ***$p < 0.0001$.
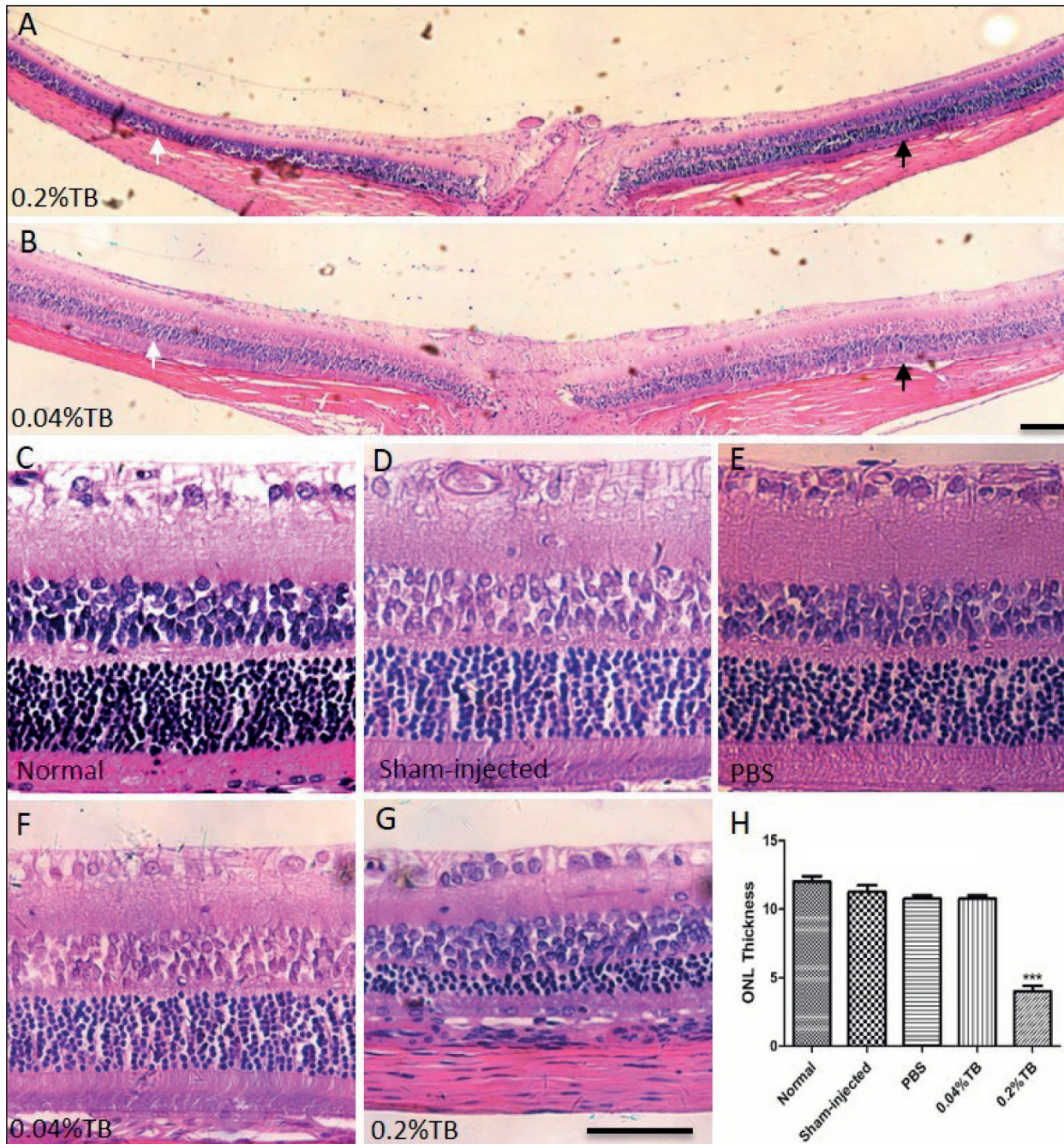
Figure 5. Histochemical analysis of the retina. ***A-B,*** Representative images of H&E stained retinal sections taken at 14 days after subretinal injection of 0.2% (***A***) or 0.04% TB (***B***). Note the thinning of the retina in0.2% TB-injected group (white arrow in A) compared to the un-injected area (black arrow in A). Scale bars: 100 μm. ***C-G,*** H&E stained retinal sections taken from the normal (***C***), sham-injected (***D***), PBS-(***E***), 0.04% TB- (***F***), and 0.2% TB-injected groups (***G***). Scale bars: 100 μm. (***H***) Quantification of ONL thickness. **$p < 0.01$, ***$p < 0.0001$ (ANOVA with Tukey's post-hoc test).

