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Dental pulp stem cells therapy overcome photoreceptor cell death and protects the retina in a rat model of sodium iodate-induced retinal degeneration

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ABSTRACT

Blindness and vision loss contribute to irreversible retinal degeneration, and cellular therapy for retinal cell replacement has the potential to treat individuals who have lost light sensitive photoreceptors in the retina. Retinal cells are well characterized in function, and are a subject of interest in cellular replacement therapy of photoreceptors and the retinal pigment epithelium. However, retinal cell transplantation is limited by various factors, including the choice of potential stem cell source that can show variability in plasticity as well as host tissue integration. Dental pulp is one such source that contains an abundance of stem cells. In this study we used dental pulp-derived mesenchymal stem cells (DPSCs) to mitigate sodium iodate ($NaIO_3$) insult in a rat model of retinal degeneration. Sprague-Dawley rats were first given an intravitreal injection of 3×10^5 DPSCs as well as a single systemic administration of $NaIO_3$ (40 mg/kg). Electroretinography (ERG) was performed for the next two months and was followed-up by histological analysis. The ERG recordings showed protection of DPSC-treated retinas within 4 weeks, which was statistically significant (* $P \leq .05$) compared to the control. Retinal thickness of the control was also found to be thinner (*** $P \leq .001$). The DPSCs were found integrated in the photoreceptor layer through immunohistochemical staining. Our findings showed that DPSCs have the potential to

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moderate retinal degeneration. In conclusion, DPSCs are a potential source of stem cells in the field of eye stem cell therapy due to its protective effects against retinal degeneration.

1. Introduction

Retinal degenerative diseases are caused by many genetic and environmental factors, and can lead to incurable blindness even in developed countries. This complete impairment of vision tends to be the major outcome when left untreated due to the loss of photoreceptors [1]. The pathological pathways of these diseases differ on onset, but the ensuing degeneration share similar mechanisms [1]. The apoptosis that occurs in the degenerating retina can involve for example, the outer nuclear layer (ONL), inner nuclear layer (INL), and photoreceptor layer [2]. Some retinal cells are capable of evading apoptosis, and this may be utilized in visual restoration with treatments such as implants [3]. Other than implants, transplanting functional retinal cells may be key to restoring vision.

In animal models, replacing dysfunctional photoreceptors was shown to have an influence on retinal function and morphology. This replacement and protection approach in preclinical research are being done with various cell types, and is a more feasible, short-term aim due to the slow-progressing nature of most clinical conditions [4]. It is postulated that this protection and maintenance of retinal function is from the release of growth factors by donor cells. Apart from retinal neurons that make up most of the retina, these methods can also improve retinal pigment epithelium dystrophy [5]. Currently, age-related macular degeneration (AMD) and retinitis pigmentosa (RP) are the main research focus in the application of a suitable stem cell therapy [4,6–8].

Dental pulp stem cells (DPSCs) are a type of stem cell that have been recently hypothesized to have regenerative and protective properties in eye diseases model [9]. The use of these neural crest-derived cells in neural regenerative medicine has gained widespread popularity because of its relative ease of isolation from adult teeth, which makes it easily accessible from patients of all ages [10]. They also surmised that the neural crest properties of DPSCs make these cells more suitable than other mesenchymal stem cells, for example bone marrow derived mesenchymal stem cells in treating central nervous system (CNS) injury [9]. However, there are contradicting evidence regarding the differentiation of DPSCs into neurons *in vitro* that either supports it [11,12] or are against it [9,13].

Interestingly, it has been emphasized that DPSCs function in neural support by way of paracrine secretions [14], which was shown in a study on spinal cord injury treatment in rodent models to improve loco motor function [15]. The paracrine mechanism was found to involve the expression of neurotrophic factors (NTFs) such as nerve growth factor (NGF), brain derived-neurotrophin factor (BDNF), and neurotrophin-3 (NT-3). In our study, the main objective is to utilize DPSCs as a stem cell source to both protect and replace the degenerating retinal cells in a Sprague Dawley rat model of retinal degeneration.

