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Original Article

Using induced pluripotent stem cell-derived conditional medium to attenuate the light-induced photodamaged retina of rats

Hua-Ming Chang [a,b,*], Kuo-Hsuan Hung [c,d], Chih-Chien Hsu [c,e], Tai-Chi Lin [c,e], Szu-Yu Chen [a]

[a] *Department of Optics and Photonics, National Central University, Chung-Li, Taiwan, ROC*
[b] *Bade Veterans Home, Taoyuan, Taiwan, ROC*
[c] *Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan, ROC*
[d] *Division of Ophthalmology, National Yang-Ming University Hospital, I-Lan, Taiwan, ROC*
[e] *Department of Ophthalmology, Taipei Veterans General Hospital, Taipei, Taiwan, ROC*

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Abstract

Background: Light injury to photoreceptor cells and retinal pigment epithelium may lead to oxidative stress and irreversible degeneration of retina, especially degeneration of the high energy-demanded macula. The model of retinal photodamage could be applied to age-related macular degeneration and other degenerative retinal diseases for exploring new treatments. Based on broadly investigated induced pluripotent stem cells (iPSC) in the field of retinal degeneration, we aimed to clarify further how the interaction progresses between iPSC-conditional medium (CM) and light-damaged retina.

Methods: iPSCs were generated from murine embryonic fibroblasts of C57/B6 mice by retroviral transfection of three factors: Oct4, Sox2, and Klf4. Cytokine array was performed to analyze the components of CM. Sprague–Dawley rats receiving white light exposure to retina were viewed as an animal model of light injury. The rats were divided into four subgroups: light-injured rats receiving intravitreal injection of iPSC-CM, apoptotic iPSC-CM, or sodium phosphate buffer (PBS); and a control group without light damage. The electroretinography and thickness of outer nuclear layer were measured to document the therapeutic effects in each condition. Apoptosis arrays for detecting annexin V and caspase 3 were performed in the retinal tissues from each group.

Results: Murine embryonic fibroblasts were induced into iPSCs and expressed the marker genes similar to embryonic stem cells. These iPSCs can differentiate into Embryoid bodies (EBs), three germ layers *in vitro* and develop teratoma in severe combined immunodeficiency mice. The quantitative polymerase chain reaction of our iPSC-CM showed significantly elevated fibroblast growth factor-2, glial cell-derived neurotrophic factor, and insulin-like growth factor-binding proteins-1, -2, and -3. Compared to rats without photodamage, the light-injured rats receiving iPSC-CM had less reduction of outer nuclear layer thickness on Day 21 than other groups treated with either PBS or apoptotic iPSC-CM. In the same animal model, both a- and b-waves of electroretinography measurement in the group treated with iPSC-CM were significantly maintained compared to the control group and others with apoptotic iPSC-CM or PBS treatment. The apoptosis assay also demonstrated lower levels of annexin V and caspase 3 in the group with iPSC-CM treatment than in other groups presenting increasing apoptotic markers.

Conclusion: The conditional medium of iPSCs contains plenty of cytoprotective, immune-modulative and rescue chemicals, contributing to the maintenance of neuronal function and retinal layers in light-damaged retina compared with apoptotic iPSC-CM and PBS. The antiapoptotic effect of iPSC-CM also shows promise in restoring damaged neurons. This result demonstrates that iPSC-CM may serve as an alternative to cell therapy alone to treat retinal light damage and maintain functional and structural integrity of the retina.

Keywords: conditional medium; induced pluripotent stem cell; light damage of retina

Conflicts of interest: The authors declare that there are no conflicts of interest related to the subject matter or materials discussed in this article.

* Corresponding author. Dr. Hua-Ming Chang, Bade Veterans Home, 1100, Rongxing Road, Bade City, Taoyuan 334, Taiwan, ROC.
E-mail address: huamingchang@gmail.com (H.-M. Chang).

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1. Introduction

Retina degeneration generally leads to visual impairment. Its major causes are associated with old age, light injury, oxidative stress, and ocular trauma. Age-related macular degeneration (AMD) is the most important and the most challenging issue to deal with, because it involves the degeneration of retinal pigment epithelium (RPE) and photoreceptor cells. AMD is clinically divided into two categories: dry and wet types. There are several therapies for the treatment of wet AMD, the neovascular and more severe type, including using argon laser photocoagulation to treat degeneration-associated choroidal neovascularization (CNV). The therapeutic aim is to destroy the neovascular membrane by photocoagulation, but such treatment also destroys the overlying retina, with a resultant scotoma.

Photodynamic therapy for wet AMD has recently been approved in the USA. It utilizes a 10-minute intravenous infusion of verteporfin (a photosensitizing drug), followed by diode laser (689 nm) exposure to destroy the neovascularization. Vascular endothelial growth factor (VEGF) has been identified in recent decades as a pivotal treatment surrogate for CNV. However, the benefits of utilizing anti-VEGF agents to prevent CNV recurrence or occult pre-CNV are still under debate. Moreover, subfoveal fibrosis will also develop or progress in neovascular AMD even though patients have no significant subfoveal hemorrhage and have already been treated with anti-VEGF agents. The presentation of antifibrotic therapeutics may be beneficial in reducing the incidence of subretinal fibrosis[1]; however, none of the types of treatment mentioned above are the perfect solution to wet AMD.

