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Mesenchymal Stromal Cell-Mediated Neuroprotection and Functional Preservation of Retinal Ganglion Cells in a Rodent Model of Glaucoma

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Running title: Mesenchymal stem cell therapy for glaucoma

Precis: The neurotrophic effects of human DPSC on RGC were analysed in glaucomatous rats. The results demonstrate significant neuroprotective and functional preservative effects of RGC elicited by DPSC, which were significantly more pronounced than those elicited by BMSC.

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Abstract

Background:

Glaucoma is a leading cause of irreversible blindness involving loss of retinal ganglion cells (RGC). Mesenchymal stromal/stem cells (MSC) have shown promise as a paracrine-mediated therapy for compromised neurons. It is however unknown if dental pulp stem cells

(DPSC) are effective as a cellular therapy in glaucoma and how their hypothesised influence compares to other more widely researched MSC sources. The present study aimed to compare the efficacy of adipose-derived stem cells, bone marrow-derived mesenchymal stem cells (BMSC) and DPSC in preventing the loss of RGC and visual function when transplanted into the vitreous of glaucomatous rodent eyes.

Methods:

Thirty five days after raised intraocular pressure (IOP) and intravitreal stem cell transplantation, Brn3a$^+$ RGC numbers, retinal nerve fibre layer thickness (RNFL) and RGC function were evaluated by immunohistochemistry (IHC), optical coherence tomography (OCT) and electroretinography (ERG), respectively.

Results:

Control glaucomatous eyes that were sham-treated with heat killed DPSC had a significant loss of RGC numbers, RNFL thickness and function compared with Intact eyes. BMSC and, to a greater extent, DPSC provided significant protection from RGC loss, RNFL thinning and preserved RGC function.

Discussion:

The study supports the use of DPSC as a neuroprotective cellular therapy in retinal degenerative disease such as glaucoma.

Key words: Stem cell transplantation, Glaucoma, Dental pulp stem cells, Mesenchymal stem cells, Neuroprotection, Retinal ganglion cells

Abbreviations: ADSC, adipose-derived mesenchymal stem cells; ADB, antibody diluting buffer; ANOVA, one-way analysis of variance; BDNF, brain-derived neurotrophic factor; BMSC, bone marrow-derived mesenchymal stem cells; BSA, bovine serum albumin; CNS, central nervous system; d, days; DPSC, dental pulp stem cells; ERG, electroretinogram;

FBS, foetal bovine serum; h, hours; IC, intracameral; IOP, intraocular pressure; ivit, intravitreal; min, minutes; MSC, mesenchymal stem cells; NGF, nerve growth factor; NT-3, neurotrophin-3; NTF, neurotrophic factors; OCT, optical coherence tomography, PBS, phosphate-buffered saline; PFA, paraformaldehyde; PDGF, platelet-derived growth factor; pSTR, positive scotopic threshold response; RGC, retinal ganglion cells; RNFL, retinal nerve fibre layer; RT, room temperature; SEM, standard error of the mean; TGF-β, transforming growth factor-beta.

1 Introduction

Glaucoma is a common cause of irreversible blindness and is characterised by a degenerative loss of retinal ganglion cells (RGC) and their axons, leading to optic disc cupping and reduced visual acuity(1). Current treatments are designed to reduce intraocular pressure (IOP) to slow disease progression whereas neuroprotective treatments that directly target the injured RGC are still in their infancy. Neurotrophic factors (NTF), in particular neurotrophins, are neuronal survival factors that are retrogradely transported along a functionally connected axon to the soma maintaining the survival of connected neurons, but unconnected neurons die by apoptosis(2). Elevation of IOP significantly inhibits retrograde transport of NTF (3) and is one of the mechanisms involved in RGC death(4). NTF, especially when delivered in combinations, promote the survival of injured RGC *in vitro*(5, 6). However their neuroprotective efficacy *in vitro* is not easily translatable to *in vivo* models as a constant delivery of multiple NTF is required for maintaining therapeutic effect(7, 8).

Mesenchymal stromal/stem cells (MSC) are multipotent self-replicating stromal cells are being evaluated as a cellular therapy for treating glaucoma based on their secretion of a wide array of NTF(9-12). MSC-derived NTF protect injured RGC, protecting them from death and ultimately preserving vision(13) and several clinical trials evaluating their neuroprotective efficacy are ongoing(14). MSC can be isolated from a variety of adult tissues

such as bone marrow (BMSC) and adipose tissue (ADSC) but here we have focused on the use of MSC-like cells from the dental pulp (DPSC). DPSC are multipotent cranial neural crest-derived stem cells(15, 16) that secrete significantly more NTF, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) than ADSC and BMSC(9, 10). We and others have demonstrated significant RGC neuroprotection by DPSC(9) and BMSC(17, 18) after traumatic optic neuropathy and shown *in vitro* that this neuroprotective effect is most likely due to NTF, including neurotrophins(9, 10) and platelet-derived growth factor (PDGF)(12). These findings indicated that MSC, and in particular DPSC, may be more effective as a treatment for neurodegenerative conditions such as glaucoma.

We have demonstrated a significantly greater neuroprotective effect of DPSC compared to BMSC/ADSC in an *in vitro* model of RGC injury, with multiple secreted NTF being the mechanism behind the effect(10). We now hypothesise that DPSC may also be a candidate cellular therapy for protecting RGC from loss in glaucoma and determined to test this using an *in vivo* model. Accordingly, three widely researched stem cells for ocular repair(14), BMSC, ADSC and DPSC were transplanted into the vitreous body of rats in which ocular hypertension was induced using exogenously administered transforming growth factor-β1 (TGF-β). Administration of TGF- β1(19) or TGF- β2(20, 21) are both accepted models that induce sustained elevations in IOP leading to significant RGC loss. In this study RGC survival was assessed using immunohistochemical quantification of $Brn3^+$ RGC and retinal imaging using optical coherence tomography (OCT) of the retinal nerve fibre layer (RNFL) thickness. Changes in retinal function were measured using electroretinography (ERG).