2. Methods

2.1. Dental pulp stem cells culture and expansion conditions

Dental pulp stem cells were obtained from a stem cell bank, CryoCord (Cyberjaya, Selangor, Malaysia) at passage 2, and stored in liquid nitrogen until further use. In brief, the 1 ml ampoule of DPSCs was thawed in a water bath at 37 °C for 1 min (min) and transferred to a 15 ml centrifuge tube containing 9 ml of complete culture medium. The medium consisted of DMEM-F12 (Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Massachusetts, USA), and 1% penicillin-streptomycin (Thermo Fisher Scientific, Massachusetts, USA). The tube was then centrifuged for 5 min at 1200 rpm. Cell count was performed after the DPSCs pellet was resuspended in 1 ml of complete culture media. A seeding density of 3000 cells per cm^2 was used for standard culture conditions, where DPSCs were incubated at 37C^o in 5% CO_2. The media was replenished every 3 days until 70% confluency. For expansion, DPSCs were trypsinized with 0.25% EDTA (Thermo Fisher Scientific, Massachusetts, USA), centrifuged for 5 mins at 1200 rpm, and reseeded accordingly.

2.2. Immunophenotyping of dental pulp stem cells

Immunophenotyping of DPSCs was performed with the FACS Aria III (Beckon Dickinson Biosciences, USA) flow cytometer. In summary, a total of 2.0×10^5 DPSCs were aliquoted into several flow tubes, and centrifuged for 7 min at 2000 rpm to form cell pellets. After aspiration, the pellets were re-suspended in 100 µl of 0.2% BSA (Sigma-Aldrich, Missouri, USA) in 1 × phosphate buffered saline (PBS). Fluorochrome-conjugated antibodies were added to the tubes and incubated at room temperature (24 °C) for 30 min. The antibodies (Abcam, Cambridge, UK) were specific to CD90, CD73, CD166, CD44, CD34, CD45, HLA-DR, and CD19. The cells were then washed with 1 × PBS and centrifuged at 2000 rpm for 7 min. The pellets were re-suspended in 400 µl of 0.2% BSA in PBS and analyzed using flow cytometry. The acquired data was analyzed using BD FACSDiva Software v6.1.2 (Becton, Dickinson Biosciences, USA).

2.3. Animal care and production of retinal degeneration model

Male Sprague Dawley rats, weighing approximately 150–300 g at 6–8 weeks old, were purchased from Sinar Scientific (Selangor, Malaysia), and housed in an animal biobubble facility (Tissue Engineering Centre, Kuala Lumpur, Malaysia). Food pellets (Altromin, Lage, Germany), water, and bedding were provided/changed *ad lib.* The housing conditions were maintained under a specific environment at 21 °C with a 12-h light/12-h dark cycle. To produce the retinal degeneration model, sodium iodate ($NaIO_3$) (Alfa Aesar, Massachusetts, USA) was dissolved in Hank's balanced salt solution (HBSS) to achieve 4% and stored at room temperature (24 °C). The solution was systemically administered at a dose of 40 mg/kg through the tail vein. The control group was given HBSS injection. All animals were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental protocols were approved by the Animal Care and Use Committee of the University Putra Malaysia through the Office of Animal Resources.

2.4. Intravitreal transplantation of dental pulp stem cells

Dental pulp stem cells were transplanted 48 h prior to $NaIO_3$ administration by first re-suspending centrifuged DPSC pellets in Hank's Balanced Salt Solution at a density of 10,000 cells/ul. The animals were anesthetized with a cocktail of ketamine (35 mg/kg, Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (5 mg/kg, TranquiVed; Vedco, Inc., St. Joseph, MO). The cornea of the right eye was anesthetized with 0.5% proparacaine hydrochloride (Alcon, USA), and a tract into the intravitreal space was made using a 30 G needle. Then, 3 ul of DPSC suspension was injected into the space using a 30 G, 10 ul blunt Hamilton syringe (Hamilton Co., Reno, NV) through the supertemporal area posterior to the lens. Taking care, to avoid lens

penetration and vein vortex damage, a special ophthalmoscope was used to view the fundus (Volk Optical Inc., Mentor, OH). The un-injected left eye served as a control.

2.5. Electroretinography of rat visual functions

Rats were first dark adapted for 12 h (h) before the test. Anesthesia was administered through intramuscular injection of ketamine (35 mg/kg, Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (5 mg/kg, TranquiVed; Vedco, Inc., St. Joseph, MO). The pupils were then dilated with topical 1% tropicamide (Alcon, USA) and the corneas anesthetized with 0.5% of proparacaine hydrochloride (Alcon, USA). Silver chloride electrodes were placed on the cornea of each eye, the reference electrode was placed on an ear while the ground electrode was placed in the tail. The scotopic ERG full-field readings were then taken under 3.0 cd.s/m^2 standard flash. Twelve separate responses were taken and averaged with the Roland Consult ERG system (RETI-port, Roland Consult; Brandenburg, Germany). Readings were taken on day 1, day 40, and day 70 after $NaIO_3$ administration.