Recent novel techniques have been able to generate induced pluripotent stem cells (iPSCs) from mouse and human adult somatic cells, such as fibroblasts, via the retrovirus-mediated transfection of four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4.[2−5] iPSCs have been further demonstrated to be indistinguishable from embryonic stem (ES) cells in morphology, proliferative abilities, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity.[4,5] iPSCs share the same features as ES cells and are capable of self-renewal and differentiation into three germ layers, offering potential for clinical cell therapies and tissue engineering.[2] Furthermore, iPSCs can be derived from the patient's own somatic cells to avoid potential immune response and ethical concerns. As a result, patient-derived iPSC therapy can provide better function in recipients than available ES cell lines can. Therefore, iPSCs are regarded as an excellent candidate for cell therapy and used in autologous transplantation without the risk of rejection.

Recent studies have shown that iPSCs present the capability of multilineage differentiation and further reduce the severity of cerebral ischemic injury in rats, endotoxin-induced acute lung injury and bleomycin-induced lung inflammation and fibrosis in mice.[6−9] We also found that both four-gene iPSCs, three-gene iPSCs, and their conditional medium (iPSC-CM) could reduce the level of inflammatory cytokines and chemokines in animals and thus decrease the inflammatory and fibrotic reaction.[8,9] Essentially, administration of iPSC-CM will not lead to the tumorigenesis that has been of greatest concern in stem cell therapy.

The treatment of light-damaged retina with iPSC-CM had not been commonly discussed before. In this study, our purpose was to demonstrate that iPSC-CM could suppress outer nuclear layer (ONL) thinning and recover impaired cellular function monitored by electroretinography (ERG) in light-injured murine retina. We hypothesized that the cytokines or chemokines in iPSC-CM might be beneficial to the light-damaged photoreceptors in the retina. Our findings may provide an alternative therapeutic strategy against light-induced retinal damage.

2. Methods

2.1. iPSCs cultured from murine embryonic fibroblasts

C57BL/6 mice were used in this study and all procedures involving animals were approved by the Animals Committee of the Taipei Veterans General Hospital. Our murine iPSCs were generated with only three introduced factors (Oct4/Sox2/Klf4) to avoid oncogene c-Myc (non-c-Myc iPSC). These iPSCs were generated from murine embryonic fibroblasts (MEFs) derived from 13.5-day-old embryos of C57/B6 mice. The reprogrammed iPSCs without c-Myc were transducted by retroviral vectors encoding three factors, Oct4, Sox2, and Klf4, as described previously.[10] Briefly, undifferentiated iPSCs were generally cultivated and expanded on mitotically inert MEFs (50,000 cells/cm^2) in six-well culture plates (BD Technology, Triangle Park, NC, USA) with 0.3% leukemia inhibitory factor in an iPSC medium consisting of Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum (FBS), 100 mM minimal essential medium nonessential amino acids, 0.55 mM 2-mercaptoethanol, and antibiotics. Every 3−4 days, colonies were separated with 0.2% collagenase IV, dissociated into single cells with 0.025% trypsin and 0.1% chicken serum in sodium phosphate buffer (PBS), and re-plated onto MEFs. The non-c-Myc iPSCs presented higher pluripotency and could be differentiated into three germ lines, including astroglial (neuro-ectodermal), osteogenic (mesodermal), and hepatocyte-like (endodermal) lineage cells.[10]

For induction of apoptosis, iPSCs were treated with 200 μM H_2O_2 for 18 hours. The definition of apoptosis was first based on a distinct sequence of morphologic features by microscopy (shrinkage of the cell and the nucleus), and further confirmed by caspase 3 activity and annexin V. After 6 hours of H_2O_2 treatment, the condition medium was collected for following experiment.

2.2. Light exposure and intravitreal treatment

All experiments were conducted in accordance with the Animal Care and Use Committee guidelines and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Four-week-old, male Sprague−Dawley rats, each weighing 150−250 g, were raised in plastic cages in a

climate-controlled animal facility and maintained under dim cyclic light (5 lux, 12 hours on/off). All animals had free access to food and water. Rats were anesthetized with intramuscular delivery of an equal-volume mixture of 2% lidocaine (Xylocaine; Astra, Södertälje, Sweden) and 50 mg/mL ketamine (Ketalar; Parke-Davis, Morris Plains, NJ, USA) by 0.15 mL/kg. Light exposure murine models were performed as previously described.[11] The rats were exposed to 10,000 lux of white light for 2 hours from 9:00AM to 11:00AM in a Plexiglas cage with mirrors on the lateral sides and the floors. Before intense light illumination, rats were dark adapted for 24 hours. Tropicamide (1% Mydriacyl; Alcon Laboratories, Hemel Hempstead, Herts, UK) was used to dilate the pupils before light exposure. The temperature of the environment was kept at 24°C. After 2 hours' exposure to light, the rats were maintained under dim cyclic light (5 lux, 12 hours on/off). After the rats were anesthetized, their corneas were anesthetized with a drop of 0.5% proparacaine hydrochloride (Alcaine; Alcon-Couvreur, Puurs, Belgium), pupils were dilated with 1% tropicamide and then the eyes were gently protruded with a rubber sleeve. Intravitreal injection of different remedies was performed 1 mm behind the limbus with a 33-gauge blunt-tip needle (Hamilton, Reno, NV, USA) and leaving the needle for 1 minute to reduce the reflux. A total of 24 rats were used in this study and were divided into four subgroups: normal control without light exposure; intravitreal injection of iPSCs and their condition medium; intravitreal injection of apoptotic iPSCs and their condition medium; and intravitreal injection of PBS after light exposure. The animals were sacrificed by carbon dioxide suffocation 21 days after the start of light exposure, and the eyes were then enucleated for histopathologic and immunohistochemical analyses.

