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14. ABSTRACT

1. Traumatic optical neuropathy (TON) results from trauma to optic nerve by head and eye injuries to both military and civilian population such as accidents, and blast related combat trauma. TON leads to irreversible blindness and represent a major public health burden with both economical and social impacts. Unfortunately, treatment is still rather limited. A large body of evidence indicates that TON affects optic nerve and its target neurons in the central nervous system, which provide vital retrograde trophic support to optic nerve. We hypothesize that systemic administration of bone marrow derived mesenchymal stem cells (MSC) to treat traumatic optic neuropathy (TON) will preserve/repair optic nerve, stabilize the unstable environment due to trauma and promote RGC regeneration and outgrowth by promoting the release of paracrine and autocrine mediators; induced Schwann cells from MSC (M-Sch) will repair the damaged RGC by remyelinating and providing multiple trophic factors. Previous studies have shown that activation in retinoic acid (RA) signalling triggers neurite outgrowth in adult mice. Here we found that intravitreal injection of retinoid X receptor agonist SR11237 not only preserved RGCs, and promoted RGC axon outgrowth at both 8 days and 14 days after TON. We have used Long Evan (LE) rats as a model for TON, MSC were isolated from LE rats, M-Sch were induced from MSC. Our main findings: (a). Using our modified forceps, a reliable and reproducible TON model was created. (b). Rat MSC and M-Sch were reliably produced for experiments. (c). Systemic administration of MSCs significantly preserved retinal ganglion cell survival after TON. (d). Systemic administration of MSCs also promote limited RGC axons regeneration. (e). Intravitreal injection of retinoid X receptor agonist SR11237 also protect RGC survival after TON and promote RGC regeneration. (f). Systemic administration of MSCs induced up expression of trophic factors in the retina (CNTF, BDNF, bFGF). (g). Intravitreal injection of retinoid X receptor agonist SR11237 combined with systemic administration of MSCs promote RGC survival and axons regeneration after TON.

This study showed that systemic administration of MSC could significantly protect retinal ganglion cells after TON. Intravitreal injection of SR11237 protect RGCs after TON and promote RGC axon regeneration. We also showed that up-regulation of trophic factors in the retina after MSC injection into TON model, which is, at least in part the mechanism of MSCs in protecting RGCs after injury.

15. SUBJECT TERMS nothing listed

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Table of Contents

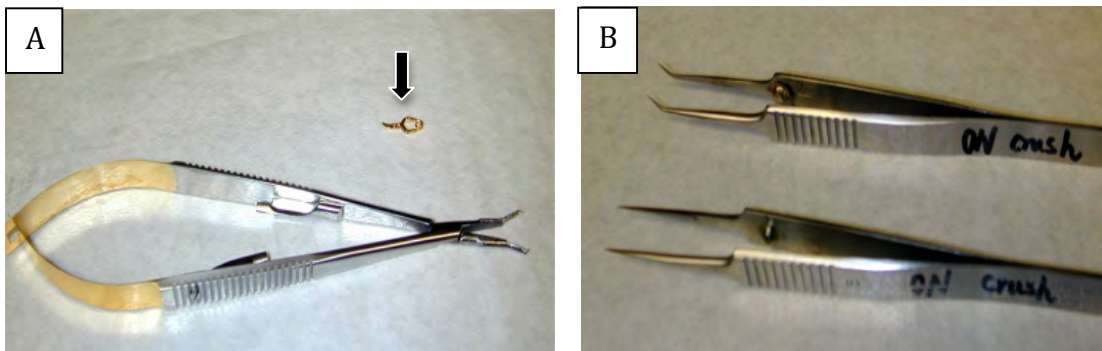
	<u>Page</u>
Introduction.....	5
Body.....	5
Key Research Accomplishments.....	11
Reportable Outcomes.....	11
Conclusion.....	11
References.....	12
Appendices.....	13

INTRODUCTION

Traumatic optical neuropathy (TON) results from trauma to optic nerve by head and eye injuries to both military and civilian population such as accidents, and blast related combat trauma [1-3]. In a military report, 82% of severe eye injuries were caused by blast and blast fragmentation. TON leads to irreversible blindness and represent a major public health burden with both economical and social impacts. Unfortunately, treatment is still rather limited. Cytokine-mediated neuroprotection has been repeatedly demonstrated, and reliably reproduced, in multiple animal models with a range of optic nerve injury conditions [4-6] and block neuronal cell death in an excitotoxicity animal model [7-9]. A significant challenge to clinical implementation of this work is that cytokines are rapidly degraded by endogenous proteases. So the effect is short lasting. A direct and reliable approach to stem cell-mediated neuroprotection is a rational approach. A large body of evidence indicates that TON affects optic nerve and its target neurons in the central nervous system, which provide vital retrograde trophic support to optic nerve [10-12]. As an alternative approach, we propose a **non-invasive, systemic delivery of stem cells to optic nerve and related target neurons in the brain**. The systemic administration of stem cells offers substantial advantages over local delivery. These cells can exert therapeutic effects over the injured optic nerve and its targeted neurons in the brain, and multiple injections can be performed if needed. Others have successfully used the intravenous administration of MSC for treating stroke, cerebral ischemia, brain injury and myocardial infarction [13-15]. Based on the extensive experience with both MSC and M-Sch as therapies for regenerative and degenerative medicine, this study will determine whether it is realistic to transfer this treatment to the clinical setting.

