

Hypoxic-Preconditioned Bone Marrow Stem Cell Medium Significantly Improves Outcome After Retinal Ischemia in Rats

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Submitted: May 31, 2015

Accepted: April 28, 2016

Citation: Roth S, Dreixler JC, Mathew B, et al. Hypoxic-preconditioned bone marrow stem cell medium significantly improves outcome after retinal ischemia in rats. *Invest Ophthalmol Vis Sci.* 2016;57:3522-3532. DOI:10.1167/iovs.15-17381

PURPOSE. We have previously demonstrated the protective effect of bone marrow stem cell (BMSC)-conditioned medium in retinal ischemic injury. We hypothesized here that hypoxic preconditioning of stem cells significantly enhances the neuroprotective effect of the conditioned medium and thereby augments the protective effect in ischemic retina.

METHODS. Rats were subjected to retinal ischemia by increasing intraocular pressure to 130 to 135 mm Hg for 55 minutes. Hypoxic-preconditioned, hypoxic unconditioned, or normoxic medium was injected into the vitreous 24 hours after ischemia ended. Recovery was assessed 7 days after injections by comparing electroretinography measurements, histologic examination, and apoptosis (TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay). To compare proteins secreted into the medium in the groups and the effect of hypoxic exposure, we used rat cytokine arrays.

RESULTS. Eyes injected with hypoxic BMSC-conditioned medium 24 hours after ischemia demonstrated significantly enhanced return of retinal function, decreased retinal ganglion cell layer loss, and attenuated apoptosis compared to those administered normoxic or hypoxic unconditioned medium. Hypoxic-preconditioned medium had 21 significantly increased protein levels compared to normoxic medium.

CONCLUSIONS. The medium from hypoxic-preconditioned BMSCs robustly restored retinal function and prevented cell loss after ischemia when injected 24 hours after ischemia. The protective effect was even more pronounced than in our previous studies of normoxic conditioned medium. Prosurvival signals triggered by the secretome may play a role in this neuroprotective effect.

Keywords: retina, hypoxia, conditioned medium, neuroprotection, stem cells

Retinal ischemia, a common cause of visual impairment and blindness, is a major contributor to tissue damage in diseases including acute angle-closure glaucoma, diabetic retinopathy, retinal vascular occlusions, and retinopathy of prematurity. Retinal ischemia is followed by retinal inflammation, tissue damage, and retinal dysfunction.¹ Rodent models of acute retinal ischemia are particularly suited for translational research, as the injury produced recapitulates that of diabetic retinopathy.² Treatment for retinal ischemic diseases is very limited. Therefore, an urgent need exists to develop strategies to enhance retinal cell survival, repair, and replacement for these and related retinal disorders.

Much research in recent years has focused on stem cell progenitor therapy for treatment of degenerative retinal diseases.³ Techniques have included transplantation of retinal progenitor cells,⁴ pigment epithelium cells, mesenchymal stem cells,⁵ neural stem cells,⁶ and sheets of retinal cells.⁷ In ischemic retinopathies, transplantation of retinal progenitor cells, induced pluripotent stem cells, mesenchymal stem cells, or neural stem

cells showed promise in repairing neural tissue and the vasculature.^{8,9} Stem cell therapy is limited by the potential for aberrant differentiation and overproliferation of the transplanted cells.^{8,10} Conditions provoked in the ischemic environment such as extracellular matrix degradation, oxidative stress, inflammation, and acute immune response further limit stem cell survival and retention rates, diminishing their benefits.¹¹

We have previously demonstrated that conditioned medium from bone marrow stem cells (BMSCs), when injected intravitreally, significantly attenuated retinal injury after ischemia.¹² In these experiments, medium was administered into the vitreous 24 hours after acute ischemia, indicating feasibility of delayed treatment. It has been reported that administration of hypoxic-preconditioned BMSCs enhanced neurogenesis after cerebral ischemia and improved ischemic renal and cardiac function.^{13,14} Moreover, culturing cells under hypoxic conditions effectively “preconditioned” them, altering the secretome and enhancing survival.¹⁵ While cell-based treatment is attractive, the disadvantages of transplanting cells into ischemic

tissue could be overcome by administering only the conditioned medium from the cells. Administration of conditioned medium is advantageous as to its easy preparation and injection into vitreous, and avoids the differentiation, proliferation, or cellular responses of transplanted cells.¹⁶ Accordingly, in the present study, we assessed hypoxic-preconditioned medium from BMSCs in our rat ischemic model as a potential therapeutic strategy for neuroprotection, and tested the hypothesis that hypoxic preconditioning of the cells enhances neuroprotection.

MATERIALS AND METHODS

Retinal Ischemia

Procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by our Animal Care Committee at the University of Chicago. Male Wistar rats (200–250 g; Harlan, Indianapolis, IN, USA) were maintained on a 12-hour on/12-hour off light cycle. For retinal ischemia, rats were anesthetized with chloral hydrate, 275 mg/kg intraperitoneally (IP). After sterile preparation, and working under an operating microscope, a 30-gauge 5/8-inch metal needle (BD PrecisionGlide; Becton-Dickinson, Franklin Lakes, NJ, USA) was placed with its tip directed away from the lens, just inside the anterior chamber of the eye. The needle was connected by plastic tubing via a three-way stopcock to a pressure transducer (Transpac 42661-04-27; Abbott, North Chicago, IL, USA) and to an elevated bag of balanced salt solution (BSS; by sterile technique, BSS was transferred from its bottle [Alcon, Fort Worth, TX, USA] to an empty 1000-mL 0.9% saline plastic bag [Baxter, Deerfield, IL, USA]). Intraocular pressure (IOP), continually displayed on an anesthesia monitor (Hewlett-Packard HP78534C; Palo Alto, CA, USA), was increased to 130 to 135 mm Hg for 55 minutes by pressurizing the bag (Smiths Medical Clear-Cuff; Smiths Medical, Dublin, OH, USA). The eyes were treated with topical Vigamox (0.5%; Alcon), cyclomydril (Alcon), and proparacaine (0.5%; Bausch & Lomb, Tampa, FL, USA). Temperature was maintained at 36°C to 37°C using a servo-controlled heating blanket (Harvard Apparatus, Natick, MA, USA). Oxygen saturation was measured with a pulse oximeter (Ohmeda, Louisville, CO, USA) on the tail. Supplemental oxygen, when necessary to maintain O₂ saturation > 93%, was administered with a plastic cannula placed in front of the nares and mouth.