2 Materials and Methods

All reagents were purchased from Sigma (Poole, UK) unless otherwise specified.

2.1 Human (h)DPSC/BMSC/ADSC cultures

hDPSC were obtained from AllCell LLC (Berkeley, CA) and both hBMSC and hADSC from Lonza (Slough, UK), Each MSC batch represented pooled samples from 3 donors. The MSC were characterised by $CD29^+/CD44^+/CD73^+/CD90^+/CD45^-$ profile confirmed by the supplier) and demonstrated multi-differentiation (osteogenic, adipogenic and chondrogenic) capability. The stem cells were cultured into T25/T75 flasks (Corning, Amsterdam, NL) in both a total volume of 5ml/15ml DMEM containing 1% penicillin/streptomycin and 10% foetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) and at a density of $1x10^6$ cells/$2x10^6$ cells, respectively. Cultures were maintained at 37°C in 5% CO_2, the supplemented medium was changed every 3d and the cells were passaged when 80% confluent using 0.05% trypsin/EDTA. One week before transplantation, cells were transfected with a *gfp* plasmid using lipofectamine 3000 (Life Technologies, Invitrogen, Paisley, UK) according to the manufacturer's protocol. This study used MSC at passage 2-4, when 80% confluent and with no observable physical differences between the 3 cell types.

2.2 Experimental design

Twelve rats (24 eyes) received bi-weekly (twice a week) bilateral intracameral (IC) injections of TGF-$\beta_{0\text{-}35d}$ (for 5 weeks) and were separated into 2 groups of 6. On 0d, Group 1 received an *ivit* transplantation of DPSC into one eye and dead DPSC (sham control) into the other eye (left and right eyes, respectively, in 3 rats, and *vice versa* in the remaining 3 rats). On 0d, Group 2 received an *ivit* transplantation of BMSC into one eye and ADSC into the other eye (left and right eyes, respectively, in 3 rats, and *vice versa* in the remaining 3 rats). All rats in Group 1 and 2 received ERG and OCT recordings on 35d, before culling and tissue processing for immunohistochemistry. Animal numbers in each group were determined using a previously published power calculation(22, 23). A separate group of 6 rats received bi-weekly unilateral IC injections of $PBS_{0\text{-}35d}$ and these rats are referred to as the Intact Group.

2.3 Animals

All animal procedures were performed in strict accordance to the UK Home Office Animals Scientific Procedures Act, 1986, and approved by the University of Birmingham Ethical Review Sub-Committee. Eighteen adult female Sprague Dawley rats weighing 150-200g (Charles River, Margate, UK) were housed in conditions of 21°C and 55% humidity under a 12h light/dark cycle with a daytime luminance of 80 lux, given food/water *ad libitum* and were monitored by welfare staff. Gaseous anaesthesia was induced with 5% Isoflurane/1.5L per minute O_2 (National Veterinary Supplies, Stoke, UK) and maintained at 3.5% during surgery and 2% during ERG recording.

2.4 Surgery for IC injections to induce ocular hypertension and *ivit* transplantation of MSC

Following anaesthetic induction, IOP were recorded for all rats using an icare tonometer (Tonolab, Helsinki, Finland). Rats were then secured in a head-holding frame for IC injections of TGF-β1 (Peprotech, London, UK) through a single corneal incision, 2mm anterior to the limbus using a 15° blade (BD Ophthalmic System, Warwickshire, UK). Using the same incision site a glass micropipette, produced in-house from a glass capillary rod (Harvard Apparatus, Kent, UK) using a Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA) was used to inject 3.5µl of 5µg/ml activated TGF-β1 IC into all 12 rats. Contemporaneously, while the animals were still anaesthetised, a glass micropipette preloaded with 150,000 MSC suspended in 5µl of PBS, was used to inject living or dead cells (killed by heating for 30min at 80°C), into the vitreous of the eye (Fig. 1). A cell dosage of 150,000 was the maximum number of cells that could be suspended in 5µl while still allowing easy passage through the fine tip of the glass micropipette. After surgery, animals were placed in warm recovery cages and monitored for recovery of normal behaviour before being returned to their home cages. IOP recordings and IC injections of TGF-β were repeated bi-weekly$_{0\text{-}35d}$ throughout the study. A separate 6 rats (Intact Group) received bi-weekly$_{0\text{-}35d}$ IC injections of PBS alone.

2.5 Optical coherence tomography (OCT) of retinal nerve fibre layer

OCT allows *in* vivo measuring of retinal thickness with RNFL being a surrogate measure of RGC density. OCT retinal nerve fibre layer analysis was performed at 35d on all rats while under inhalation anaesthesia using a Spectralis HRA3 confocal scanning laser ophthalmoscope (Heidelberg Engineering, Heidelberg, Germany). OCT images were taken of the retina around the optic nerve head and the in-built software was used to segment the images and quantify the RNFL thickness.

2.6 Electroretinography

ERG records the electrical function of retina in response to a known intensity of light, with different intensities eliciting responses in different retinal cell populations. By eliciting a RGC dependant response, visual function of the glaucomatous rats can be assessed. ERG (HMsERG; Ocuscience, Kansas City, MO) were recorded at 35d. Rats were dark adapted for 12 hours overnight and prepared for ERG recording under dim red light (>630nm). Scotopic flash ERG were recorded from -5.5 to +1 log units with respect to standard flash in half log-unit steps, using DTL fiber (Unimed Electrode Supplies, Farnham, UK) corneal electrodes with pressure-molded Aclar contact lenses and needle skin recording electrodes (Unimed Electrode Supplies).