BODY

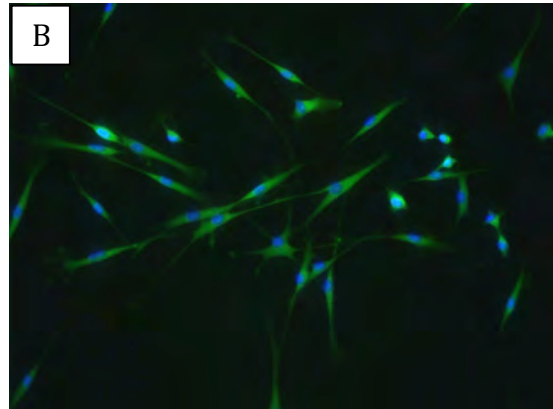
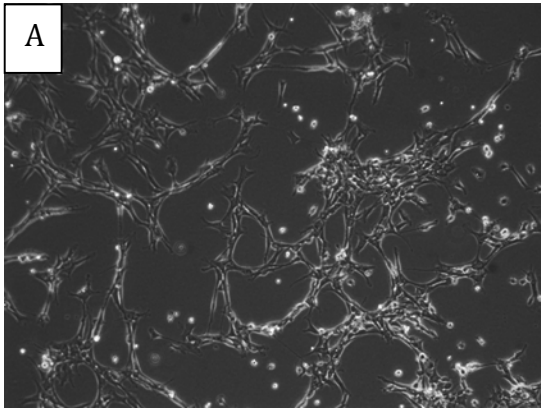
1. Animal models: Long Evan rats are used throughout the study. Traumatic axonal injury (TAI) model was first created with a 60-g Yasargil aneurysm clip (Aesculap AG &C0, Tutlingen, Germany, arrow in image A) according to published protocol (16). However, we found it is impossible to use the Yasargil aneurysm clip, since it cannot perform 'dissecting', which is critical for free blood vessels from optic nerve bundle. We made a special clip by modifying a fine forceps (see image B). A reliable TAI model is created by using this modified forceps.



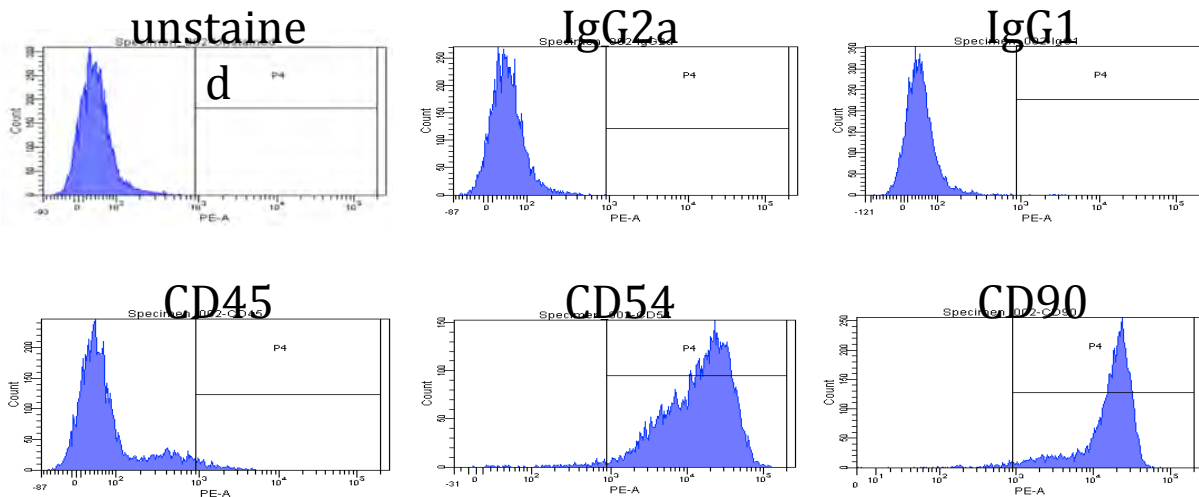
2. Isolation rat bone marrow derived mesenchymal stem cells (MSC, see image A); induction of Schwann cells from MSC (M-Sch, see image B) and MSC were purified with BD IMagnet combine with FACS analysis.

Isolation MSC from Long Evans rats using our published protocols (17), Image A showed MSC at passage 0.

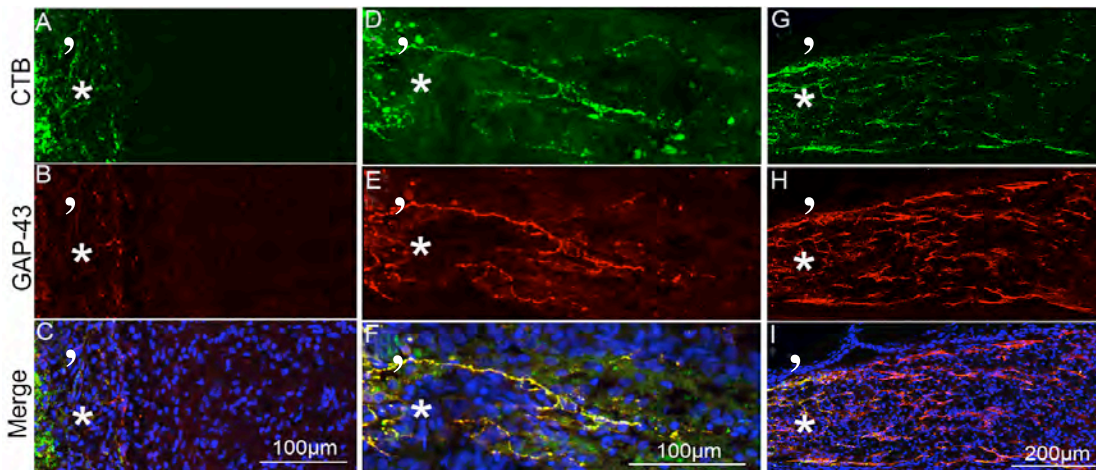
Induction of Schwann cells from MSC according to published protocol (18-19). Image B showed antibody against S100 staining of Schwann cells induced from MSC. Graph C showed FACS analysis after CD54/CD90 selection.