Electroretinography

Procedures have been described in detail previously.^{17–19} Animals were dark adapted for at least 2 hours before electroretinogram (ERG) recordings. For baseline and postischemic follow-up ERG, rats were injected IP with ketamine (35 mg/kg) and xylazine (5 mg/kg) every 20 minutes to maintain anesthesia.

For recordings, custom Ag/AgCl electrodes were fashioned from 0.010-inch Teflon-coated silver wire (Grass Technologies, West Warwick, RI, USA). Approximately 10 mm was exposed and fashioned into a small loop to form the corneal/positive electrodes while ~20 mm formed a hairpin loop, the sclera/negative electrodes loop. To maintain moistness of the cornea and to ensure electrical contact, eyes were treated intermittently with Gonisol (Alcon). Electrodes were referenced to a 12-mm × 30-gauge stainless steel needle electrode (Grass) inserted two-thirds down the length of the tail. Stimulus-intensity ERG recordings were obtained from both eyes using a UTAS-E 4000 ERG system and a full-field model 2503D Ganzfeld (LKC Technologies, Gaithersburg, MD, USA) as previously

described.^{12,20,21} Low-pass filter was 0.05 Hz and high-pass 500 Hz. Flash intensities varied electronically from 3.39 to 1.40 log cd-s/m². Responses of 3 to 10 flashes delivered 4 to 27 seconds apart were averaged, with flash numbers decreasing and time between them increasing with intensity. Settings were confirmed by photometry (EG & G Model 550 photometer; Electro-Optics, Boulder, CO, USA). To prevent attenuating dark adaptation, flash series were progressively delivered from the lowest to the highest intensity, and at least 1 minute elapsed between each series of flashes.

Recorded amplitude, time course, and intensity were exported and analyzed in Matlab 2011a (MathWorks, Natick, MA, USA). For each rat, the waveforms were averaged across each flash series, and the a-wave, b-wave, and P2 were taken at each intensity. The a-wave values were calculated as the absolute value of the minimum amplitude following the flash stimulus, while the b-waves were calculated as the difference between the negative a-wave value and the maximum amplitude recorded thereafter. The P2 was derived by first fitting the Hood and Birch phototransduction model to the leading edge of the a-wave, as we previously described.¹² The oscillatory potential (OP) values were the sum of the root mean squares (sum RMS) of the amplitudes of the OP waves.

Hypoxic-Conditioned Medium From BMSCs

Bone marrow stem cells were harvested as previously described.¹² To ensure uniformity, cells were collected from a single male donor. Briefly, bone marrow was flushed from femoral cavities with phosphate-buffered saline (PBS). The resulting cell suspension was centrifuged at 300g for 5 minutes. The pellet was then resuspended and cultured in RPMI medium containing 10% fetal bovine serum, antibiotics, and L-glutamine. The following day, nonadherent cells were removed and the medium was replaced. The adherent, spindle-shaped MSC population was expanded within approximately four to five passages after the cells were first plated to obtain the BMSCs for experiments. Bone marrow stem cells (1 × 10⁵ cells/mL) were plated onto a T75 flask to reach 70% to 80% confluency prior to subjecting the cells to hypoxia. The hypoxic BMSC-conditioned medium was collected from cells incubated in a hypoxic chamber at 1% O₂ for 72 hours. Similarly, hypoxic unconditioned medium was collected from cell-free medium incubated under similar hypoxic conditions for the same length of time. Normoxic cells were not exposed to hypoxia.

Administration of Hypoxic-Conditioned Medium From BMSCs

The conditioned medium collected from each group was centrifuged and filtered to remove the cells and evaluated using a standard pH meter, and protein concentration was determined (BCA protein assay kit; Pierce, Rockford, IL, USA). Hypoxic- or normoxic conditioned medium or hypoxic unconditioned medium (4 μL each) was injected into the vitreous of both the ischemic and nonischemic eyes 24 hours after retinal ischemia as previously described.¹² The normal/nonischemic left eye served as a control. Rats were subjected to ERG recordings at baseline, prior to ischemia, and 7 days post injections of the conditioned medium, that is, 8 days after ischemia. We have previously reported the minimal impact of intravitreal injections, including conditioned medium, on the nonischemic eye.¹²

Histology

Eyes enucleated after the last ERG recordings were placed in Davidson's fixative (11% glacial acetic acid, 2% neutral buffered

formalin, and 32% ethanol in H₂O) for 24 hours, then transferred to 70% ethanol for 24 hours and stored in PBS at 4°C. Eyes were embedded in paraffin, sectioned to 4 μm, and stained with hematoxylin and eosin (H&E); they were then examined by light microscopy and cell counts were quantitated using ×40 optics. Specifically, the number of cells in the retinal ganglion cell (RGC) layer was counted in a standardized region in all of the retinae centered 1280 μm from the thinning of the neurofilaments arising from the optic nerve head. Counts were made, in both directions from the optic nerve head, in a region spanning 128 μm. The average number of cells in the RGC layer is reported as previously described.²¹ Cell numbers in the inner nuclear layer (INL) and outer nuclear layer (ONL) were determined after image capture using Micron (Westover Scientific, Mill Creek, WA, USA). The numbers of cells in the INL and ONL were manually counted and determined per area for three to five regions per section, as previously described.¹²