2.6. Enucleation and histopathology of stem cell-treated retina

Upon completion of final ERG, the rats were euthanized by lethal injection of sodium pentobarbital (150 mg/kg). The eyes were enucleated and snap-frozen in liquid nitrogen. After OCT-embedding and equilibration at −20 °C, the tissues were then sectioned into slices of 4 um and mounted on histological slides. After fixation with 4% paraformaldehyde for 15 min, standard Hematoxylin & Eosin (H&E) (Sigma Aldrich: Steinheim, Germany) staining was performed. The stained slides were mounted and a coverslip was placed before being observed under a light microscope.

2.7. Immunofluorescent staining of transplanted dental pulp stem cells in the retina

The sections were permeabilized with 0.15% triton X-100 in 1 × PBS and washed with 0.025% triton X-100 in 1 × PBS, three times. The sections were then blocked with 10% fetal bovine serum (FBS) and 1% bovine serum albumin in 1 × PBS and incubated for 2 h at room temperature. Retinal primary antibodies were added to the sections— 1:250 rabbit anti-PKCα (cat no. AB32376, Abcam, Cambridge, UK), 1:250 mouse anti-rhodopsin (cat no. sc-57,432, Santa Cruz Biotechnology, Texas, USA), 1:500 mouse anti-RPE65 (cat no. AB78036, Abcam, Cambridge, UK), or mouse anti-stem 121 (cat no. Y40410, TaKaRa, Shiga, Japan), and incubated overnight. After that, the sections were washed three times and secondary antibodies were added— 1:400 anti-mouse AF594 (cat no. AB150116, Abcam, Cambridge, UK) or 1:400 anti-rabbit AF594 (cat no. AB150080, Abcam, Cambridge, UK) to the retinal sections and incubated for 1 h at room temperature. The slides were washed again, and mounting medium (Santa Cruz Biotechnology, Texas, USA) was added with a coverslip. The sections were then viewed under a fluorescent microscope (Olympus, Tokyo, Japan).

2.8. Statistical Analysis

Mean values ± SD were calculated from the data obtained from 6 rats per experimental group using Statistical 5.0 software (Chicago, IL, USA). The statistical analysis for ERG recordings was performed using one way-ANOVA with Tukey's multiple comparisons test. The retinal thickness analysis was performed using an unpaired *t*-test. P values < .05 were considered significant.

3. Results

3.1. Immunophenotyping of dental pulp stem cells

Dental pulp stem cells exhibit fibroblast-like morphology in standard culture conditions (Fig. 1). The DPSC immunophenotype consisted of highly expressed CD90 (99.3%), CD73 (99.7%), CD44 (99.3%), and a slightly lower expression of CD166 (60.9%). On the other hand, there were no expressions of endothelial and hematopoietic cell markers – CD19, HLA-DR, CD34, and CD45 (Fig. 2).

3.2. Electroretinographic and histological analysis of DPSC-treated retinas

Electroretinography was performed throughout the study. The scotopic response at 3.0 cd.s/m^2 was detected and recorded for two months following cell transplantation. The b-wave amplitude detected in DPSC-treated rats and control rats (HBSS) displayed differences on day 1, day 40 and day 70. For both DPSC-treated rats and control rats, b-waves were shown to decrease with time but on day 40, the former demonstrated a slower deterioration of retinal functions and yielded a statistically significant result in comparison with the HBSS control group (Fig. 3A). The difference could be observed in the ERG waveforms (Fig. 3B). On day 70, there were no obvious changes between both the treated retina and the control. Interestingly, histological analysis of the retinal sections showed that DPSC-treated retinas were significantly thicker than the control after one month following stem cell treatment (Fig. 3C). Sodium iodate, when administered, causes retinal degeneration particularly in the RPE and layer of rods and cones (Fig. 3D).