C

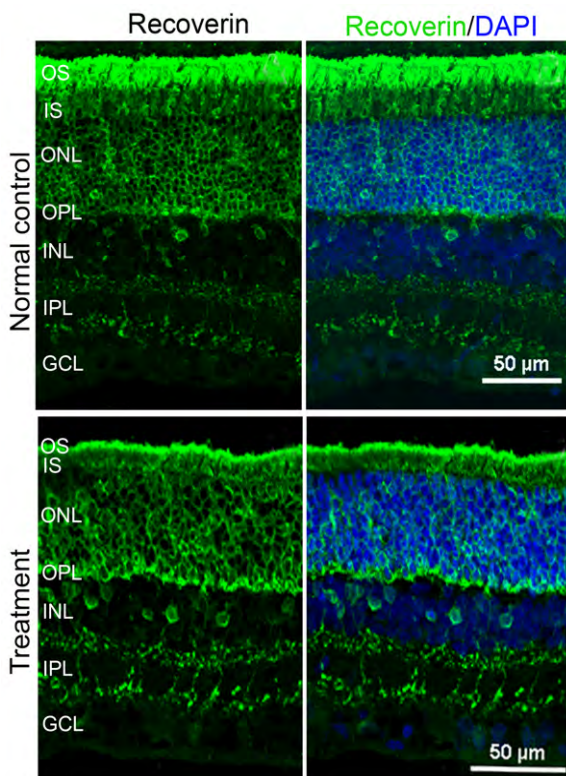


3. RGC axon regrowth after injection of SR11237 following TON

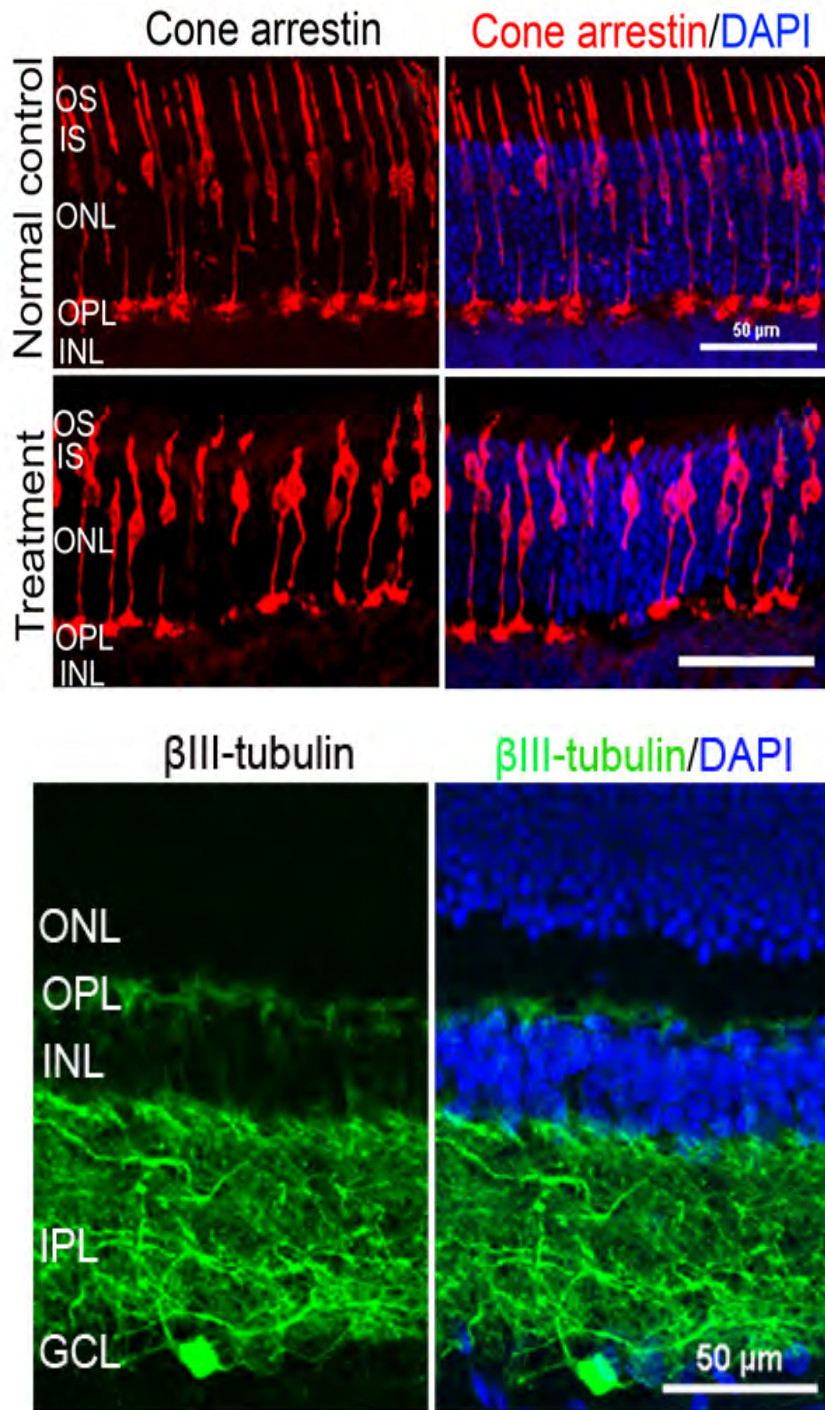


Immediately after ONC, retinoid X receptor agonist SR11237 (4 μ M) was injected vitreously. CTb (green) was injected 3 days before animal euthanization. Optic nerves were collected at 7 and 14 days after ONC and stained with growth-associated protein 43 (GAP-43, red), counterstained with DAPI (blue). These images show that ONC without treatment, there is hardly regeneration of RGC axons; while in SR11237 treated eyes; there is regrowth of RGC axons at 7 and 14 days after ONC.