Fluorescent TUNEL

Eyes were removed from euthanized rats 24 hours after injection of medium (i.e., 48 hours after ischemia).^{12,18,22} Enucleated eyes were fixed at room temperature in 4% paraformaldehyde for 3 hours. After removal of the anterior segment, the posterior eye portion was postfixed in the same fixative overnight at 4°C before being placed in 25% sucrose for a second overnight period at 4°C for cryoprotection. Eye cups were embedded in optimum cutting temperature (OCT) compound (Sakura Finetec, Torrance, CA, USA) and cut into 10-μm-thick cryosections. Fluorescent TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay) was performed using a Fluorescein FragEL DNA Fragmentation Detection Kit (Calbiochem, La Jolla, CA, USA) on frozen retinal sections from retina at 24 hours after medium injection (48 hours after ischemia) as we described previously.¹² Timing of the measurement is consistent with the time course of apoptosis after ischemia (peaking 24–48 hours after ischemia) as previously reported.^{12,23,24} Accordingly, the timing of these measurements was earlier than for those used for histology (at 8 days after ischemia). For imaging TUNEL,¹² we utilized a fluorescence microscope (Olympus IX81 inverted microscope; Melville, NY, USA), a Fast Firewire Retiga EXi chilled CCD camera (QImaging, Surrey, BC, Canada), and a ×40 oil lens. Excitation/dichroic/emission settings were 530 to 550 nm and 570DM to 590LP for fluorescein.

Cytokine Antibody Array

We utilized a 34-cytokine preconfigured sandwich ELISA Rat Cytokine Array G2 (RayBiotech, Inc., Norcross, GA, USA). The subarrays (containing antibody supports, in duplicate, for 34 cytokines, 4 positive, and 10 negative controls) were incubated with either hypoxic BMSC-conditioned medium ($n = 4$), hypoxic unconditioned medium ($n = 6$), or normoxic conditioned medium ($n = 6$), then incubated with a cocktail of biotin-conjugated anti-cytokine antibodies followed by fluorescent-labeled streptavidin.²⁵ The microarray glass chip was returned to RayBiotech for laser scanning (GenePix 4000B; Molecular Devices, Sunnyvale, CA, USA); then fluorescence intensity from the individual spots was determined with S02-AAR-CYT-G2 RayBiotech software (<http://www.raybiotech.com>; in the public domain). The program determined the median pixel fluorescence values at 532 nm, subtracted local background, and then averaged duplicate spots to determine the average local background-subtracted 532-nm median fluorescent signal. Individual wells were normalized to each other by normalizing the positive control spot signals from array to array and factoring in the normalization factor across

all slides. To be considered a measurable and significant difference in expression required a ≥ 1.5 -fold increase or ≤ 0.65 -fold decrease in signal intensity for a single analyte between groups, also requiring that the levels exceeded background (i.e., mean background ± 2 standard deviations [SD], accuracy $\approx 95\%$). The data are presented as mean intensities \pm SD after positive control normalization and background subtraction.

Bioinformatic Data Analysis and Protein Network Modeling

We used PANTHER (Protein Analysis THrough Evolutionary Relationships, <http://pantherdb.org>; in the public domain)²⁶ to examine the categories of biological process, molecular function, cellular components, and protein class for the array results of the 21 significantly increased proteins in hypoxic compared to normoxic conditioned medium. Next, the 21 proteins were input into DAVID Bioinformatics (<http://david.ncifcrf.gov>; in the public domain).^{27,28} Functional annotational clustering charts were generated using the following parameters: Similarity score = 4, similarity threshold = 0.95, initial and final group membership = 4, multiple linkage threshold = 0.95, class stringency = high, EASE score (a modified Fisher's exact P value, for gene-enrichment analysis, where Fisher's exact P value = 0 represents perfect enrichment) = 0.05.

Data Handling and Statistical Analysis

The postischemic a-waves, b-waves, OPs, and P2-waves were expressed as normalized intensity-response plots with stimulus intensity on the x -axis, and corresponding percent recovery of baseline on the y -axis, as we previously reported. The normalization process accounted for differences in wave amplitudes between the identically injected ischemic and nonischemic eyes, as well as day-to-day diurnal variation in either eye. Recorded amplitude, time course, and intensity were exported and analyzed in Matlab 2011a (MathWorks).^{12,21} Electroretinogram data were compared by ANOVA and t -test. Analyses were performed using Stata version 10.0 (StataCorp, College Station, TX, USA).

RESULTS

Functional Effects

Electroretinographic responses were tested at baseline prior to ischemia and at 7 days after injection of medium (i.e., 8 days after ischemia). In the scotopic ERG stimulus-intensity responses, the injection of medium from hypoxic-preconditioned BMSCs 24 hours after ischemia remarkably enhanced postischemic recovery of the amplitudes of the b-wave, P2, and oscillatory potentials (approximately 4-fold, $P < 0.05$ for all stimulus intensities) compared to the recovery in ischemic eyes injected with hypoxic unconditioned medium (Figs. 1A–D); there was no significant difference in recovery of the a-wave amplitudes. When compared to normoxic medium, the medium from the hypoxic-preconditioned BMSCs significantly enhanced recovery of the b-wave and the P2 (approximately 2-fold; $P < 0.05$ for all stimulus intensities), with a trend for a difference in the OP and no difference in the a-wave amplitude (Figs. 1E–H). These results can also be seen in the representative ERG traces from the three groups (Fig. 2).