3.3. Immunohistochemical staining of DPSCs in the treated retina

Immunohistochemical staining of sectioned retinas were performed to identify localized DPSCs in the treated retina. The retinal sections were stained with antibodies specific to retinal cell markers- rhodopsin (Rho) and RPE65. Anti-STEM121 antibodies were used to track the transplanted DPSCs two months after intravitreal transplantation. 4′,6-diamidino-2-phenylindole (DAPI) was used as a counter stain in the retina for nucleus visualization. In a healthy retina, rho and RPE65 expression is highly specific, and is confined to the photoreceptor layer and RPE layer, respectively (Fig. 4A). The administration of sodium iodate resulted in the degeneration of the layers and hence, a loss of rho and RPE65 expression. When stained with cytoplasmic anti-STEM121 antibodies, the tissue section revealed a positive staining of DPSCs in

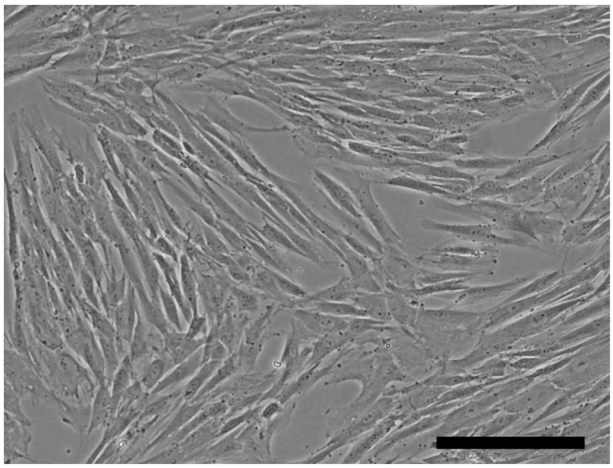

Fig. 1. Dental pulp stem cell morphology in standard culture conditions. The stem cells exhibited fibroblast-like morphologies. The cells were viewed under a phase-contrast microscope at a total magnification of 100 ×, where the scale bar denotes 200 μm.