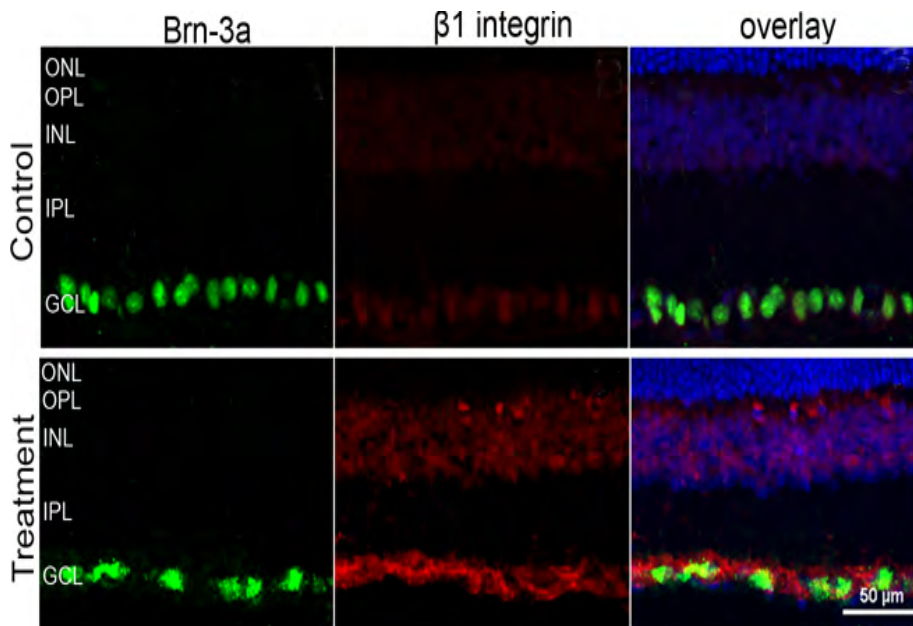
4. Inner retinal changes after intravitreal injection of SR11237 following TON



TON also affects photoreceptors: one week after TON, photoreceptor outer segments were obviously reduced in length even after retinoid X receptor agonist treatment compared with wild type untreated control.