Histology

Histologic examination at 7 days after injection of medium (i.e., 8 days after ischemia) showed cell loss in the RGC layer in the normoxic conditioned medium group and in the hypoxic

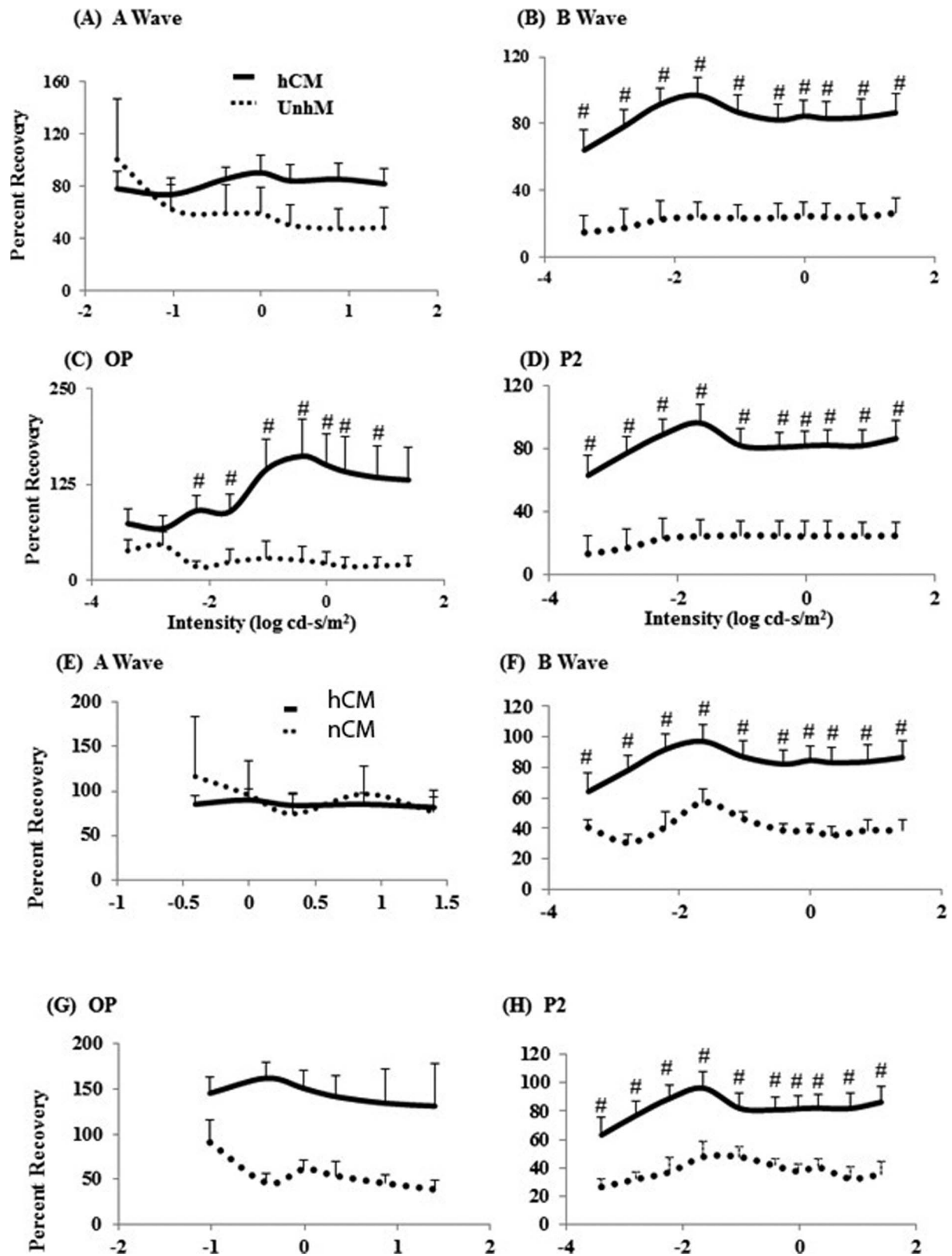


FIGURE 1. Stimulus-intensity responses for a-wave, b-wave, oscillatory potentials (OP), and P2 from electroretinograms in rats subjected to retinal ischemia with their eyes injected 24 hours later with conditioned medium from normoxic (nCM, $n = 6$) or hypoxic BMSC-conditioned medium (hCM; $n = 6$) or hypoxic unconditioned medium (unCM; $n = 6$). The recordings were at baseline (prior to ischemia) and after ischemia. At 24 hours after ischemia, the animals received intravitreal injection of nCM, hCM, or unhCM. Seven days later, the ERG was recorded again. ERG data for the waves over a range of flash intensities are shown as mean \pm SEM. (A–D) compares hCM to unhCM; (E–H) compares nCM to hCM. Note that the y-axis scales differ on some of the graphs. There was significant improvement with injection of hypoxic CM 24 hours after ischemia. # $P < 0.05$ for hCM versus unhCM, and for hCM versus nCM.