2.7 ERG analysis

By analysing the amplitude of the ERG trace, the visual function of the rat can be quantified. ERG traces were analysed using ERGVIEW (Ocuscience). Oscillatory potentials were removed by filtering waveforms above 20Hz from the trace before analysis. Traces at a light intensity of 1 and 3 mcd/s were chosen for analysis as they gave a clean, unambiguous positive scotopic threshold response (pSTR) with a mean latency of 100ms. The amplitude of the pSTR was measured from baseline. A representative, unfiltered trace is shown in Figure 5C.

2.8 Tissue preparation

Rats were given an intraperitoneal injection of 1ml sodium pentobarbital (National Veterinary Supplies) at 35d and perfused intracardially with 4% paraformaldehyde (PFA; TAAB, Reading, UK) in PBS while under terminal anaesthesia. Eyes were removed and immersion fixed in 4% PFA in PBS for 2h at 4°C before cryoprotection in 10%, 20% and 30% sucrose solution in PBS for 24h with storage at 4°C. Eyes were then embedded using optimal cutting temperature embedding medium (Thermo Shandon, Runcorn, UK) in peel-away mould containers (Agar Scientific, Essex, UK) by rapid freezing under crushed dry ice and stored at -80°C. After embedding, eyes were sectioned on a cryostat microtome (Bright, Huntingdon, UK) at -22°C at a thickness of 20 µm and mounted on positively charged glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA). Radial eye sections were left to dry on slides overnight at 37°C before storage at -20°C. Standard eye sections containing the optic nerve head were used to account for variation in RGC density.

2.9 Immunohistochemistry

Mounted tissue sections were equilibrated to room temperature (RT), hydrated in PBS for 2 X 5min, permeabilized in 0.1% triton x-100 in PBS for 20min at RT and washed for 2 X 5min in PBS before isolation with a hydrophobic PAP pen (Immedge pen; Vector Laboratories, Peterborough, UK). Non-specific protein binding sites in sections were blocked by incubation in blocking buffer (75µl; 0.5% bovine serum albumin (g/ml), 0.3% Tween-20, 15% normal goat/donkey serum (Vector Laboratories) in PBS) in a humidified chamber for 30min at RT and then sections were drained and incubated with either Brn3a primary antibody (1:200; Santa Cruz, CA, #SC-31984) or Stro1 primary antibody (1:100; R&D Systems, MN, #MAB1038) diluted in antibody diluting buffer (ADB; 0.5% bovine serum albumin, 0.3% Tween-20 in PBS) overnight at 4°C. The following day, slides were washed for 3 X 5min in PBS. Tissue sections were then incubated with Goat IgG Alexa 594 secondary antibody (1:400; Molecular Probes, Paisley, UK; #A-11058) diluted in ADB for 1h in a hydrated

incubation chamber at RT. After 1h, slides were washed for 3 X 5min in PBS, mounted in Vectorshield mounting medium containing DAPI (Vector Laboratories) and stored at 4°C before microscopic analysis.

2.10 Microscopy and analysis

Fluorescently stained sections were analysed by an operator blinded to treatment groups, using a Zeiss Axioplan-2 fluorescent microscope (Carl Zeiss Ltd, Hertfordshire, UK). For immunohistochemistry, $Brn3a^+$ RGC were counted in 20µm thick standard sections of the retina, along a 250µm linear region of the ganglion cell layer either side of the optic nerve, as described previously(22). Four sections per retina and 6 retinae from 6 different animals per treatment group were quantified.

2.11 Statistics

All statistical tests were performed using SPSS 17.0 (IBMM SPSS, Inc., Chicago, IL) and data were presented as mean ± standard error of the mean (SEM). The Shapiro-Wilk test was used to ensure all data were normally distributed before parametric testing. IOP data were measured using the generalised estimated equations (autoregressive correlation matrix). RGC survival and function data were tested for significance using 1-way ANOVA for >2 group comparisons ± SEM and Tukey *post-hoc* test. Statistical difference was considered significant at p values < 0.05.

3 Results

3.1 IC injections of TGF-β significantly raised IOP over 35d

IC injections of TGF-β induced a significant increase in IOP by 7d (16.9 ± 2.3mmHg) compared to Intact (PBS injected) eyes (10.2 ± 0.7mmHg) which was maintained between 14d (13.8 ± 0.7mmHg) and 35d (13.9 ± 0.9mmHg) compared to PBS (9.3 ± 0.2mmHg and 11.4 ± 1.1mmHg, respectively; Fig. 2). No significance differences were seen at 3d and 10d. IOP measurements between the four separate MSC treatment groups IC injected with TGF-

β were not significantly different from each other at any time point (not shown). These data show that IC TGF-β injections raised IOP and that IOP was not affected by the IC injection of stem cells.

3.2 MSC protected eyes from elevated IOP-induced RNFL thinning

The RNFL comprises axons belonging to the RGC and is lost concomitantly with the loss of the RGC cell body. TGF-β-induced elevations in IOP led to a significant RNFL thinning by 35d (27.0 ± 1.8μm) compared to Intact controls (55.5 ± 0.6μm; Fig. 3). Intravitreal (*ivit*) transplantation of both BMSC and, to a greater degree, DPSC, preserved RNFL thickness (37.3 ± 0.6μm and 41.3 ± 1.3μm, respectively) compared to sham-treated (dead DPSC transplanted) eyes (27.0 ± 1.8μm). *Ivit* transplantation of ADSC did not affect IOP-induced loss of RNFL thickness (29 ± 0.8μm) compared to sham-treated eyes (27.0 ± 1.8μm). RNFL thickness in Intact eyes was significantly greater than the RNFL thickness in all treated/sham-treated eyes.