ineffective at staining the retina; whereas, 0.04% of TB was both effective and safe to the injected retinal area, without causing any adverse effect. A higher dose of TB at a concentration of 0.08% induced retinal cytotoxicity following injection as revealed by TUNEL, while 0.2% of TB caused further damage, including reduction of ONL thickness and impairment retinal function. Our results thus indicate an effective and safety dose of TB in the rat retina.

A toxic effect of TB has been suggested previously in *in vitro* and *in vivo* experiments. Although an acute exposure (less than or equal to 5 min) of RPE cells to TB seemed safe[37,38], even at a relatively high concentration (0.30%)[39], the safety doses of TB to RPE cells became much lower (less than 0.05%) under the chronic exposure (> 3 days)[23]. Retinal neurons, however, are more susceptible to TB toxicity than RPE cells. A short exposure (within 30 min) to 0.06% TB or a 3-day exposure to low concentrations (0.01%-0.025%) of TB induced cytotoxicity in RGCs[40]. In clinic, injection of 0.15% TB in patients caused retinal thinning and chorioretinal atrophy in areas showing TB migration, as revealed by optical coherence tomography[12]. Saeed and Heimann[14] also noted an association between TB administration and retinal lesion that included increased retinal pigmentation and RPE atrophy over a 2-week period after vitrectomy. In rabbit studies, Maia et al[41] and Penha et al[42] also showed that subretinal injection of 0.15% TB caused photoreceptor damage within 12 h after surgery, and the toxic effect of TB was observable even after 14 days. In the present study, we demonstrated that TB induced retinal damage in a dose-dependent manner in rats.

The reported safety dose of TB *in vitro* was slightly lower than that was seen *in vivo*. Likely, this is because the concentration of TB in the subretinal space is inaccurate or diluted due to fluid diffusion and outflow, while the TB concentration in cell cultures is more controllable. Second, cells embedded in the retinal structure might be more resistant to TB toxicity than dissociated cells in a culture dish. Presence of Müller glia and other cell types in the retina may serve to maintain retinal homeostasis and provide structural and trophic support to the surrounding cells.

In our work, 0.04% is a safe concentration for subretinal injection of TB. We assessed cell apoptosis with TUNEL. A large number of TUNEL+ cells were detected in the ONL, INL, and GCL, as well as the RPE layer of 0.08% and 0.2% TB-injected groups, even at 7 days after injection. These results are consistent with the ERG assessment, in which the amplitudes of both a- and b-waves were significantly reduced after exposure to high concentrations of TB. In contrast, TUNEL+ cells were barely detected in the 0.04% TB-injected group. Notably, subretinal injection of high concentrations of TB (0.08% and 0.2%) also showed toxic effects on RGCs *in vivo*. We suggested that TB might have leaked into the vitreous cavity through the retinal hole generated by the syringe that entered *via* the vitreous. Together, the results of our study indicate that high concentrations of TB (0.08% and 0.2%) have a toxic effect on retinal cells, including ONL, INL, GCL, and RPE cells.

The cytotoxic effect of TB is likely to exist in humans. Currently, we do not yet know if the safety dose of TB in the retinas of humans and rats is the same. It is clear that low concentrations (e.g., less than or equal to 0.04%) of TB are likely safer than the higher concentrations (greater than 0.08%). Thus, if a vitreous or subretinal injection of a high concentration of TB (greater than or equal to 0.08%) is unavoidable, we suggest that it should be removed as soon as possible. While our study used only albino rats, it is most likely that TB induces a similar toxicity effect in the retinas of pigmented rats, just as it has been reported for many ophthalmic drugs in albino and pigmented rats[43-45].

Conclusions

Since TB which has been initially introduced to the clinic, particularly vitreoretinal surgery, as a remarkable biocompatible dye, many researches[24,37,38,40] have reported a toxic effect of TB in the retina in a dose- and time-dependent manner. Our study in rats indicates that 0.04% TB is a safe and effective dose of TB for subretinal injection to localize the treated area under a light microscope. Currently, more long-term investigations are required for further verification of TB's safety in the retina.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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