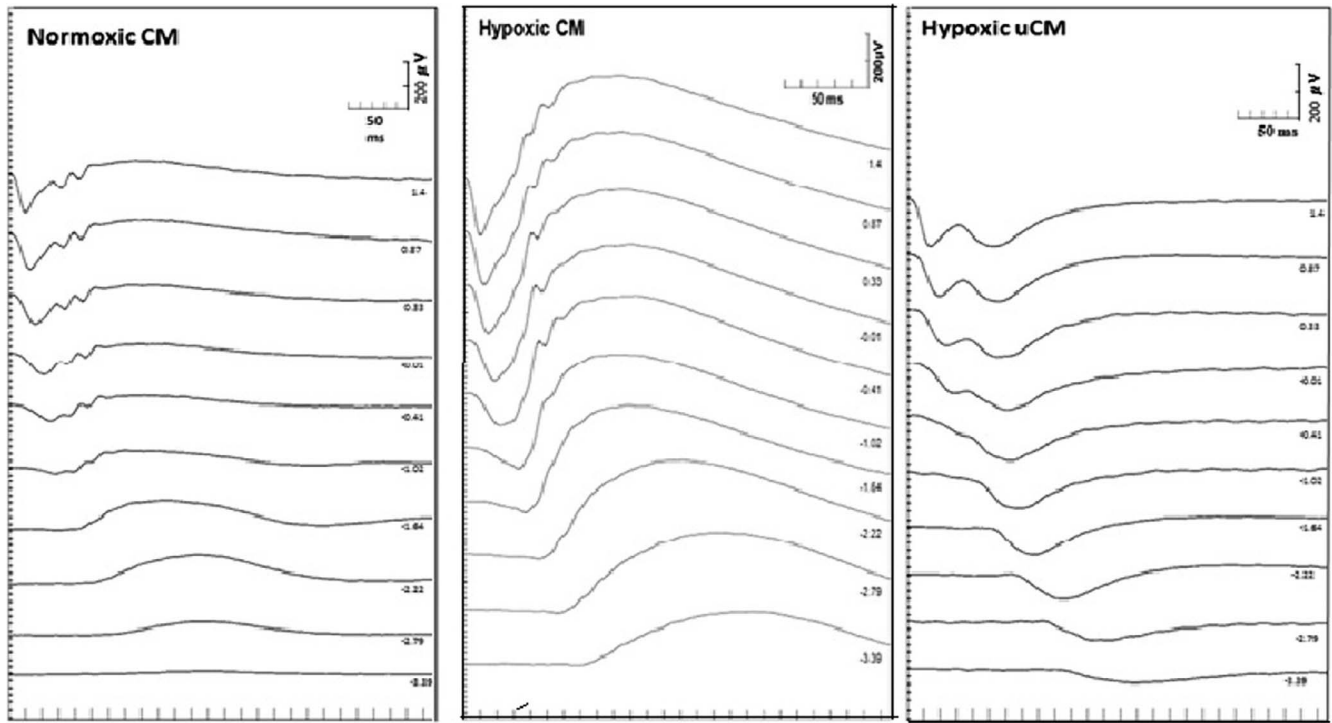


FIGURE 2. Representative ERG traces. Traces at each flash intensity from -3.39 to 1.40 log cd-s/m² are shown for normoxic CM (left), hypoxic CM (middle), and hypoxic unconditioned medium (right) upon posts ischemic follow-up. There was very low recovery of the b-waves and absence of OP in the hypoxic unconditioned medium group.

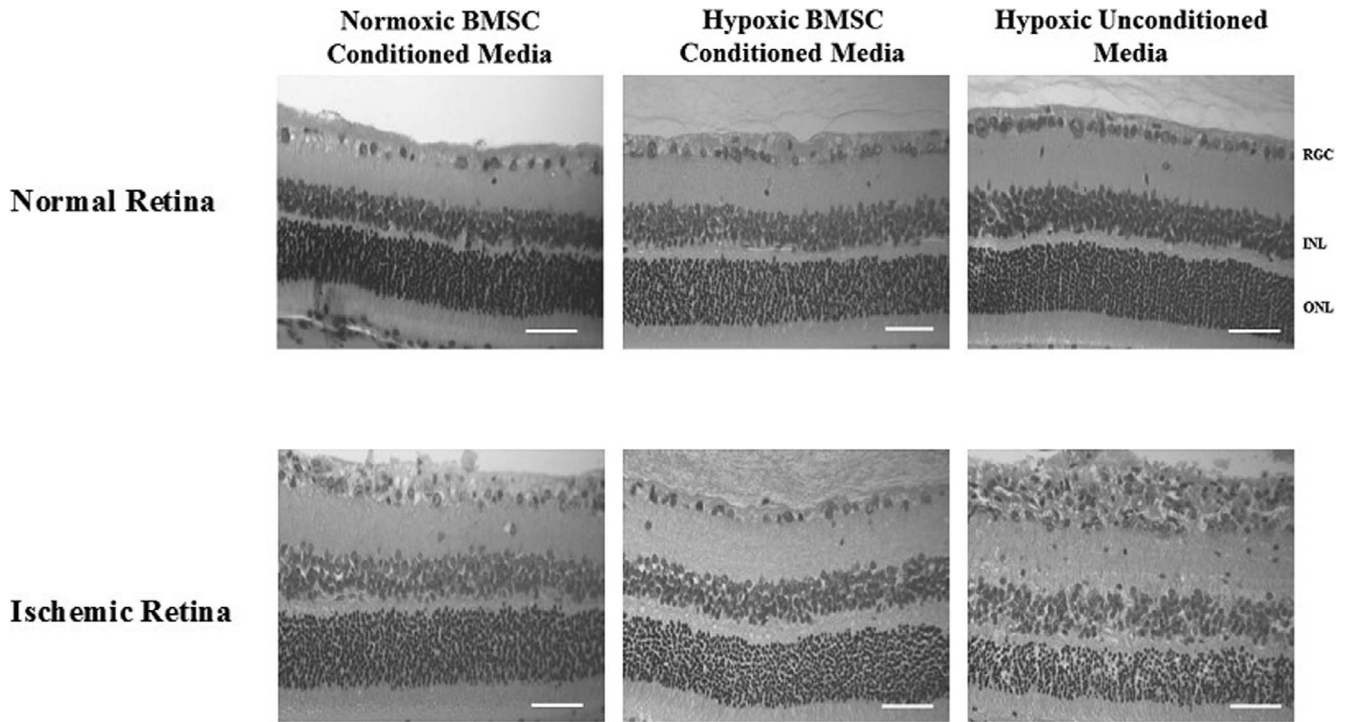


FIGURE 3. Representative histologic preparations of retina 7 days after ischemia and injection of normoxic or hypoxic CM unconditioned medium. The ischemic retinae from the hypoxic CM group showed greater retention of cells in the RGC layer as well as less disorganization versus normoxic and hypoxic unhCM. Normal retinae (top row) and ischemic retinae (bottom row). Scale bar: 50 µm.