2.3. Investigation of cytokines

To identify components of each CM, 1 mL of CM was analyzed by the Human Cytokine Array Kit (Panel A; R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions. The pixel density was identified using ImageJ software (NIH, Bethesda, MD, USA). Signals were normalized to the average of the positive controls on each array and the background was eliminated.

2.4. Measurement of ONL thickness

To compare the trophic effect of different CMs, the thickness of the outer nuclear layer in photodamaged murine retina, treated under variable conditions, was directly measured on histologic section on Day 21 after intravitreal injection. After removing the contents including cornea, lens, and vitreous, the remaining parts of the eye were fixed in 4% (W/V) paraformaldehyde in 100 mM PBS, pH 7.3 overnight at 4°C, before cryoprotection in sucrose and being mounted in an optimal counting temperature compound (OCT Tissue-Tek; Sakura, Alphen aan den Rijn, The Netherlands). Retinal sections were then cut at 0.5-mm thickness along the vertical meridian using a cryostat. The sections were stained with hematoxylin and eosin (H&E) and examined by light microscope. Six different measurements were recorded in each harvested retina, and the average was calculated at the same time.

2.5. ERG

The animals were kept in dark for at least 1 day overnight, and ERGs were recorded at 1 day, 3 days, 7 days, 14 days, or 21 days after light exposure, as previously described, with some modifications.[12] Briefly, the anesthesia of animals was done with intramuscular injections of 50 mg/kg ketamine/0.15 mg/kg lidocaine, their corneas were anesthetized with a drop of 0.5% proparacaine hydrochloride, and 1% tropicamide was used to dilate the pupils. The rats were placed on a heating pad that maintained their body temperature at 35–36°C during the experiment. The ground electrode was a subcutaneous needle through the tail, and the reference electrode was placed between the eyes subcutaneously. The contact lens electrodes were put over the cornea. Responses were amplified differentially, light pulses of 800 cd/m^2, bandpass filtered at 0.3–500 Hz, digitized at 0.25–0.5-ms intervals by a commercial system (RETIport ERG laptop version, Acrivet, Germany), and stored for processing. The implicit times of the a- and b-waves were measured from the onset of stimuli to the peak of each wave. The a-wave amplitude was measured from the baseline to its trough, and b-wave amplitude was determined from the trough of the a-wave to the peak of the b-wave.

2.6. Caspase 3 activity and annexin V

The cell lysates from the retina of the animal model were harvested for apoptotic assay. Retinal caspase-3 activity was identified with a commercial colorimetric kit (EnzChek Caspase 3 Assay Kit # 1; Molecular Probes, Inc. Eugene, OR, USA) according to the manufacturer's instructions. Briefly, equal aliquots of retinal homogenate were heated at 37°C for 30 minutes with caspase-3-specific substrate Z-DEVD-AMC in the kit reaction buffer. The absorbance of each sample was read at 441 nm and the color reaction shown was directly proportional to caspase-3 activity levels. Chick retinas (embryonic stage 11) were used as positive control of caspase-3 activity. Results were expressed as the percentage change in the absorbance. For retinal cells, apoptosis was assessed with flow cytometry using an annexin V-fluorescein isothiocyanate apoptosis detection kit (BD Technology) according to the manufacturer's instructions. Briefly, cells were washed, pooled, and suspended in 500 μL binding buffer, followed by addition of 5 μL annexin V-fluorescein isothiocyanate. Cells were incubated at room temperature avoiding light for 15 minutes, and analyzed with flow cytometry (Beckman Coulter EPICS XL/MCL). Viable cells were unstained with annexin V, while apoptotic cells were stained with annexin V.

2.7. Statistical analysis

The data are expressed as mean ± standard error of the mean. Differences between groups were compared using Student t test. Values of $p < 0.05$ were considered to indicate statistical significance.

3. Results

3.1. Quantitative polymerase chain reaction for investigation of mRNA expression level in CM

We hypothesized that if components of CM from iPSCs support survival of retinal neurons after light injury, growth or neurotrophic factors should be found therein. Compared with the murine fibroblasts, components of CM from iPSCs showed significantly elevated vital cytokines for neurons by quantitative polymerase chain reaction, including fibroblast growth factor (FGF)-2, glial cell-derived neurotrophic factor (GDNF), insulin-like growth factor-binding protein-1 (IGFBP-1), IGFBP-2, and IGFBP-3 (Fig. 1). All of these increased cytokines potentially enhance retinal neurons to survive against apoptosis.