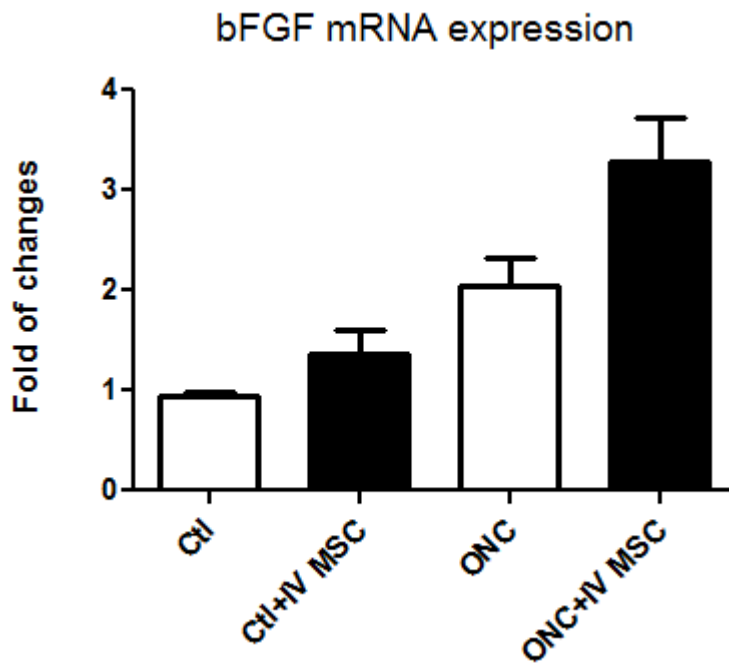


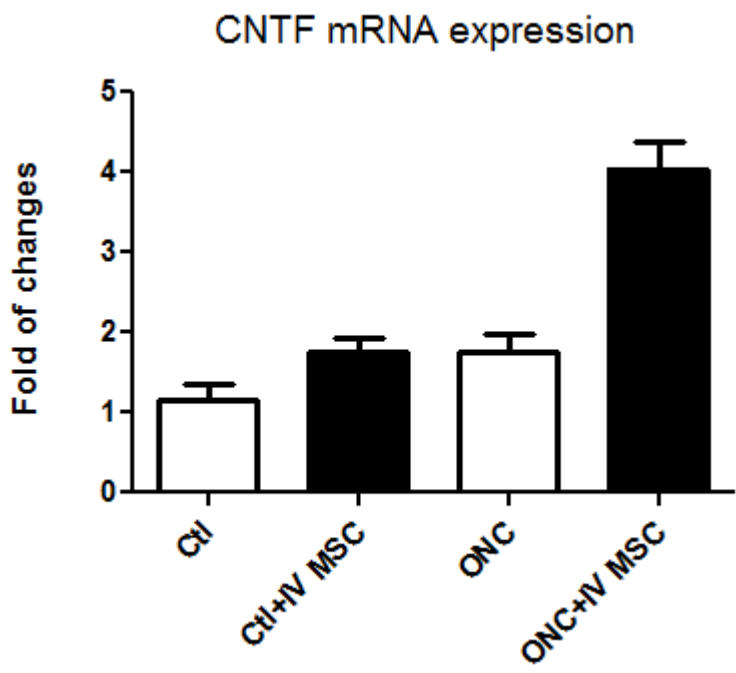
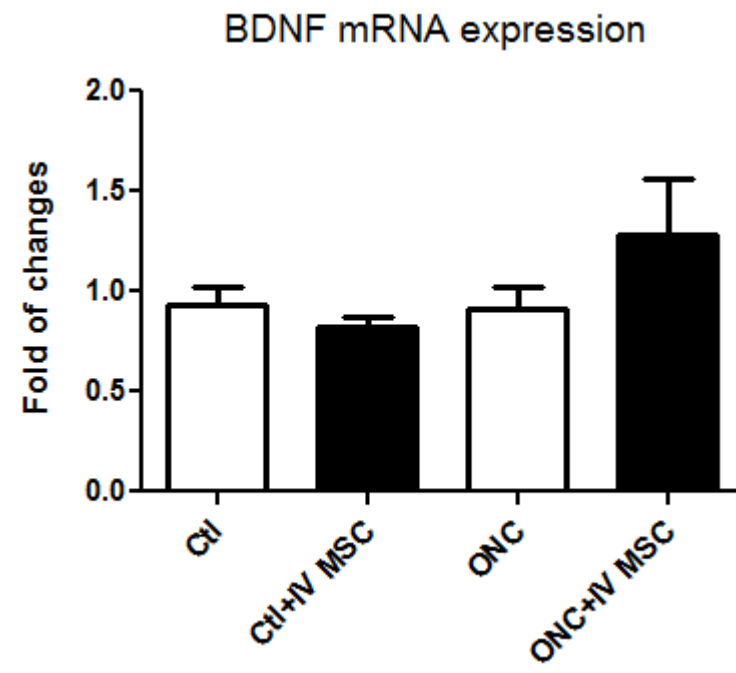
Retinal sections were stained with antibody against cone arrestin after injection of SR11237 following ONC. At one week, cone morphology is preserved compared with normal control; while in untreated retina, both inner and outer segments were further reduced in length. Further examination showed that RGC dendrites as revealed by β III-tubulin (green) are also preserved



Retinal sections from SR11237 treated after ONC showed that there is up-regulation of $\beta 1$ integrin (red) in the inner nuclear and RGC layers compared with normal untreated control.

5. Trophic factor expression after intravenous injection of MSCs following TON





Total RNA was extracted from retinas 7days after TON following intravenous injection of 2 millions of MSCs; control samples were from untreated and TON without intravenous injection of MSCs. We found the expression of CNFT and bFGF was significantly higher in retina receiving MSC treatment compared with controls.

6. Functional evaluation

Electroretinogram (ERG) measure full-field retinal potential from the cornea. Under scotopic conditions, a-wave is generated by photoreceptor phototransduction; b-wave is mainly generated by depolarization of ON-bipolar cells and Müller cells. The Scotopic threshold responses (STR) are created from the innermost retina, where the RGC bodies are located.

Our study showed that at one week after ONC without treatment, both a-and b-waves reduced compared with untreated wild type rats. Further study with measuring STR did not detect significantly difference between TON and TON followed by MSC treatment.

Luminance threshold recording (LTR) from the superior colliculus

The functional state of the retina was evaluated by recording the multi-neuronal responses in multiple (16–18) microelectrode penetrations into the unilateral superior colliculus (SC) of anesthetized rats. At each recording site, the receptive field was located by presenting flashes of the light spot of 3° in diameter. Response luminance threshold was then measured and defined as a minimal luminance of the stimulating light spot eliciting criterion multi-unit response (of amplitude twice of the level of the background activity). This procedure results in a map of focal luminance thresholds over the whole visual field of the eye contralateral to the tested SC. Based on these recordings, the cumulative curve of the luminance thresholds across the retina was calculated, which showed the percent of retinal area (y-axis) where the visual thresholds were less than the values indicated at the x-axis. Our LTR showed that there was no signal recorded after optic nerve crush, indicating the integrity of retina is needed for luminance threshold recording. We did not perform LTR during the second year.

KEY RESEARCH ACCOMPLISHMENTS

- Reliable create TAI rat model by using our modified forceps
- Reliable isolate rat MSC and induction of Schwann cells from MSC (M-Sch)
- Worked out a new protocol for MSC purification with BD IMagent combine FACS analysis
- Reliable anterogradely label retinal ganglion cells and axons by injecting CTB into vitreous cavity; prepare for retinal whole mount
- Reliable retrogradely label retinal ganglion cells by applying fluorogold onto the superior colliculus
- Worked out protocol for quantifying retinal ganglion cells on retinal whole mount preparation
- Intravenous administration of MSC protect retinal ganglion cells after TAI and promote axons regeneration
- Intravenous injection of retinoid x receptor agonist protects RGCs and promote RGC axon regrowth after TON
- Intravenous injection of retinoid x receptor agonist also preserve inner retina
- Trophic factors-bFGF, CNTF and BDNF were up-regulated after TON following intravenous injection of MSCs
- Applying M-Sch to optic nerve crush site preserve retinal ganglion cells after TAI
- The Scotopic threshold responses (STR) are the better measurement for TAI model
- Optokinetic response provides non-invasive measurement for TAI model

REPORTABLE OUTCOMES:

The research was presented at ARVO, 2014, Orlando, FL.