TABLE 1. Histologic Results After Ischemia

	Number of Cells in RGC Layer	P Value vs. Hypoxic BMSC-Conditioned Medium
Hypoxic BMSC-conditioned medium	10.3 ± 0.6	
Normoxic conditioned medium	8.3 ± 0.5	0.04
Hypoxic unconditioned medium	8.3 ± 0.7	0.001
	INL cells/area	P value vs. hypoxic BMSC-conditioned medium
Hypoxic BMSC-conditioned medium	2.3 ± 0.1	
Normoxic conditioned medium	2.2 ± 0.1	0.47
Hypoxic unconditioned medium	2.5 ± 0.1	0.45
	ONL cells/area	P value vs. hypoxic BMSC-conditioned medium
Hypoxic BMSC-conditioned medium	5.2 ± 0.2	
Normoxic conditioned medium	4.7 ± 0.2	0.66
Hypoxic unconditioned medium	6.0 ± 0.2	0.18

Number of cells in the RGC (see text for explanation of measurement), inner nuclear (INL cells/area, $\mu\text{m}^2 \times 100$), and outer nuclear (ONL cells/area, $\mu\text{m}^2 \times 100$) layers in the ischemic retinae 8 days after ischemia in normoxic conditioned ($n = 5$), hypoxic ($n = 6$), and hypoxic unconditioned medium ($n = 6$) injected eyes. Comparisons are shown for hypoxic unconditioned versus normoxic medium; and hypoxic-conditioned versus normoxic medium. Results are expressed as mean \pm SEM.

unconditioned medium retina, cell loss, disorganization, and infiltration of inflammatory cells. Retinal structure appeared relatively intact in the hypoxic BMSC-injected group (Fig. 3). Table 1 demonstrates significant differences between the number of cells in the RGC layer in the hypoxic BMSC-conditioned medium-treated ischemic retina (10.3 ± 0.6 ; $n = 6$) versus normoxic conditioned medium treated (8.3 ± 0.5 , $n = 6$, $P = 0.04$), and hypoxic unconditioned medium treated (8.3 ± 0.7 ; $n = 6$; $P = 0.001$). There were no differences for INL and ONL cell density between hypoxic BMSC medium versus hypoxic unconditioned medium- or versus normoxic conditioned medium-injected ischemic eyes.

Fluorescent TUNEL

Fluorescent TUNEL (Fig. 4) was performed at 48 hours after ischemia, consistent with previous experiments in which

TUNEL peaked at 24 to 48 hours after ischemia.^{23,24} Injection of hypoxic-conditioned medium 24 hours after ischemia significantly attenuated the percentage of apoptotic cells in the RGC layer in ischemic retinae ($9.7 \pm 2.2\%$; $n = 7$) versus the hypoxic unconditioned medium-injected ischemic retinae ($29.0 \pm 5.6\%$; $n = 6$; $P = 0.01$).

Cytokine Assay

Comparing hypoxic-conditioned medium ($n = 6$) and normoxic conditioned medium ($n = 4$), there were significant increases in 21 cytokines (Table 2, listed in descending order of % difference). RAGE (4902%; $P = 0.04$), ICAM-1 (596%; $P = 0.02$), prolactin-R (286.3%; $P = 0.001$), IL-1 R6 (252%, $P = 0.0005$), and CINC-2 α (230%, $P = 0.001$) showed the largest differences. Medium from hypoxic-preconditioned BMSCs compared to unconditioned hypoxic medium showed signifi-

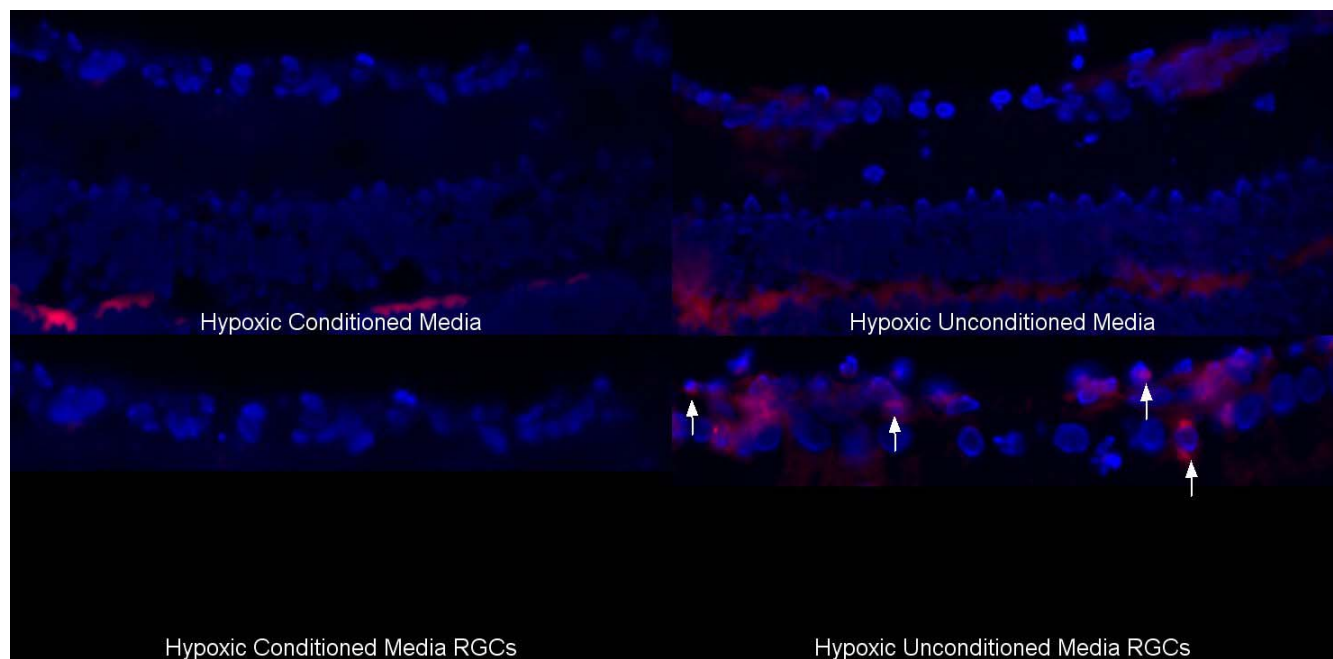


FIGURE 4. Fluorescent TUNEL. Apoptosis was attenuated in the ischemic retinae 24 hours after hypoxic CM injection ($n = 7$) as compared to hypoxic unhCM injection ($n = 6$), that is, at 48 hours after ischemia. Blue, DAPI; red, TUNEL. White arrows show TUNEL cells. Top image is a close-up of the retinal ganglion cell layer; bottom image shows larger view of retina. Pink indicates overlap of DAPI and TUNEL.