3.3 MSC protected eyes from elevated IOP-induced loss of RGC

TGF-β-mediated elevations in IOP induced a significant 33% loss of RGC by 35d (49.0 ± 1.6/mm of retina; sham-treated group) compared to Intact controls (73.2 ± 2.5/mm of retina; Fig. 4). *Ivit* transplantation of both BMSC and to a greater degree, DPSC, protected against this RGC loss and thus exhibited greater numbers of RGC (60.2 ± 1.3/mm of retina and 69.7 ± 1.9/mm of retina, respectively) compared with sham-treated eyes (49.0 ± 1.6/mm of retina). DPSC-induced RGC survival was significantly greater than that achieved by BMSC. Notably, RGC numbers in the DPSC treatment group was not different from RGC numbers in Intact healthy eyes. Interestingly, *Ivit* transplantation of ADSC did not affect RGC survival (52.8 ± 1.9/mm of retina) compared with sham-treated eyes (49.0 ± 1.6μm/mm of retina). $GFP^+/Stro1^+$ MSC were detectable on the vitreal side of the inner limiting membrane in all animals at select regions throughout the eye (Fig. 4B). No evidence of migration into the retina was seen.

3.4 MSC protected eyes from elevated IOP-induced loss of RGC function

The pSTR represents the compound action potentials of RGC in response to the light dependant electrochemical signal originating from photoreceptors and thus its amplitude provides a read out of RGC function. TGF-β-mediated elevations in IOP induced a significant 85% loss of pSTR amplitude by 35d (21.2 ± 11.8μv) compared to Intact controls (140.8 ± 6.3μv; Fig. 5A). *Ivit* transplantation of both BMSC and to a greater degree, DPSC, protected against the RGC dysfunction associated with raised IOP and thus promoted greater preservation of pSTR (60.5 ± 18.3μv and 75.5 ± 17.4μv, respectively) compared to ADSC/sham-treated eyes (25.3 ± 7.8μv/21.2 ± 11.8μv, respectively), although this was only statistically different for DPSC treated eyes.

Recordings at a light intensity stimulus of 1000mcd/s as opposed to 3000mcd/s gave lower recordings of amplitude but the same relative differences (Fig. 5B)

4 Discussion

This study demonstrates for the first time that in a model of open angle glaucoma, DPSC promotes neuroprotection of injured RGC following *ivit* transplantation, preserving both their $Brn3a^+$ cell body within the ganglion cell layer, axons within the RNFL and electrical function as measured using ERG. Of note DPSC had the greatest therapeutic effect for RGC in this model of glaucoma whereas ADSC had the least. DPSC thus represent a potential effective cellular neuroprotective therapy for glaucoma patients.

Open angle glaucoma is the most common form of glaucoma and is characterised by elevated IOP related to trabecular meshwork fibrosis that perturbs the aqueous outflow pathway, as opposed to physical obstruction of the pathway by the iris i.e. angle closure glaucoma. The TGF-β model of glaucoma used in this study is based on the observation that patients with open angle glaucoma have elevated levels of TGF-β in the aqueous humour(24). This, coupled with the observations that TGF-β is a potent pro-fibrotic cytokine

and that fibrosis of the trabecular meshwork is a pathology that underpins the elevation in IOP in open angle glaucoma, makes this model a suitable representation of the human pathology. As seen in previous studies both from our laboratory and others(20, 21), TGF-β induced a reliable chronic rise in IOP and thus may be considered a suitable model to test neuroprotective cellular therapies relevant to glaucomatous RGC loss.

Our findings are consistent with our previous study that showed DPSC to be RGC neuroprotective in an optic nerve crush injury model(9). Optic nerve crush causes a rapid loss of RGC with approximately 90% dead by 3 weeks. DPSC promoted some neuroprotection of RGC when *ivit* transplanted, however, there was still approximately 60% RGC death after complete RGC axotomy. We postulated that the slow consistent release of NTF from MSC, would be better suited as a therapy for a chronic neurodegenerative condition, such as glaucoma where the retrogradle NTF supply is attenuated but still present, as opposed to an acute traumatic condition such as traumatic optic neuropathy, where the retrogradle NTF supply is completely ablated.

The present study is the first to demonstrate both the potential of DPSC to treat glaucomatous eyes and also to show that their therapeutic neuroprotective effect is significantly greater than BMSC and ADSC transplantation. RGC survival increased from 67% after treatment with dead DPSC to 95% after treatment with living DPSC; notably a RGC count not significantly different from that in Intact animals. This result corroborates the neuroprotective effects observed after optic nerve crush(9), as well as in transplantation studies after spinal cord injury in the rat(25), both of which demonstrated that DPSC promoted greater neuronal survival than BMSC. Similar to the studies described above, we also found that the transplanted GFP^+ MSC (stained for the MSC marker Stro1) survived within the vitreous space for the full length of the study with no evidence of migration into retinal tissue (Fig. 4B).

Previous studies by Johnson *et al,* have explored the use of BMSC as a treatment for glaucoma(13, 26). In their earliest study, BMSC were *ivit* transplanted into a laser-induced ocular hypertensive glaucoma model. The model yielded a 40% loss of RGC axons 28d after induction of glaucoma, which was reduced to a 10% after *ivit* BMSC transplantation. In the present study, our model yielded a Brn3a$^+$ RGC loss of 33% which was reduced to an 18% loss when eyes received *ivit* transplantation of BMSC. Possible explanations for why BMSC-mediated neuroprotection was 30% in a previous study(13) and only 15% in the present study may be that: A), the present study used a different model of glaucoma which results in less RGC death and thus less potential for neuroprotection, and B), the present study counted Brn3a$^+$ RGC whereas the Johnson *et al,* counted axons within the optic nerve, whose death precedes the loss of the RGC soma and will therefore yield greater disparities between treatment and control groups. A more recent study(27) has explored the use of BMSC as a treatment for open angle glaucoma and demonstrated a 17% increase in the survival of RGC, similar to the 15% neuroprotection observed in this study. However, the authors injected hyaluronic acid IC to elevate IOP instead of TGF-β (and thus this was a model of acute rather than chronic open angle glaucoma) and used animal-derived BMSC instead of human-derived as was used in the present study.