Manuscript is in preparation.

- **CONCLUSION:** We have reliably created rat model for TAI; reliably isolated MSC and induction of Schwann cells. We have found that non-invasive administration of MSC can protect retinal ganglion cells after TAI and local administration Schwann cells derived from MSC also protect retina ganglion cells. Systemic administration of MSC promotes axon regeneration, however axon regrowth is rather limited. To evaluate retinal function after TAI and intervention, the scotopic threshold responses (STR) are the better measurement for TAI model; Optokinetic response also provides valuable indication of retinal function; and luminance threshold recording for the superior colliculus fails to record any retinal activities after TAI. Intravenous injection of retinoid X receptor agonist protects RGC survival and promotes RGC axon regrowth after TON. Trophic factors bFGF, CNTF and BDNF were up-regulated after intravenous injection of MSCs following TON. Future study: long-term evaluation of retinal ganglion cell protection and axon regeneration after intervention; new regents that have approved to promote axon regeneration combine with systemic administration of MSCs.

Future study

Current study showed that intravenous injection of MSCs can protect RGCs and preserve retinal integrity after TON. Intravenous injection of retinoid X receptor agonist promotes RGC survival and RGC axon regrowth after TON. However, the RGC axon regrowth is rather limited. Future study will use optic nerve injury model that is mild (without optic nerve transection), such as trauma. The critical step to preserve vision after optic nerve injury is to protect RGCs from dying even for 1-2 weeks, which will provide a window for other therapeutic intervention.

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APPENDICES: PI's biosketch

Principal investigator (Wang, Shaomei)

BIOGRAPHICAL SKETCH

NAME Shaomei Wang	POSITION TITLE Associate Professor		
eRA COMMONS USER NAME Wangsha			
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education,</i>			
INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
Jinzhou Medical College, PR China	B.M. (M.D)	1984	Medicine
Chinese Medical University, PR China	Masters	1987	Neuroscience
University of Sheffield, UK	Ph.D.	1997	Visual neuroscience
Institute of Ophthalmology, UCL, London	Post-doc	2001	Cell-based therapy

A. Personal Statement

Our laboratory has a long history of applying cell-based therapy for retinal degeneration. We have explored the efficacy of a range of different cell types (i) cells to replace defective retinal pigment epithelial (RPE) cells such as human embryonic stem cell derived –RPE cells, (ii) cells that appear to function by releasing growth factors such as peripheral nerve ensheathing cells (Schwann cells) or cells genetically modified to release growth factors and (iv) cells with multiple functions such as stem cells and progenitors. Our studies have provided the pre clinical data for three prominent first in man human clinical trials for retinal degeneration using either human embryonic stem cells (Advanced Cellular Therapeutics) or adult mesenchymal stem cells (Johnson and Johnson) or central nervous system derived neural stem cells (StemCell inc). Recently, my laboratory has pioneered a new approach to treatment involving systemic administration of mesenchymal stem cells and shown extensive morphological and functional preservation in rodent models of retinal disease. I was recruited to the Cedars-Sinai Regenerative Medicine Institute where we are collaborating with the director, Dr Clive Svendsen who has a long history of using stem cells to model and treat diseases of the CNS, Dr Alexander Ljubimov, director in eye program, and immunologists with an interest in transplantation. I will continue to work with our long-term collaborators Dr Gamm (U of Wisconsin) using retinal progenitors/stem cells to limit retinal degeneration. Our preclinical studies will be focused on the efficacy, long-term survival of donor cells, mechanism of action and immunological responses after cell-based therapy. The object of our translational research program is to treat retinal degeneration and optic nerve repair with cellular therapy.

B. Positions and Honors.

Positions and Employment

2012-present Associate professor, Cedars-Sinai Regenerative Medicine Institute, LA, CA

2006 - 2011 Assistant Professor, Casey Eye Institute, OHSU, Portland, USA
 2005- 2006 Assistant Professor, Moran Eye Center, Utah, USA
 2001 - 2005 Senior lab specialists, Moran Eye Center, Utah, USA
 1997 - 2001 Post-doctoral fellow, Institute of Ophthalmology, UK
 1994 - 1997 PhD student, University of Sheffield, UK.
 1991 - 1993 Visiting Scholar, University of Sheffield (supported by British Council).
 1987 – 1991 Lecturer, JinZhou Medical College, PR China.

Honors

1984-1987 Graduate studentship, Jinzhou Medical college, PR China
 1991-92 British Council and Chinese government award (visiting scholar to University of Sheffield, UK)
 1992 Overseas Research scholarship
 2004 Permanent US residency awarded in the US National Interest on the basis of outstanding researcher
 2010 Paper was selected as Paper of the Month (March) at OHSU med school.

Other Experience and Professional Memberships

Member of ARVO's Global Presence Pillar Steering committee
 Association for Research in Vision and Ophthalmology
 Society for Neuroscience
 Editorial member of Transplantation & technology and research
 Editorial member of International Journal of Ophthalmology
 Investigative Ophthalmology and Visual Science
 Experimental Eye Research
 Vision Research
 Current Eye Research
 Expert Reviews
 Stem cells international
 Plus ONE

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Shaomei Wang; YuChun Tsai; Benjamin Bakondi; Sergey Girman; Lin Shen; Bin Lu. Systemic administration of MSCs preserves RGC survival after optic nerve crush. 2014. ARVO, B0139.