TABLE 2. Cytokine Levels Compared Between Hypoxic BMSC CM ($n = 6$) and Normoxic BMSC CM ($n = 4$) (Significantly Changed Proteins Listed First With P Value in Bold)

Protein Name	UniProt Accession No.	Gene Name	Normoxic CM, Mean \pm SEM	Hypoxic CM, Mean \pm SEM	% Change	P Value
RAGE	Q63495	<i>AGER</i>	41 \pm 12	2072 \pm 663	4954	0.04
ICAM-1	Q00238	<i>CD54</i>	333 \pm 104	2318 \pm 534	596	0.02
Prolactin-R	P05710	<i>Prl</i>	256 \pm 102	987 \pm 93	286	0.001
IL-1 R6	Q62929	<i>IL1RL2</i>	297 \pm 137	1047 \pm 88	253	0.0005
CINC-2 α	Q10746	<i>Cxcl3</i>	366 \pm 171	1208 \pm 108	230	0.001
Leptin	P50596	<i>lep</i>	72 \pm 33	225 \pm 17	213	0.0002
Thymus Chemokine-1	Q99Me0	<i>ppbp</i>	328 \pm 133	984 \pm 90	200	0.001
IL-1 α	P16598	<i>IL1a</i>	596 \pm 270	1690 \pm 121	184	0.0002
L-selectin	P30836	<i>CD62/sell</i>	552 \pm 246	1561 \pm 98	183	0.0001
IL-10	P29456	<i>IL10</i>	436 \pm 174	1188 \pm 117	172	0.002
IL-6	P20607	<i>IL6</i>	310 \pm 106	821 \pm 72	165	0.001
IL-4	P20096	<i>IL4</i>	491 \pm 217	1296 \pm 89	164	0.0003
IL-2	P17108	<i>IL2</i>	696 \pm 284	1755 \pm 130	152	0.0004
CINC-3	P30348	<i>cxcl2</i>	93 \pm 30	228 \pm 38	145	0.02
GM-CSF	P48750	<i>CSF2</i>	384 \pm 153	920 \pm 54	140	0.0002
LIX	P97885	<i>Cxcl5</i>	665 \pm 277	1584 \pm 124	138	0.001
IL-1 β	Q63264	<i>IL1b</i>	120 \pm 55	279 \pm 28	133	0.003
B7-2/CD86	O35531	<i>CD86</i>	230 \pm 101	525 \pm 27	128	0.0001
β -NGF	P25427	<i>NGF</i>	307 \pm 125	641 \pm 63	109	0.003
VEGF	P16612	<i>VEGFA</i>	35808 \pm 20222	63259 \pm 1225	77	0.02
TNF α	P16599	<i>TNF</i>	207 \pm 89	361 \pm 22	74	0.001
Fas ligand	P36940	<i>FASLG</i>	97 \pm 35	3104 \pm 1178	3100	0.07
MMP-8	O88766	<i>MMP8</i>	131 \pm 30	243 \pm 53	85	0.30
IFN- γ	P01581	<i>IFNG</i>	147 \pm 138	441 \pm 123	200	0.09
IL-13	P42203	<i>IL13</i>	44 \pm 10	71 \pm 31	61	0.46
TIMP-1	P30120	<i>TIMP1</i>	8801 \pm 3461	11127 \pm 1561	26	0.38
CNTF	P20294	<i>CNTF</i>	235 \pm 123	285 \pm 16	21	0.09
MIP-3 α	P97884	<i>CCL20</i>	937 \pm 157	1126 \pm 71	20	0.79
MCP-1	P13844	<i>CCL2</i>	19545 \pm 10416	20524 \pm 6165	5	0.92
Agtrin	P25304	<i>AGRN</i>	2449 \pm 803	1923 \pm 148	-21	0.59
CINC-1	P14095	<i>CXCL1</i>	9022 \pm 14320	6239 \pm 933	-31	0.71
Activin A	P18331	<i>INHBA</i>	1240 \pm 217	825 \pm 452	-33	0.73
PDGF-AA	Q6P7C3	<i>PDGFA</i>	5580 \pm 195	2935 \pm 711	-47	0.65
Fractalkine	Q6IRF7	<i>CXCL3</i>	1081 \pm 58	441 \pm 86	-59	0.55

cant increase in nine proteins, with the five highest being VEGF (7982%; $P = 0.0000001$), TIMP-1 (4123%; $P = 0.001$), MCP-1 (1330%; $P = 0.04$), ICAM-1 (614%; $P = 0.02$), and CINC-1 (335%; $P = 0.01$). There were significant decreases in leptin (-69%; $P = 0.01$) and TNF α (-81%; $P = 0.05$) (Table 3). The media from all conditions exhibited pH in the range 7.0 to 8.0 (normal control 7.4, normoxic conditioned 8.0, hypoxic unconditioned 8.25). Normoxic unconditioned medium, normoxic conditioned, and hypoxic unconditioned medium had ~ 2.5 $\mu\text{g}/\mu\text{L}$ protein, and hypoxic-conditioned medium ~ 4 $\mu\text{g}/\mu\text{L}$.