As well as counting RGC somata we used OCT to measure the thickness of the RNFL which reflects RGC axon loss. The RNFL is comprised of axons of the RGC as they course over the inner surface of the retina and towards the optic disc. Although axonal loss precedes RGC loss, the resolution of OCT is not as high as direct fluorescent microscopy making direct comparisons of the two measurements difficult. In addition, the RNFL contains astrocytes and retinal blood vessels and thus is not an absolute measure of RGC axon numbers. Despite this, we have previously shown that RNFL thickness is a reliable measure of moderate axonal loss but becomes inaccurate when the RNFL is substantially thinned (9). In the present study, RNFL thickness was reduced by 51% in the sham-treated glaucomatous eyes when compared to Intact controls. This is higher than the 33% loss in

RGC reported, and can be explained by fact that in glaucoma, RGC axonal loss precedes RGC loss and will thus is expected to be higher(28, 29). Indeed, although the pattern of neuroprotection as inferred from the RNFL thickness (i.e. DPSC being the most efficacious followed by BMSC and ADSC showing no neuroprotection) is the same as determined from $Brn3a^+$ RGC counts, there is more retinal cell "death" recorded by RNFL thickness measurements than $Brn3a^+$ RGC counts of the same group for the above reason.

The present study did not explore the mechanisms by which MSC elicit neuroprotection of RGC in glaucomatous eyes, or why DPSC have greater therapeutic efficacy than BMSC/ADSC. However, previous published studies by us and others provided strong evidence for paracrine-dependent effects. Perturbations in retrograde axonal transport of NTF have been implicated in the pathology of glaucoma(3, 4) and explains the success of NTF supplementation as a neuroprotective strategy(9-13). We have previously shown that DPSC, BMSC and ADSC secrete multiple NTF including NGF, BDNF, NT-3, glial cell line-derived neurotrophic factor, vascular endothelial growth factor and PDGF(10), whereas a recent paper demonstrated that PDGF(12) was a significant contributor to the neuroprotective effects elicited by BMSC on injured RGC. Our results corroborate this, demonstrating a significant ablation of the neuroprotective effects of DPSC, BMSC and ADSC when NTF receptors are blocked in culture(9, 10). As after optic nerve crush, the present study found that DPSC were the most RGC neuroprotective stem cells out of the tested MSC in our model of glaucoma which can be explained by their enhanced NTF profile in comparison to BMSC and ADSC(10), possibly due to the neural crest origin of DPSC (16).

Our study did not find a neuroprotective effect of ADSC in this model of glaucoma. This is unsurprising given the reduced neurotrophic profile of ADSC in comparison to BMSC/DPSC, particularly since the neurotrophins BDNF and NT-3 showing no detectable expression(10). However, a recent previous study demonstrated a 19% RGC neuroprotective effect of ADSC after transplantation into the vitreous of an animal model of glaucoma(27). The disparity between the finding of this study and ours could be explained by: A), the use of a different

model of glaucoma focusing on closed angle glaucoma rather than open angle glaucoma; B), study duration of only 4 weeks in comparison to our 5 weeks; and C), the study used ADSC derived from rats whereas the present study used ADSC derived from humans.

Finally, we measured the amplitude of the pSTR after ERG recordings on rats, using this as a measure of RGC function. A previous study(30) induced ocular hypertension in rats by injecting hypertonic saline into the episcleral vein, 5 times per week for 5 weeks. They demonstrated a 50% decrease in pSTR amplitude which correlated with both raised IOP and optic nerve damage. Our study demonstrated an 80-85% decrease in RGC function in sham-treated glaucomatous eyes compared to Intact eyes, a more profound effect than the previous study, which could be due to the difference in the animal model used. Interestingly, pSTR amplitude showed a similar pattern to the Brn3a survival and OCT RNFL thickness data, with DPSC and BMSC promoting the most significant RGC survival/functional preservation and ADSC/sham-treated eyes showing the least. There is a profound neuroprotective effect of DPSC on RGC, with RGC numbers no different from that of an Intact eye, but less functional preservation, with pSTR amplitude in the DPSC group significantly lower than in Intact eyes. This observation suggests that DPSC significantly protect RGC from death and dysfunction in glaucoma, however a portion of surviving RGC may still be dysfunctional or destined to die at a later time point, likely because of the underlying raised IOP. Indeed, a previous study(31) demonstrated a significant 50% decrease in pSTR after only 75min of acute ocular hypertension (from 12mmHg to 60mmHg) suggesting, together with this study, that RGC function is more sensitive to raised IOP than is RGC survival. The data highlights the fact that IOP lowering drugs may still be a useful adjunct to a cellular neuroprotective therapy.

Future work will focus on unravelling the precise mechanism for the paracrine-mediated neuroprotection of RGC and ensuring the safety of DPSC as a cellular therapy, for example, through the encapsulation of cells to prevent unwanted migration/proliferation(32), or the

genetic integration of a “suicide gene” to ensure cells can be selectively removed with ganciclovir after transplantation(33).

5 Conclusions

We demonstrate here for the first time the potential of DPSC to act as a cellular therapy for glaucomatous visual loss by protecting RGC and their axons from death and preserving RGC function. In comparison to BMSC and ADSC, DPSC were the most efficacious and thus may potentially represent an “ideal” cell to be trialled as a neuroprotective treatment for glaucoma.