C. Current Grant Support

1RO1EY020488 Wang (PI)

09/01/2011-08/31/2016

NIH/NEI

Development of non-invasive cell-based therapy for retinal degeneration and associated vascular pathology. The overall objective of this research proposal is to preserve vision and limit vascular pathology using non-invasive MSC therapy in rodent models for retinal degeneration. The MSCs have been widely used in both regenerative and degenerative medicine. The proposed research will be critical in determining whether systemic administration of MSCs offers a realistic likelihood of translation to the clinic for the treatment of retinal degeneration and ocular vascular pathology.

Role: PI

CS-RMI (start-up fund) Wang (PI)

06/01/2011-05/31/2015

Applying stem cell therapy for retinal degenerative disease

Department of Defense Wang (PI)

09/01/12-08/31/14

Non-invasive cell based therapy for traumatic optic neuropathy

Janssen Research & development, LLC Wang (PI)

08/01/13-07/31/15

Preclinical research with umbilical derived stem cell therapy for RCS rodent model for retinal degeneration.

Pending

NEI (R24) Wang (PI)

Preclinical program for Treating Retinitis Pigmentosa by Neural Progenitor Cells

Poster board Number: 2440 - B0139



Systemic Administration of MSCs Preserves RGC Survival after Optic Nerve Crush

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Abstract

Purpose: Traumatic optic neuropathy (TON) results from trauma to optic nerves by head and eye injuries to both military and civilian population such as accidents, recreation and blast related combat trauma. TON leads to irreversible blindness and represents a major public health burden with both economical and social impacts. Unfortunately, treatment is still rather limited. A large body of evidence indicates that TON affects optic nerve and its target neurons in the central nervous system, which provide vital retrograde trophic support to optic nerve. Here we used systemic administration of bone marrow derived mesenchymal stem cells (MSC) to treat TON in a well-established rat model-optic nerve crush (ONC).

Methods: Long Evans rats, both sex at 6-8 weeks old were used for creating ONC model (one side) with customized forceps. On the next day, 2 millions of MSC-derived from Long Evans rats were injected via tail vein. Visual function was tested by optokinetic response, luminance threshold recording from the superior colliculus and electroretinograph at 8 and 14 days after ONC. Survival retinal ganglion cells (RGCs) were quantified by applying Fluoro-Jade to the superior colliculus and antibody-BrdU staining on retinal whole-mount preparation. Optic nerve axons were examined by injecting CTb intravitreally and antibody-RT97 staining. Stereology was used to count the number of RGC survival.

Results: There was about 60% RGC survival in animals with intravenous injection of MSC after ONC, compared with 40% RGC survival in control animals (no treatment after ONC). This difference is significant ($p < 0.05$). The visual acuity was also preserved in MSC-treated animals compared with control. Luminance threshold recordings revealed there was no signal detected after ONC, indicating the integrity of retina is required for luminance threshold recording. On all the MSC-treated animals, there were some RGC axons regeneration, future manipulations to enhance axons grow is needed.

Conclusions: Systemic administration of allogeneic MSC promotes RGC survival and enhances RGC axon regeneration after optic nerve crush. Long-term study using this approach to preserve both RGC and its axons in under investigation.

Introduction

Traumatic optic neuropathy (TON) is an acute injury of the optic nerve with disruption of visual function. TON results from trauma to optic nerve by head and eye injuries to both military and civilian population such as accidents, recreation and blast related combat trauma. In a military report, 42% of acute eye injuries were caused by blast and blast fragmentation. TON leads to irreversible blindness and represents a major public health burden with both economical and social impacts. Unfortunately, treatment is still rather limited. The optic nerve is fixed within the sheath of optic canal, which contributes to the vulnerability of the nerve in this location. Visual restoration of the globe can result in partial or complete optic nerve avulsion. However, it is unknown how much swelling is needed to compress the nerve. Despite this uncertainty, swelling is commonly considered as a potential for performing optic canal decompression surgery. So far there is no convincing evidence that this treatment improves the outcome of patients' vision.

Material and Methods

MSCs were isolated from bone marrow of Long Evans rats (6-8 weeks old) according to the method described (Wang et al. 2010). MSCs were purified with BD MagSort combined with FACS analysis. **Brown cells** were cultured according to D'Souza et al. 2004.

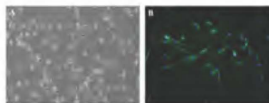
Optic nerve crush (ONC) Long Evans (LE) rats at 6-8 weeks were used to create ONC model with customized forceps.

Intravenous injection. 2 millions of MSCs were injected via tail vein at 24 hours and 1 day after ONC. Retinal X-ray against SR11277 (Sigma) was injected into vitreous cavity 24 hours after ONC.

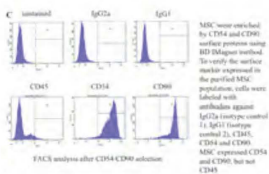
Quantification of survival and outgrowth of RGC. Retrograde tracer Fluoro-Jade was applied to the superior colliculus. Slides before fixation animals. Retinal whole-mount will be prepared to quantify RGC survival with our published protocol. Anterograde tracer CTb was injected into vitreous cavity to examine RGC regeneration histologically.

Visual function was tested by optokinetic response (OKR) and Luminance threshold recording (LTR) with our published protocol.