3.2. Effects of iPSC-CM in the histopathology and thickness of light-injured murine retina

The iPSC-CM was able to maintain structure of light-injured murine retina, as assessed by histopathology. Fig. 2A-I shows normal histology of murine retina without light exposure in the H&E stain. Light exposure can cause significant damage and apoptosis to retinal cells. These light-injury consequences included apoptosis, edema, and degeneration of RPE cells. To explore the role of iPSC with CM in photodamaged retina, we compared retinal sections from light-injured rats that had undergone PBS, apoptotic iPSC-CM, and iPSC-CM treatment. These rats were sacrificed on the 21st day after treatment, at which time all retina sections were evaluated with H&E stain. Rat retinas that received the intravitreal injection of apoptotic iPSC-CM (Fig. 2A-III) showed no improvement in the severity of retina damage induced by light exposure as compared with intravitreal injection of PBS (Fig. 2A-II). In contrast, rats that received the iPSC-CM treatment showed improvement of retinal damage in the retinal layers (Fig. 2A-IV).

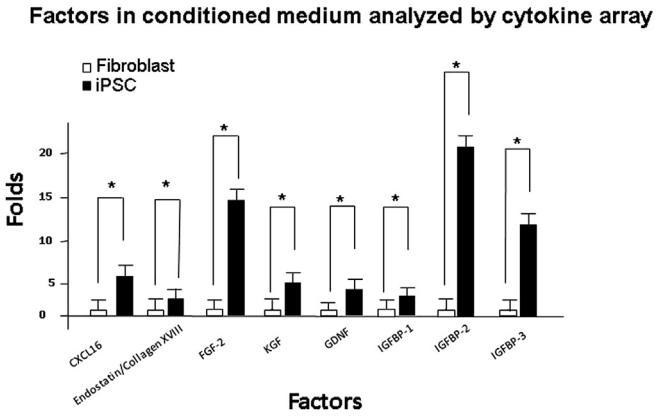

Fig. 1. Factors in conditional medium analyzed by quantitative polymerase chain reaction. The quantitative polymerase chain reaction for detecting cytokines in conditioned medium (CM) of induced pluripotent stem cells (iPSCs) showed significantly increased expression of fibroblast growth factor-2 (FGF-2), glial cell-derived neurotrophic factor (GDNF), and insulin-like growth factor-binding proteins (IGFBP)-1, IGFBP-2, and IGFBP-3, comparing with that from murine fibroblast. Statistically significant results are indicated by an asterisk (*).

3.3. iPSC-CM suppressed ONL thinning in light-injured retina

To evaluate the trophic effect of iPSC-CM on light-injured retinas of rats, we measured the change of ONL thickness in six areas on the 21st day after light exposure. The ONL thickness of light-injured retina in PBS or apoptotic iPSC-CM rats were significantly reduced, as compared to that in rats receiving no light exposure (Fig. 2B). As shown in Fig. 2C, the average retina thickness in normal rats without light injury was 117.5 μm. Compared to the average ONL thicknesses of photodamaged retinas under iPSC-CM (88.33 μm), apoptotic iPSC-CM (70.3 μm) and PBS (70.1 μm) treatment on the 21st day, we found that the iPSC-CM treatment was able to significantly suppress ONL thinning in the light-injured rats. These findings indicate restorative effects on light-injured murine retina following treatment with iPSC-CM.

3.4. iPSC-CM recovered impaired ERG

RPE is essential for maintaining the physiology of the neurosensory retina. Injection of iPSC-CM may restore the function of damaged neurons. The amplitudes of ERG decreased in several retinal dysfunctions, such as AMD and light-damaged retina. Light exposure to murine retina on the first day led to significant reduction in average a- and b-wave amplitudes ratio (0.4 and 0.4, respectively; Fig. 3) in photodamage rats than normal rats without light exposure. We wanted to investigate the therapeutic effect of intravitreal iPSC-CM application on light-induced retinal dysfunction in rats. The changes of ERG response were observed for 21 days after light exposure (Fig. 3). Our findings showed that application of iPSC-CM to light-exposed rats on the 14th day significantly minimized the reduction of the average amplitudes ratio in a-wave (0.68; Fig. 3A) and b-wave (0.6; Fig. 3B), as compared to apoptotic iPSC-CM treatment in light-exposed rats (0.45 and 0.41, respectively) and PBS treatment in light-exposed rats (0.4 and 0.39, respectively).

Similar conditions were found on the 21st day in light-injured rats. iPSC-CM application to light-exposed rats significantly restored the reduction of average ratio in a-wave (0.7) and b-wave (0.65) compared to apoptotic iPSC-CM treatment in light-exposed animals (0.45 and 0.4, respectively) and PBS treatment in light-exposed animals (0.4 and 0.39, respectively). There ERG data demonstrate that iPSC-CM recovered the amplitude ratio of a- and b-wave in light-injured murine retina.