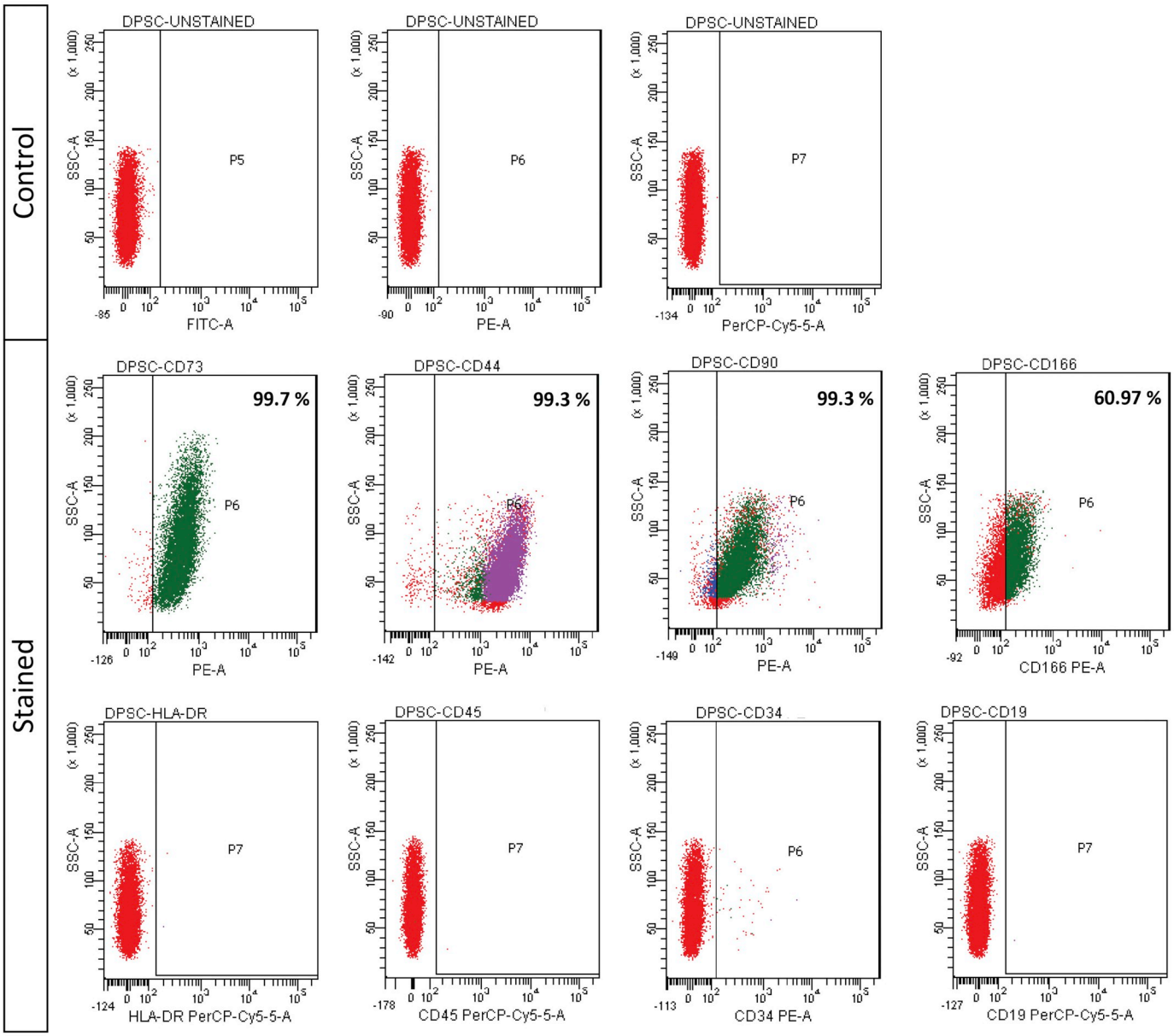

Fig. 2. Flow cytometric results showing DPSCs expression of mesenchymal and endothelial- hematopoietic stem cell markers. The cultured DPSCs showed positive expression of mesenchymal markers (CD166, CD90, CD73, CD44) and negative expression of endothelial-hematopoietic stem cell markers (CD19, CD45, CD34, HLA-DR).

the photoreceptor layer of treated retinas (Figure 4B).

4. Discussion

This study provides the first evidence showing human DPSC-mediated rescue of the retina in a rodent model of retinal degeneration. Stem cells are unspecialized cells that have the potential to differentiate into various cell lineages. DPSC, also known as postnatal DPSC, is a type of MSC that was first isolated by Gronthos et al. (2002) [16]. These cells are known to have high proliferative and clonogenicity levels, and are able to generate highly calcified colonies [16]. In standard culture conditions consisting of DMEM supplemented with antibiotic and FBS, DPSCs exhibit a morphology that is typical of fibroblast-like cells and are plastic-adherent (Fig. 1). Identifying these cells through immunophenotyping revealed positive expressions of CD90 (99.3%), CD73 (99.7%), CD44 (99.3%), and a slightly lower expression of CD166 (60.9%) (Fig. 2). These known markers are positively expressed in human DPSCs, and are crucial for stem cell verification [17]. There were no expressions of CD19, CD45, CD34, and HLA-DR, which indicated no contaminating cultures of non-dental pulp stem cells, such as hematopoietic stem cells [18].

In this study, the application of DPSCs in the treatment of retinal degeneration was explored. The model used was chemically-induced by $NaIO_3$ to cause retinal degeneration. It selectively reacts with RPE to induce cytotoxicity, which in turn leads to apoptotic photoreceptors due to the disruption of homeostatic relationships between both cell layers [19–21]. Several dose ranges have been studied from 20 mg/kg up to 75 mg/kg, and a $NaIO_3$ dose of 25 mg/kg was the least needed to produce a retinotoxic effect [21–23]. An optimum dose of 40 mg/kg was used in this study so as to reduce any possible risks of systemic damage [24]. Following DPSC-transplant and $NaIO_3$ administration, there were no statistically significant differences in the ERG b-wave amplitude between the treated and control groups within 24 h (Fig. 3A). After one month, the control group revealed a larger