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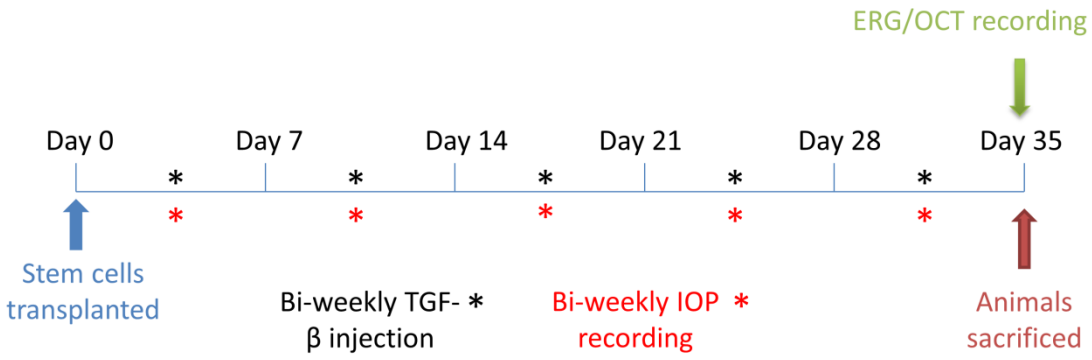

Group number	Number of animals	Injury	Treatment Left eye (1-3) Right eye (4-6)	Right eye (1-3) Left eye (4-6)
1	6	TGF-β-induced glaucoma	Intravitreal DPSC transplant	Intravitreal dead DPSC transplant
2	6	TGF-β-induced glaucoma	Intravitreal BMSC transplant	Intravitreal AMSC transplant
3	6	PBS control injection	No treatment	No treatment

Figure 1

Experimental design for the study. Time line of the *in vivo* experiment detailing when the stem cells were transplanted, glaucoma induction using bi-weekly injections of TGF-β and control animals using bi-weekly injections of PBS, ERG/OCT recording and day of animal culling. The group numbers and treatment regime for each eye are also given.

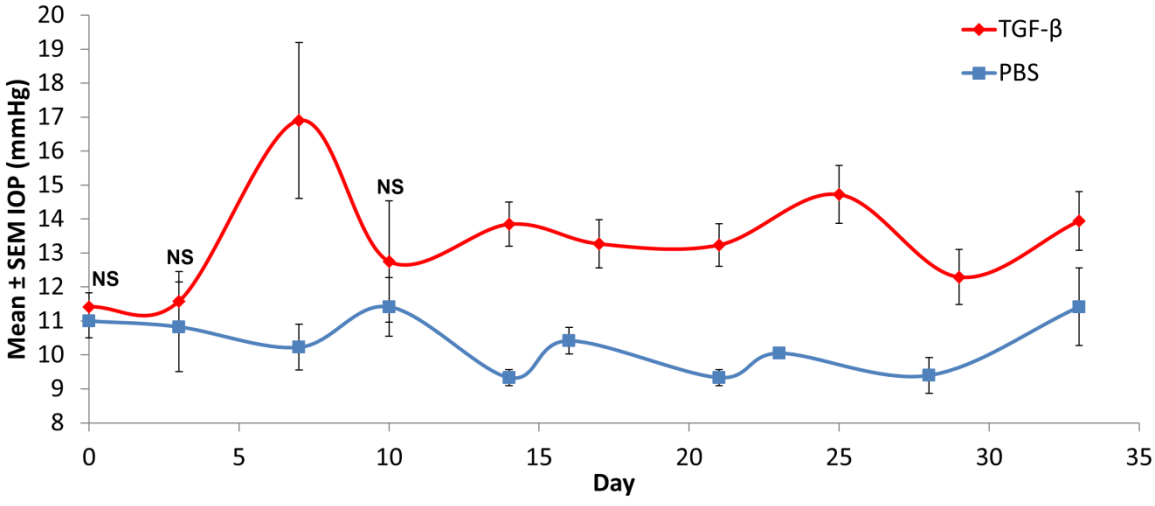

Figure 2

IOP in rats injected with either TGF-β or PBS. IOP measurements (mmHg) from rats IC injected with bi-weekly TGF-β (*red line*) or PBS (*blue line*) and *ivit* injected with stem cells (see Figure 1.) Note the acute peak rise in IOP at 7d and the chronically maintained elevation from 14d and beyond, all time points are significantly different from each other ($p<0.05$) unless otherwise stated (NS; Not Significant), as determined using the generalised estimated equations (autoregressive correlation matrix). The PBS data is from 6 eyes in 6 rats, whereas the TGF-β data is consolidated from the 4 treatment groups (12 rats, 24 eyes). Note that no significant differences in IOP were seen between the TGF-β injected groups transplanted with different MSC (not shown).