3.5. Apoptosis assay for light-injured murine retina in different treatment protocols

Loss of neuronal cells from light damage to retina leads to thinning and atrophy of retinal layers. To estimate the cell loss under various rescuing treatments, we used apoptosis assay to

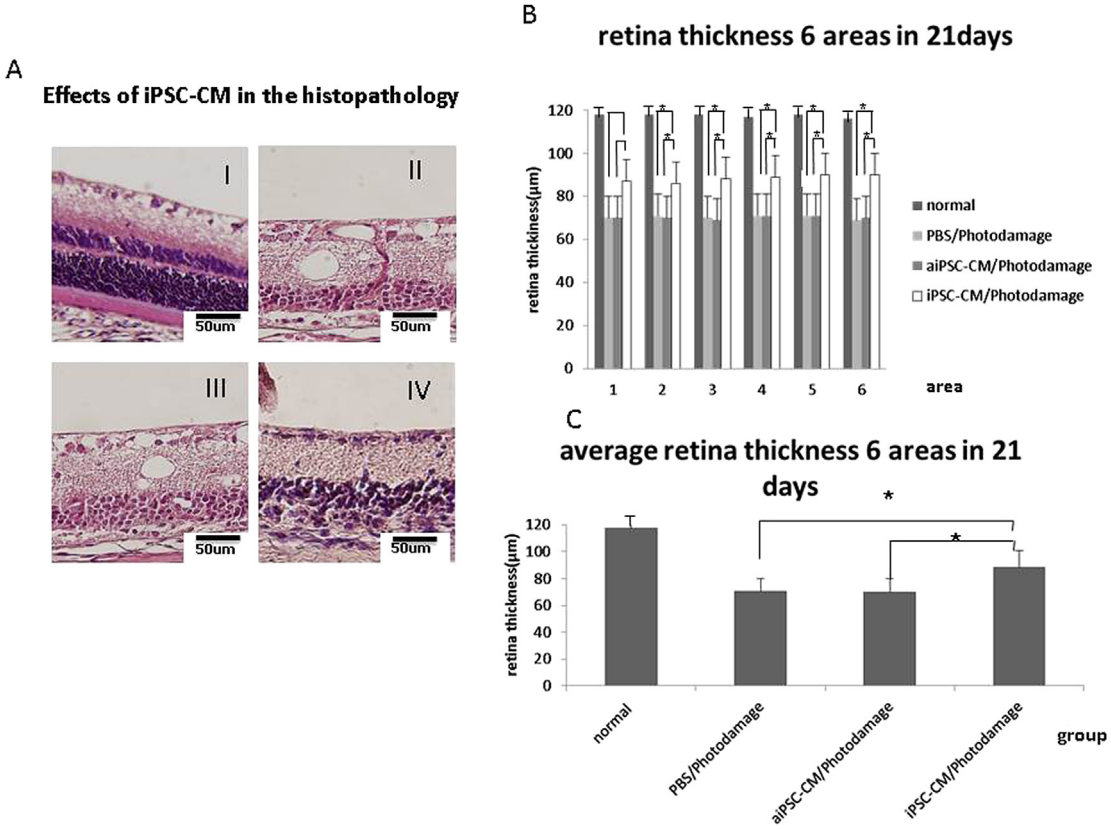

Fig. 2. Changes of outer nuclear layer thickness in normal control and each damaged retina under variable treatments. (A) Retinal tissue sections stained with hematoxylin and eosin (H&E): normal retinal structure (A-I) and photodamaged retina treated with intravitreal PBS (A-II), conditioned medium (CM) of induced apoptotic pluripotent stem cells (iPSCs) iPSC-CM (A-III), iPSC-CM (A-IV) injection after 21 days. The group treated with iPSC-CM (A-IV) showed maintained outer nuclear layers and whole integrity layers of retina. (B) Retinal thickness was measured at six areas and then the data were averaged. Significant maintained retinal thickness was noted between groups of photodamaged retina treated with iPSC-CM and those treated with PBS or apoptotic (a)iPSC. (C) iPSC-CM successfully restored the retinal thickness significantly, as compared to PBS and aiPSC-CM groups, after 21 days. Statistically significant results are indicated by an asterisk (*).

detect the caspase 3 and annexin V as markers of apoptotic change in retinal cells. The results of assay showed that after light exposure, annexin V and caspase 3 were increasingly detected in groups under treatment of PBS and apoptotic iPSC-CM rather than in those receiving treatment of CM-iPSCs or normal control (Fig. 4). This result revealed that iPSC-CM was capable of maintaining the homeostasis of damaged cells and preventing them from further injury and apoptosis.

4. Discussion

Induced pluripotent stem cells and associated cell therapies have been widely investigated in applications to retinal degenerative diseases instead of ES cells to avoid ethical problems, immune rejection, and shortage of cell sources.[13–15] Although efficacy and safety of subretinally transplanted iPSC-derived RPE graft has been guaranteed in a murine retinal model of retinitis pigmentosa,[16] directly transplanting the iPSC-derived neural progenitor cells into the vitreous cavity or subretinal space of patients with retinal degeneration may lead to unpredictable cellular response and complications from the intervention. The encouraging response of utilizing conditional medium for treatment of diseases may provide an alternative safe and effective way to restore the dysfunctional retina.[17,18]

As shown in published *in-vivo* and *in-vitro* studies, conditional medium of stem cells contains several supplements nourishing to cells, such as tissue inhibitor metalloproteinase-1, secreted protein acidic and rich in cystein, ciliary neurotrophic factor (CNTF), basic FGF, and insulin-like growth factor, which may inhibit photoreceptor and retinal ganglion cell degeneration.[17,19] At the same time, the supernatants from light-induced retina may stimulate mesenchymal stem cells (MSCs) to secret neurotrophin, such as basic FGF, CNTF, and brain-derived neurotrophic factor, and reduce DNA fragmentation, downregulate bax, and upregulate bcl-2 in the rat white light-induced retinal injury model.[20] These results indicate that damaged retina may elevate rescue signals to recruit more neurotrophins from the surrounding microenvironment for survival, and that MSCs have the ability to provide such factors in abundance.