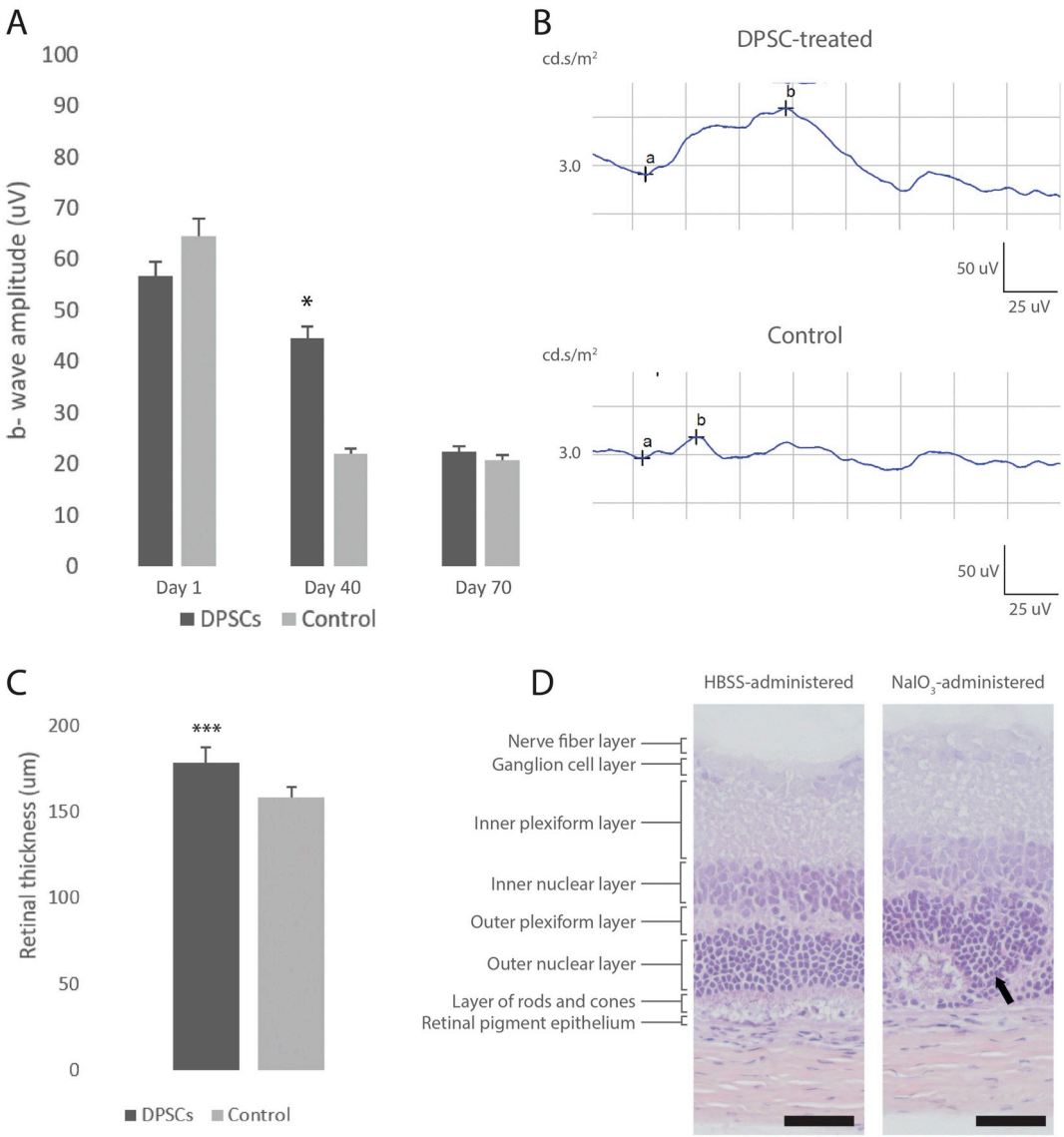

Fig. 3. Electroretinographic and histological analysis of DPSC-treated retinas. A. ERG b-wave assessment of retinal functions at 3.0 cd.s/m^2 light intensity, where day 40 yielded a statistically significant result between the DPSC-treated and HBSS-control $NaIO_3$ group, but not day 70. B. Representative images of ERG waveforms produced by the DPSC-treated and control groups. The results are presented as mean +/− SD. The *P* value was obtained using one-way ANOVA and Tukey's multiple comparisons test, * $P \leq .05$. C. Histological analysis on the treated and control retina showed that there was a significant change in the thickness of the retinal layer after one month following stem cell treatment, which correlated with the ERG findings. The results are presented as mean +/− SD. The P value was obtained using an unpaired *t*-test, *** $P \leq .001$. D. The reduction in retinal thickness is due to sodium iodate acting on the RPE and subsequently, the layer of rods and cones. The representative images of healthy and sodium iodate-induced retinas were viewed at 40 × total magnification. The black arrow indicates a lesion with degenerated RPE, rods and cones photoreceptor layer. The scale bar denotes 250 um.

reduction of the amplitude in comparison with the treated group. The difference was immediately visible in the ERG waveforms (Fig. 3B). This showed a protective role of DPSCs in the retina. Histological evaluation of the morphological changes in DPSC-treated rat retina and control showed a statistically significant difference in retinal thickness after one month (Fig. 3C). This finding corroborated with the electrophysiological data obtained earlier with ERG and further cements the potential role of DPSC-mediated rescue of the retina. The overall thinning of the tissue was due to the death of RPE and photoreceptors by $NaIO_3$, which causes rosette-patterned lesions to form in the outer retinal layers (Fig. 3B).