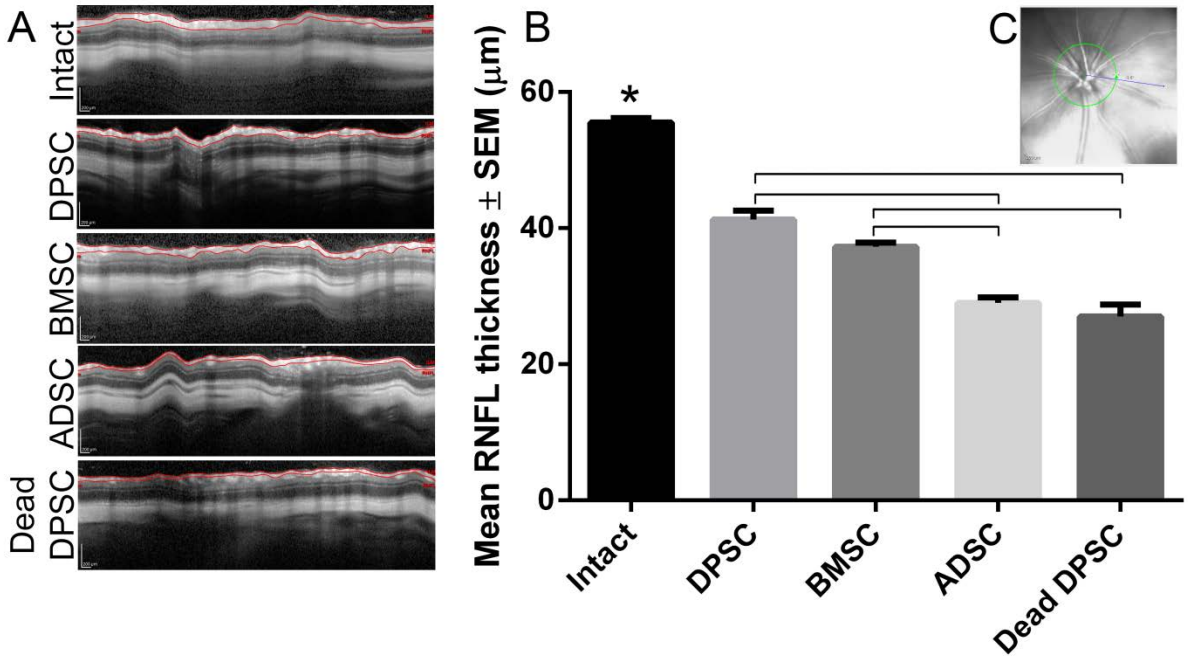

Figure 3

RNFL thickness after TGF-β-induced glaucoma in rats. OCT images of retina from an uninjured eye and a TGF-β-induced glaucomatous eye treated with *ivit* DPSC, BMSC, ADSC and dead DPSC (sham-treated) are shown (**A**) with *red lines* outlining the RNFL. The graph (**B**) depicts the RNFL thickness (µm) at 35d after glaucoma induction for the above

treatment groups as well as for uninjured eyes. *Asterisks* indicate significant difference from all groups where as *black lines* indicate significant difference between particular groups ($p<0.05$), as determined using a 1-way ANOVA and Tukey *post-hoc* test. Measurements were taken from images of sections containing the optic nerve head, indicated by the *green line* (**C**). Images and data representative from 5 animals/eyes per treatment group (*scale bar*: 200µm).

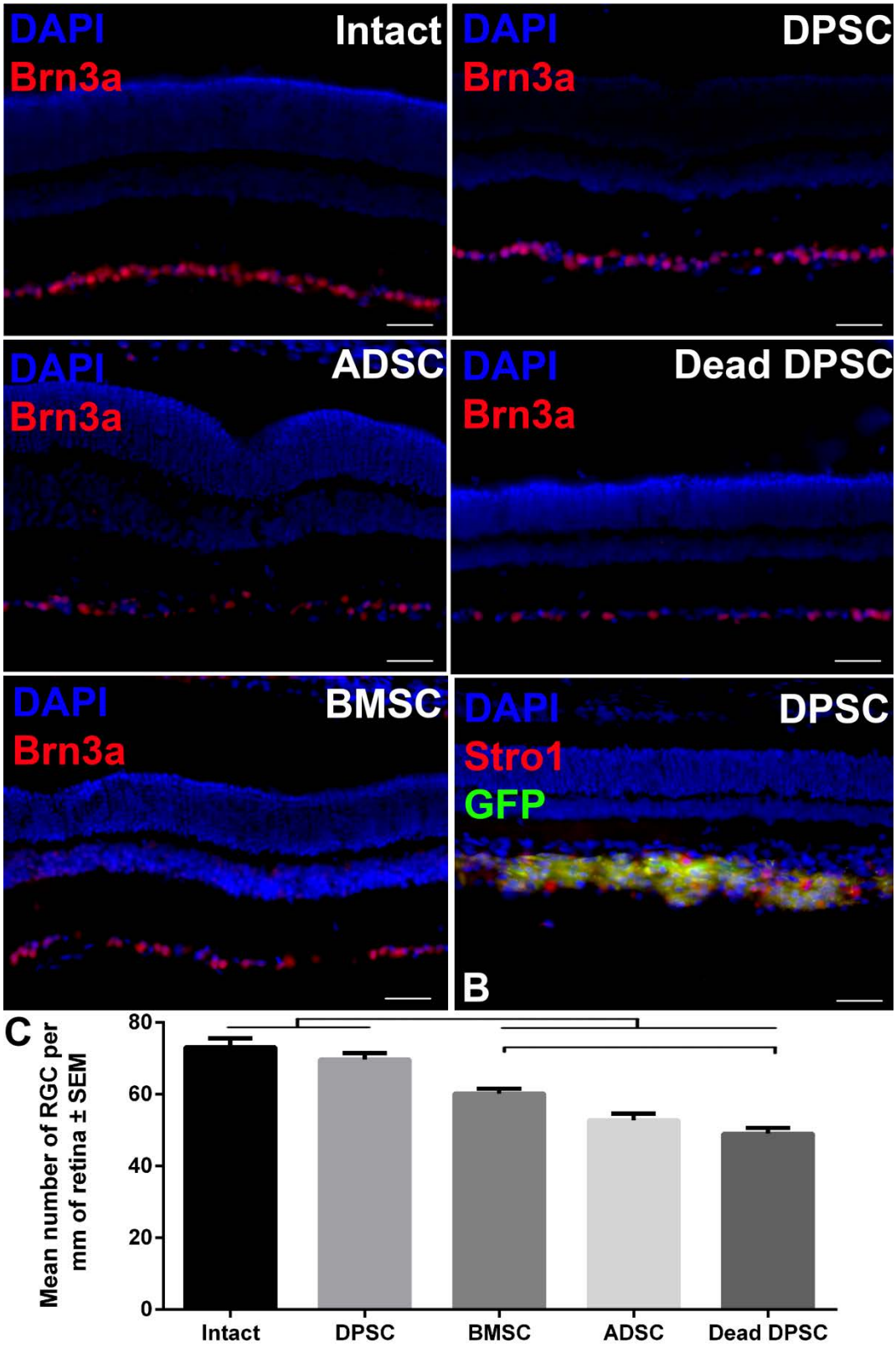
DAPI
Brn3a
Intact
DAPI
Brn3a
DPSC
DAPI
Brn3a
ADSC
DAPI
Brn3a
Dead DPSC
DAPI
Brn3a
BMSC
DAPI
Stro1
GFP
DPSC
B
C
Mean number of RGC per mm of retina ± SEM
80
60
40
20
0
Intact
DPSC
BMSC
ADSC
Dead DPSC