The cytokine array of our CM showed that factors, FGF-2, GDNF, IGFBP-1, IGFBP-2, and IGFBP-3 were significantly elevated compared with those from the MEFs. These factors may prevent photoreceptors cells and retinal ganglion cells

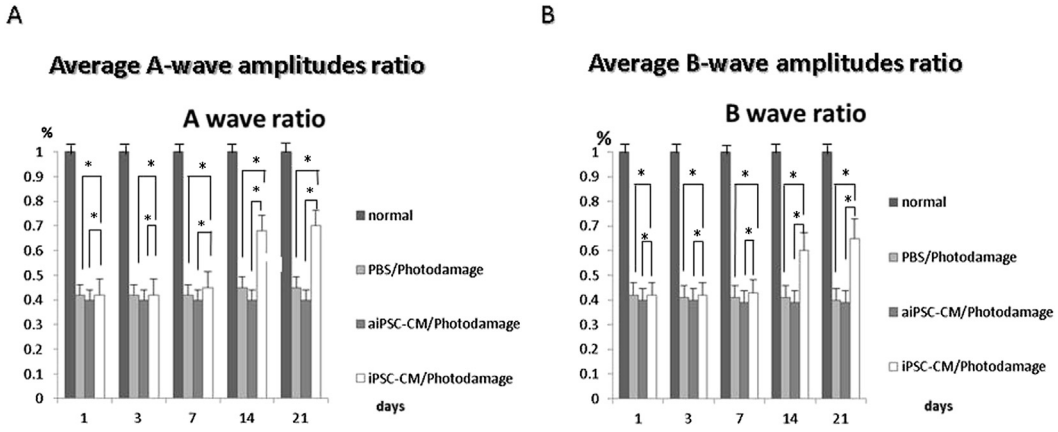

Fig. 3. The average ratios of (A) a-wave and (B) b-wave amplitude in the four groups were measured on Days 1, 3, 7, 14, and 21. Conditioned medium (CM) of induced pluripotent stem cells (iPSCs) restored the reduction of average ratio in a-wave and b-wave significantly, as compared to apoptotic (a)iPSC-CM and PBS groups, on Days 14 and 21. Statistically significant results are indicated by an asterisk (*).

from neurodegeneration after light damage due to their neurotrophic effects.[17,19] Instead of protective effect in ischemic retinal diseases,[21] *in-vivo* and *in-vitro* evidence has shown benefit of increased FGF-2 in photodamaged retina by protein kinase B and extracellular signal-regulated kinase 1/2 (ERK 1/2) pathway to support neurons.[22–24] GDNF has also demonstrated nourishing effect on and promising protection of retinal neurons from apoptosis in murine models of ischemia, diabetes, and retinitis pigmentosa.[25–27] This neuroprotection effect may be in part due to upregulated glutamate-aspartate transporter.[26] Finally, the IGFBP family is involved in conjugation with IGF and maintaining IGF concentration in target tissues, meaning that upregulated IGFBPs could extend the trophic effect of IGF on degenerating retinal neurons under oxidative stress. Both markers of apoptosis: annexin V and caspase 3, were elevated in neurons after photodamage in our apoptosis assay, but only the group receiving iPSC-CM presented the same low grade of apoptosis as the control group without light injury.

As reported in our previous study, subretinal delivery of Oct 4/SirT1 gene in advance could prevent following oxidative stress damage from the light injury.[28] In the current study, giving iPSC-CM treatment to light-damaged mice in a sequential process also showed promising benefit in maintaining a more significant ratio of a- and b-waves in ERG than other conditions. This means that the paracrine effect of iPSC-CM may support the neurons to function normally and fight against apoptosis, although they could not completely recover from the light damage during our short study period. The a-wave of ERG is composed of signals from photoreceptor cells, bipolar cells, amacrine cells, and Muller cells; the cytoprotective effects of iPSC-CM may be attributed to all of the closely interacting retinal neurons, not merely photoreceptor cells. Both FGF receptor and CNTF receptor-a were found in the photoreceptor cells that directly benefit from neurotrophins.[29] A question remains: if CM was harvested from different cell origins of iPSC, such as neuron-derived iPSC, the paracrine effects might be more specific and multiplied