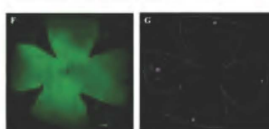
Retinal whole mount. At the end of experiment, retinal whole mount was prepared, antibody against BrdU was used to identify RGCs, stereology images were taken. Stereology was used to count the number of RGCs using an Axiovert 5MOT Zeiss microscope with a x2.5 objective lens. A simultaneously, stereology counts to count the area of interest, and a x40 magnification to count RGCs. Retinal sections were examined for photoreceptors and inner retinal changes after ONC and treatment.



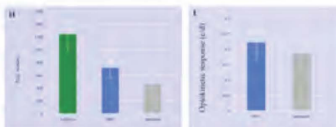
MSCs were isolated from Long Evans rats (6-8 weeks old) according to our published protocol (Wang et al. 2010). Passage 2-4 was used in this study. A. MSCs at Passage 1. B. Isolation of Schwann cells from MSCs according to D'Souza et al. 2004. The image showing S100 staining of Schwann cells isolated from MSCs.



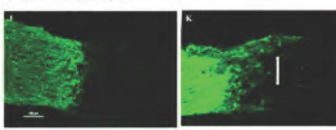
D. 60% Young's laminar clip was reported to create optic nerve crush (ONC) model. However, it turned out the clip was too big to be inserted into the optic nerve. It turned out that the clip was too big to be inserted into the optic nerve. It turned out that the clip was too big to be inserted into the optic nerve. It turned out that the clip was too big to be inserted into the optic nerve.



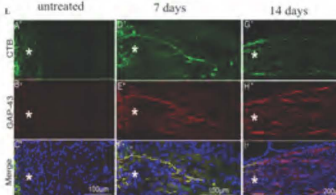
F. Retrograde labeling retinal ganglion cells by applying Fluoro-Jade to the superior colliculus. Here is the retinal whole mount image showing Fluoro-Jade labeled retinal ganglion cells and high power image of Fluoro-Jade labeled retinal ganglion cells. G. Optic Nerve transection was used to create RGCs.



F. Optic Nerve transection was used to create RGC survival in control and MSC-treated groups. The bar graph shows RGC survival percentage in control and MSC-treated groups. The bar graph shows RGC survival percentage in control and MSC-treated groups. The bar graph shows RGC survival percentage in control and MSC-treated groups.

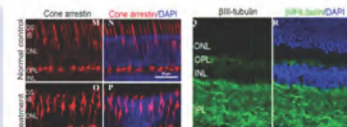


G. Choline tracer (CTb) was injected into vitreous cavity after ONC 3 days before euthanasia of animals to anterogradely label RGC axons. 3 weeks after ONC without treatment, there is limited optic nerve regeneration. 4 weeks after ONC with intravenous injection of Schwann cells, there are some optic nerve regeneration (double arrows).

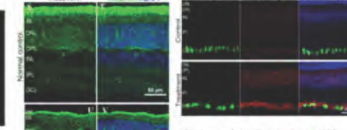


H. Immediately after ONC, retinal X-ray against SR11277 (Sigma) was injected intravitreally. CTb (Sigma) was injected 3 days before animal euthanasia. Optic nerve was collected at 7 and 14 days after ONC and stained with growth-associated protein 43 (GAP-43, only counterstained with DAPI). These images show that ONC without treatment, there is hardly regeneration of RGC axons (A-C) while in SR11277 treated eyes, there is growth of RGC axons at 7 (D-F) and 14 (G-I) days after ONC.

Abbreviations: CTb: Choline tracer; SR11277: SR11277 (Sigma); GAP-43: Growth-associated protein 43; DAPI: 4',6-diamidino-2-phenylindole; MSC: mesenchymal stem cells; ONC: optic nerve crush; OKR: optokinetic response; LTR: luminance threshold recording; RGC: retinal ganglion cell; RPE: retinal pigment epithelium; SR11277: Retinal X-ray against; SRW-1: Schwann cell derived factor.



I. Retinal sections were stained with antibody against choline acetyltransferase (ChAT) following ONC. At one week, some morphology is preserved compared with normal control, while in untreated retina, both inner and outer segments were further reduced in length. Further examination showed that RGC dendrites in control (B) inhibit (arrows) are also preserved (C&D).



J. Optic nerve crush also affects photoreceptors. SR11277: Normal retinal sections stained with antibody against: One week after ONC, photoreceptor outer segments were severely reduced in length from after SR11277 treatment (ARVs) compared with normal retina (ARVs). B. Retinal sections from SR11277 treated after ONC showed that there is up-regulation of P1 integrin (red) in the inner nuclear and RGC layers compared with normal untreated control.

Results

1. Intravenous injection of bone marrow derived mesenchymal stem cells (MSC) can preserve retinal ganglion cells after optic nerve crush.
2. Intravenous injection of MSC also promotes limited regeneration of RGC axons following ONC.
3. Retinal X-ray against choline acetyltransferase (ChAT) survival and promote RGC axon regeneration.
4. Visual function was not improved with MSC treatment after ONC.
5. ONC also affects morphology of photoreceptors.

Further study

1. Enhance RGC survival and promote RGC axon regeneration by combined treatment such as local injection of SR11277 and intravenous injection of MSC.
2. To investigate the mechanism of action of MSC in protecting RGCs.
3. Local delivery trophic factors by Schwann cells combined with systemic injection of MSC.
4. Preserving photoreceptors and retinal integrity should be taken into consideration when applying therapy for optic nerve damage.

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Commercial relationship disclosure: None