To date, no study has been done on the underlying mechanisms of DPSC-mediated retinal rescue from $NaIO_3$ insult however, a study done by Mao et al. (2018) showed that MSCs were able to prevent $NaIO_3$-induced RPE cell death *in vitro* through the deactivation of NLRP3 inflammasome by NF-κB upregulation [25]. DPSCs are a subset of MSCs, and hence may share similar cellular reparative mechanisms. Interestingly, it has been implied that DPSCs are more suitable candidates for neural regeneration [26–29]. The retina is composed mostly of specialized retinal neurons. Several studies have been done to show that DPSCs possess increased neurogenic and neuroprotective capabilities over other types of human MSCs in *in vitro* and *in vivo* studies [30–32]. This could be explained by the heightened expressions of neurotrophic factors (NTF) by DPSCs [30]. Recently, Mead et al. [33] were able to show DPSC-mediated neuroprotection as well as functional preservation in a rodent model of glaucoma [33]. The authors suggested NTFs as the main contributor. However, it is important to note that the model used in their study was induced by transforming growth factor beta 1 (TGF-β1), not $NaIO_3$. On day 70, the DPSC treated and control groups in our study showed equally reduced b waves. This was not explored further, but the reason could be due to the persistent toxicity of $NaIO_3$ in the rodent retina. Optimizing the number of cells needed per