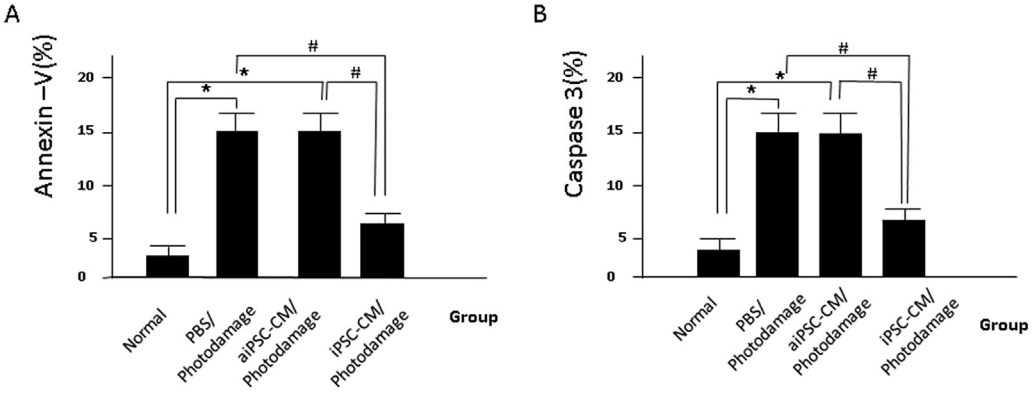

Fig. 4. Levels of apoptosis markers in each treatment group. Apoptosis assay demonstrated lower levels of (A) annexin V and (B) caspase 3 in the group with conditioned medium (CM) of induced pluripotent stem cells (iPSCs) treatment, similar to the control group. However, significantly increased apoptotic markers were detected in the other two groups with apoptotic (a)iPSC-CM and PBS treatment. Statistically significant results between groups treated with PBS/apoptotic iPSC-CM and normal control are marked by *; statistically significant results between groups treated with iPSC-CM and those treated with PBS/aiPSC-CM are indicated by a hash sign (#).

during the cell–cell interaction rather than those of MEFs shown in our study. Otherwise, further experiments should be carried out to confirm whether the iPSC-CM totally recovers the function of photoreceptor cells after repeated therapies or higher-dosage applications. The rescue signals from light-damaged retinal cells should also be identified by microarray or proteinomic methods, and the results of analysis could be combined with CM to enhance the therapeutic effect.

AMD is the most important issue to investigate because it involves the degeneration of RPE and photoreceptor cells. In AMD, apart from oxidative stress of retinal photoreceptors and subsequent surge of VEGF, another parallel pathogenesis is inflammation that could be clinically suppressed by retrobulbar, peribulbar, or intravitreal injection of steroid. It is believed that stem cells possess the ability immunomodulate in microenvironments by several pathways, such as suppressing T cells.[30] If the immunomodulative effects also worked in iPSCs-CM, they might be proved in the treatment of AMD to eliminate local inflammation and prevent extension of cell apoptosis. In the present study, we confirmed that apoptotic iPSC-CM inhibits light-induced apoptosis, such as activation of caspase 3 and annexin V, indicating that iPSC-CM is capable of maintaining the homeostasis of damaged cells and preventing them from further injury and apoptosis. This novel findings may provide a new strategy for treating AMD.

The vitreous cavity is a relatively closed system. If iPSC-CM were going to be used clinically, intravitreally injected CM might affect all cells of the intraocular tissue. Thus, it is important to clarify which route is most appropriate for delivery of the therapeutic medium and at which clinical stage of retinal degeneration one should receive that therapy. In the future, cell therapy combined with specific CM may provide another therapeutic modality for treatment of degenerative ocular diseases. However, because iPSC-CM is composed of abundant neurotrophic factors, including VEGF,[31] adverse and mixed effects during delivery of such medium into ocular tissue should be evaluated more definitely.

In conclusion, the conditional medium of iPSCs comprises variable trophic factors, such as FGF-2, GDNF, and IGFBP, that support retinal neurons and maintain neuronal function with integrity of retinal structure after light damage. Further detailed studies should be conducted to identify how to integrate these components of CM into the treatment of clinical diseases and their long-term efficacy.