Figure 4

Brn3a$^+$ RGC counts in standard radial eye sections. Immunohistochemically stained radially sectioned retina, stained for Brn3a (*red*), from Intact rats and rats IC injected with TGF-β for 35d and also *ivit* transplanted with DPSC, BMSC, ADSC and dead DPSC (sham-treated). All images are representative of the 8 images taken per retina from 6 different animals (*scale bar*: 50μm). All images show tissues counterstained with the nuclear marker DAPI (*blue*). In (**B**), GFP$^+$ MSC stained for the MSC marker Stro1 identified in the vitreous, adhered to the inner limiting membrane. In (**C**), the mean number of Brn3a$^+$ RGC in a 1mm region of retina either side of the optic nerve head is shown from each of the above groups. *Black lines* indicate significant difference between groups ($p<0.01$), as determined using a 1-way ANOVA and Tukey *post-hoc* test.

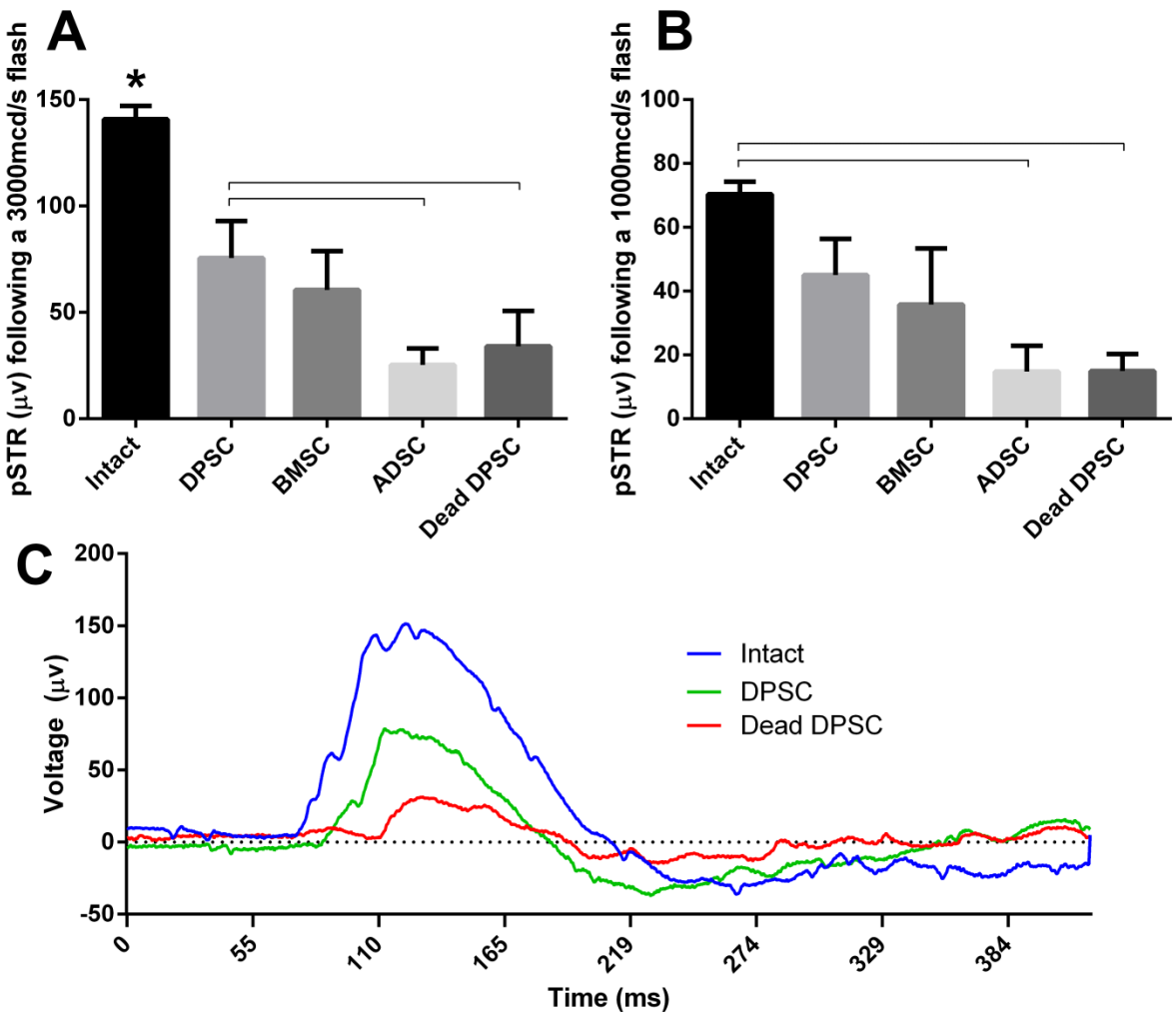

Figure 5

pSTR response after TGF-β-induced glaucoma. The mean amplitude of the pSTR response from the eyes of Intact rats and rats IC injected with TGF-β for 35d and also *ivit* transplanted with DPSC, BMSC, ADSC and dead DPSC (sham-treated) after a receiving a flash intensity of 3000mcd/s (**A**) and 1000mcd/s (**B**). *Asterisks* indicate significant differences between all groups whereas *black lines* indicate significant difference between groups ($p<0.05$), as determined using a 1-way ANOVA and Tukey *post-hoc* test. In (**C**), a representative image is shown of the ERG traces with an observable pSTR response from Intact rats and from rats IC injected with TGF-β for 35d with *ivit* transplantation of DPSC or dead DPSC, 6 different animals per group.