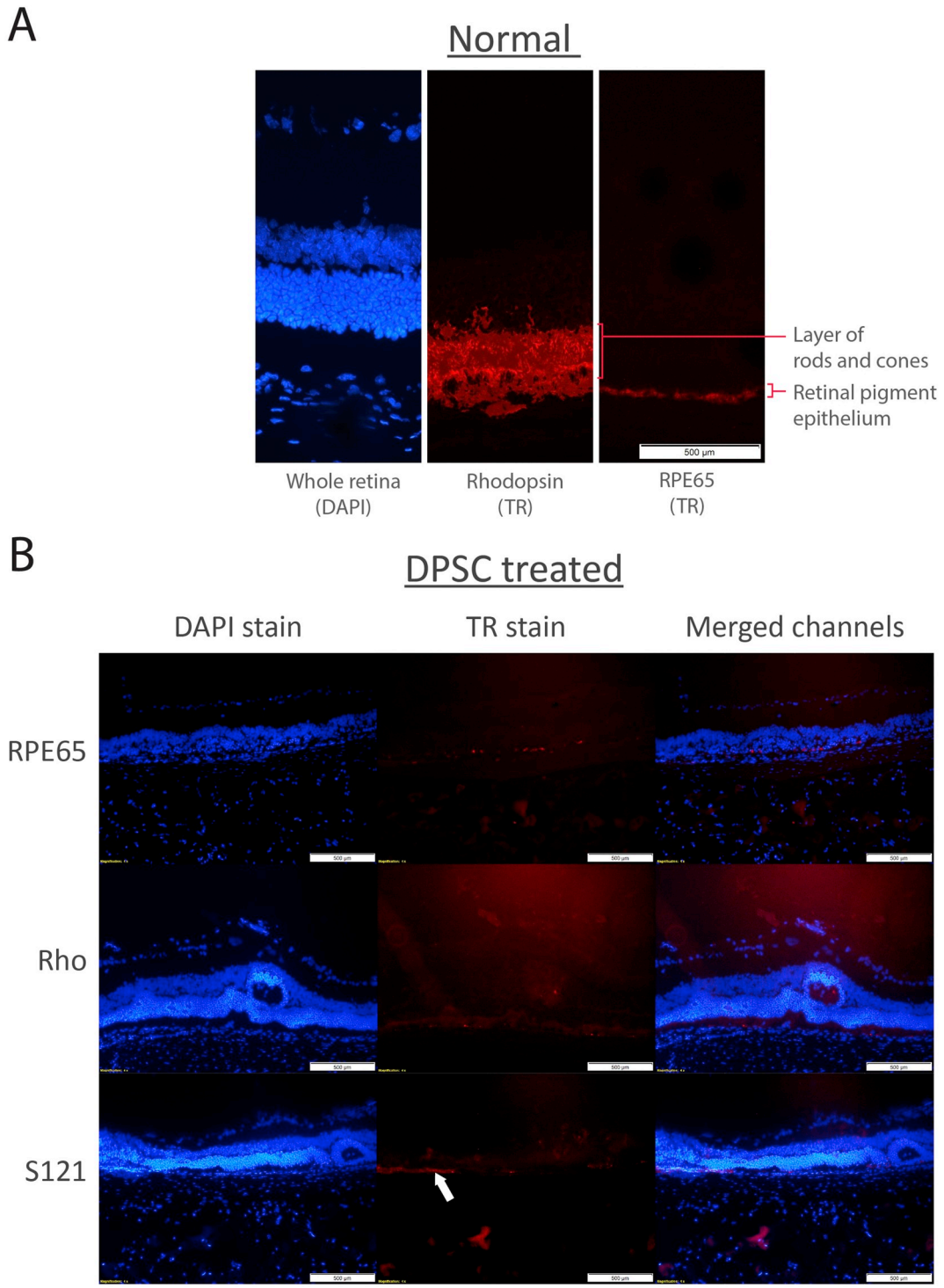

Fig. 4. Immunostaining assay of retinas treated with DPSCs, and visualized by fluorescence microscopy. 4′,6-diamidino-2-phenylindole (DAPI) was used as a counter stain to the anti-rhodopsin (rho), RPE65, and STEM121 (S121) antibodies. A. Healthy retinas constitutively express rho and RPE65 expression. It is highly specific and is confined to the photoreceptor layer and RPE layer, respectively. B. Staining of Texas red (TR)-conjugated antibodies specific to rho, RPE65, and STEM121. A marked reduction of RHO and RPE65 was observed in the DPSC-treated retina but positive STEM121 staining was detected in the photoreceptor layer, as indicated by the white arrow. The images were taken under a fluorescent microscope at 40 × total magnification. The scalebars denote 500 um. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

transplant could also improve this result.

To observe whether DPSCs have localized in the retina after intravitreal transplantation, immunohistochemistry was performed with anti-STEM121 antibodies, which specifically targets human cells. Positive staining of DPSCs were observed in the retina one month after transplantation (Fig. 4). The cells were found in the photoreceptor layer, which suggests that integration of localized DPSCs from the intravitreal space has occurred. Other studies however, contrasted with this finding by showing that most DPSCs and MSCs remain in the vitreous with only a few cells integrating in the RGC layer. The study by Mead et al. [33] did not observe any localization of DPSCs within the retinal layers [33]. Similarly, recent studies on MSCs that were

transplanted into the vitreous space reflected this [34,35]. However, the models used in those studies were not induced by $NaIO_3$, so it is hypothesized that the dysregulated retinal layers caused by the chemical could allow DPSCs migration into the photoreceptor layer. This was supported by studies involving the $NaIO_3$ model, where transplanted stem cells were observed to preferentially migrate to sites of retinal injury [36,37]. The integrated DPSCs were not found to express RHO and RPE65 at the end of this study, but a separate study done by Bray et al. (2014) has shown that human DPSCs are able to express the photoreceptor marker rhodopsin when co-cultured with rat retinal explants [38]. As such, further studies are needed to investigate the differentiation potential of transplanted DPSCs in the retina.

5. Conclusion

Stem cell therapy has a high potential in enhancing the life quality of patients experiencing ocular dysfunctions. In this study, mesenchymal stem cells taken from dental pulp (DPSCs) have been shown to possess therapeutic capabilities in protecting the retina from retinal degeneration. DPSCs were able to protect the visual functions and, to a certain extent, the retinal morphology of $NaIO_3$-induced rats within two months. The cells were also found to have localized in the photoreceptor layer. However, the protective effect is limited, and it could be due to the persistent toxicity of $NaIO_3$. In our study, we used DPSCs to recover the RPE and photoreceptor cells to restore vison in our rat model. Even so, DPSCs are a potential source in stem cell therapy for treating retinal degenerative diseases.

Author contributions

Suresh Kumar Subbiah and Pooi Ling Mok conceived the experimental study design; Hiba Amer Alsaeedi and Avin Ee-Hwan Koh equally contributed in carrying out the research study. Munirah binti Abd Rashid, Chenshen Lam, and Mohd Hairul Nizam Harun, aided in the electroretinographic study; Muhamad Fakhri bin Mohd Saleh aided in the histological study; Hiba Amer Alsaeedi, Avin Ee-Hwan Koh, and Seoh Wei Teh composed the manuscript and prepared the figures; Kong Yong Then, Suresh Kumar Subbiah, Catherine Mae-Lynn Bastion, Min Hwei Ng, Sue Ngein Leow, Hazlita Mohd Isa, Chi Luu, Bala Sundaram Muthuvenkatachalam, Antony V Samrot, K.B. Swamy, and Jaikumar Nandakumar supported the study design, analyzed and commented on the data; Suresh Kumar Subbiah and Pooi Ling Mok edited the manuscript. All authors were involved in reviewing the manuscript.

Declaration of Competing Interest

The authors declare no competing or financial interests. The funding sponsors played no role in the study design; in the data collection and analysis; in the manuscript writing; and in the decision to publish the results.

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