References

1. Hwang JC, Del Priore LV, Freund KB, Chang S, Iranmanesh R. Development of subretinal fibrosis after anti-VEGF treatment in neovascular age-related macular degeneration. *Ophthalmic Surg Lasers Imaging* 2011;**42**:6–11.
2. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006;**126**:663–76.
3. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature* 2007;**448**:313–7.
4. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;**131**:861–72.
5. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007;**318**:1917–20.
6. Kao CL, Tai LK, Chiou SH, Chen YJ, Lee KH, Chou SJ, et al. Resveratrol promotes osteogenic differentiation and protects against dexamethasone damage in murine induced pluripotent stem cells. *Stem Cells Dev* 2010;**19**:247–58.
7. Chen SJ, Chang CM, Tsai SK, Chang YL, Chou SJ, Huang SS, et al. Functional improvement of focal cerebral ischemia injury by subdural transplantation of induced pluripotent stem cells with fibrin glue. *Stem Cells Dev* 2010;**19**:1757–67.
8. Yang KY, Shih HC, How CK, Chen CY, Hsu HS, Yang CW, et al. IV delivery of induced pluripotent stem cells attenuates endotoxin-induced acute lung injury in mice. *Chest* 2011;**140**:1243–53.
9. How CK, Chien Y, Yang KY, Shih HC, Juan CC, Yang YP, et al. Induced pluripotent stem cells mediate the release of interferon gamma-induced protein 10 and alleviate bleomycin-induced lung inflammation and fibrosis. *Shock* 2013;**39**:261–70.
10. Li HY, Chien Y, Chen YJ, Chen SF, Chang YL, Chiang CH, et al. Reprogramming induced pluripotent stem cells in the absence of c-Myc for differentiation into hepatocyte-like cells. *Biomaterials* 2011;**32**:5994–6005.
11. Wenzel A, Grimm C, Samardzija A, Reme CE. Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration. *Prog Retin Eye Res* 2005;**24**:275–306.
12. Peng YJ, Wen CW, Chiou SH, Liu SJ. Sustained release of ganciclovir and foscarnet from biodegradable scleral plugs for the treatment of cytomegalovirus retinitis. *Biomaterials* 2010;**31**:1773–9.
13. Kvanta A, Grudzinska MK. Stem cell-based treatment in geographic atrophy: promises and pitfalls. *Acta Ophthalmol* 2013;**92**:21–6.
14. Lewallen M, Xie T. Cell-based therapies for retinal degenerative diseases: a thousand strategies. *J Glaucoma* 2013;**22**(Suppl 5):S42–5.
15. John S, Natarajan S, Parikumar P, Shanmugam PM, Senthikumar R, Green DW, et al. Choice of cell source in cell-based therapies for retinal damage due to age-related macular degeneration. *J Ophthalmol* 2013;**2013**:465169.
16. Li Y, Tsai YT, Hsu CW, Erol D, Yang J, Wu WH, et al. Long-term safety and efficacy of human-induced pluripotent stem cell (iPS) grafts in a preclinical model of retinitis pigmentosa. *Mol Med* 2012;**18**:1312–9.
17. Sugitani S, Tsuruma K, Ohno Y, Kuse Y, Yamauchi M, Egashira Y, et al. The potential neuroprotective effect of human adipose stem cells conditioned medium against light-induced retinal damage. *Exp Eye Res* 2013;**116C**:254–64.
18. Inoue Y, Iriyama A, Ueno S, Takahashi H, Kondo M, Tamaki Y, et al. Subretinal transplantation of bone marrow mesenchymal stem cells delays retinal degeneration in the RCS rat model of retinal degeneration. *Exp Eye Res* 2007;**85**:234–41.
19. Satarian L, Javan M, Kiani S, Hajikaram M, Mirnajafi-Zadeh J, Baharvand H. Engrafted human induced pluripotent stem cell-derived anterior specified neural progenitors protect the rat crushed optic nerve. *PLoS One* 2013;**8**:e71855.
20. Xu W, Wang X, Xu G, Guo J. Light-induced retinal injury enhanced neurotrophins secretion and neurotrophic effect of mesenchymal stem cells in vitro. *Arq Bras Oftalmol* 2013;**76**:105–10.
21. Unoki K, LaVail MM. Protection of the rat retina from ischemic injury by brain-derived neurotrophic factor, ciliary neurotrophic factor, and basic fibroblast growth factor. *Invest Ophthalmol Vis Sci* 1994;**35**:907–15.
22. Zhang Y, Wang W. Effects of bone marrow mesenchymal stem cell transplantation on light-damaged retina. *Invest Ophthalmol Vis Sci* 2010;**51**:3742–8.
23. O'Driscoll C, O'Connor J, O'Brien CJ, Cotter TG. Basic fibroblast growth factor-induced protection from light damage in the mouse retina in vivo. *J Neurochem* 2008;**105**:524–36.
24. Abe T, Saigo Y, Hojo M, Kano T, Wakusawa R, Tokita Y, et al. Protection of photoreceptor cells from phototoxicity by transplanted retinal pigment epithelial cells expressing different neurotrophic factors. *Cell Transplant* 2005;**14**:799–808.

25. Wu WC, Lai CC, Chen SL, Sun MH, Xiao X, Chen TL, et al. GDNF gene therapy attenuates retinal ischemic injuries in rats. *Mol Vis* 2004; **10**:93−102.
26. Wang L, Deng QQ, Wu XH, Yu J, Yang XL, Zhong YM. Upregulation of glutamate-aspartate transporter by glial cell line-derived neurotrophic factor ameliorates cell apoptosis in neural retina in streptozotocin-induced diabetic rats. *CNS Neurosci Ther* 2013;**19**:945−53.
27. Dalkara D, Kolstad KD, Guerin KL, Hoffmann NV, Visel M, Klimczak RR, et al. AAV mediated GDNF secretion from retinal glia slows down retinal degeneration in a rat model of retinitis pigmentosa. *Mol Ther* 2011;**19**:1602−8.
28. Peng CH, Cherng JY, Chiou GY, Chen YC, Chien CH, Kao CL, et al. Delivery of Oct4 and SirT1 with cationic polyurethanes-short branch PEI to aged retinal pigment epithelium. *Biomaterials* 2011;**32**: 9077−88.
29. Valter K, Bisti S, Gargini C, Di Loreto S, Maccarone R, Cervetto L, et al. Time course of neurotrophic factor upregulation and retinal protection against light-induced damage after optic nerve section. *Invest Ophthalmol Vis Sci* 2005;**46**:1748−54.
30. Liang X, Ding Y, Zhang Y, Tse HF, Lian Q. Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplant* 2013;**23**:1045−9.
31. Grote K, Petri M, Liu C, Jehn P, Spalthoff S, Kokemüller H, et al. Toll-like receptor 2/6-dependent stimulation of mesenchymal stem cells promotes angiogenesis by paracrine factors. *Eur Cell Mater* 2013;**26**: 66−79.