PANTHER classified the 21 proteins increased in the hypoxic BMSC medium relative to the normoxic group. They were signaling, receptor, cell adhesion, defense, hydrolases, proteases, and transfer/carrier proteins. The biological processes (Fig. 5) included apoptosis, adhesion, regulation, immune system, localization, locomotion, metabolic process, and response to stimulus. DAVID found 13 GO (gene ontology) annotation clusters; for simplicity, the highest three enrichment scoring clusters are shown in Table 4; the remainder are in Supplementary Table S1. The highest enrichment score (6.33) was for negative regulation of cell death (cluster 1); this included GM-CSF, IL-1 β , IL-10, IL-2, IL-4, IL-6, NGF, TNF, and VEGF-A. Cluster 2 (enrichment score 5.38) included six proteins, identified as positive regulation of phosphorylation, GM-CSF, IL-1 β , IL-2, IL-4, IL-6, and TNF. Cluster 3 (enrichment

score 5.08) was for positive regulation of transcription, with ICAM-1, IL-1 β , IL-10, IL-4, and TNF. Cluster 5 included IL-1 α , IL-10, IL-2, IL-6, and TNF for positive regulation of secretion, and cluster 6, leukocyte activation/proliferation, IL-1 β , IL-2, IL-4, IL-6, and IL-10.

DISCUSSION

Bone marrow stem cells can exert beneficial effects either by differentiating into new cell types, or, indirectly, via release of factors in a paracrine role.²⁹ Transplanted cells generally survive poorly in tissues,⁶ while penetration of cells into the retina remains limited.³⁰ In vivo, the cells reside under hypoxic conditions between 4% and 7%.³¹ Yet culture of MSCs under normoxia (21% oxygen) causes premature senescence and reduction in MSC differentiation capacity with each subsequent population doubling or passage.³² As a result, recent attention has focused on MSC isolation and expansion under hypoxic or relatively hypoxic conditions in order to "precondition" the cells to the subsequent in vivo hypoxia.¹¹ We reasoned that the secretome would be altered in the hypoxic-preconditioned BMSCs, leading to release of paracrine factors that could enter the retina more effectively as injected medium rather than relying upon release from cells that mainly remain in the vitreous.³⁰

TABLE 3. Cytokine Levels Compared Between Hypoxic-Conditioned and Hypoxic Unconditioned Medium ($n=6$ for Both) (See Table 2 for UniProt Annotation Terms and Gene Names; Significantly Changed Proteins Listed First and P Value in Bold)

Protein Name	Hypoxic Unconditioned Medium, Mean \pm SEM	Hypoxic-Conditioned Medium, Mean \pm SEM	% Change	P Value
VEGF	793 \pm 61	63259 \pm 1225	7877	0.0000001
TIMP-1	270 \pm 24	11127 \pm 1561	4021	0.001
MCP-1	1669 \pm 83	20524 \pm 6165	1130	0.04
ICAM-1	378 \pm 29	2318 \pm 534	513	0.02
CINC-1	1860 \pm 245	6239 \pm 933	235	0.01
Fractalkine	200 \pm 18	441 \pm 86	121	0.05
β -NGF	426 \pm 16	641 \pm 63	50	0.03
TNF α	447 \pm 26	361 \pm 22	-19	0.05
Leptin	325 \pm 24	225 \pm 17	-31	0.01
Activin A	244 \pm 52	825 \pm 452	238	0.30
PDGF-AA	1724 \pm 87	2935 \pm 711	70	0.18
Agrin	1362 \pm 194	1923 \pm 148	41	0.06
CNTF	234 \pm 25	285 \pm 16	22	0.16
MIP-3 α	1013 \pm 74	1126 \pm 71	11	0.34
IL-1 R6	1070 \pm 65	1047 \pm 88	-2	0.86
L-sselectin	1595 \pm 199	1561 \pm 98	-2	0.89
IL-2	1801 \pm 80	1755 \pm 130	-3	0.79
CINC-2 α	1255 \pm 68	1208 \pm 108	-4	0.75
IL-1 α	1787 \pm 60	1690 \pm 121	-5	0.53
IL-10	1260 \pm 114	1188 \pm 117	-6	0.70
MMP-8	259 \pm 70	243 \pm 53	-6	0.87
IL-4	1383 \pm 26	1296 \pm 89	-6	0.42
IL-6	882 \pm 58	821 \pm 72	-7	0.56
IL-13	77 \pm 11	71 \pm 31	-8	0.89
Prolectin-R	1165 \pm 30	987 \pm 93	-15	0.15
B7-2/CD86	624 \pm 57	525 \pm 27	-16	0.19
IFN- γ	525 \pm 129	441 \pm 123	-16	0.68
Thymus Chemokine-1	1187 \pm 54	984 \pm 90	-17	0.11
Fas ligand	3831 \pm 1437	3104 \pm 1178	-19	0.73
IL-1 β	345 \pm 41	279 \pm 28	-19	0.26
LIX	1970 \pm 121	1584 \pm 124	-20	0.07
CINC-3	297 \pm 34	228 \pm 38	-23	0.25
GM-CSF	1222 \pm 111	920 \pm 54	-25	0.06
RAGE	3023 \pm 1055	2072 \pm 663	-31	0.51

We examined the effect of delayed, 24 hours post retinal ischemic injection of the conditioned medium from hypoxic bone marrow mesenchymal stem cells on the outcome in a rat model. Our hypothesis, that hypoxic BMSC-conditioned medium protects against functional and histologic damage in retinal ischemia, was substantiated. Moreover, the functional improvement in retinal recovery after ischemia was enhanced compared to that with delivery of normoxic medium. Stem cell-based treatment of retinal diseases, while attractive, is limited by the number of cells that can be delivered, their ability to penetrate into the retina, immune reactions, side effects of impurities or dead cells, and the risk of tumor transformation.³³ Pharmacologic approaches are limited by drug concentration, specificity, and side effects. By eliminating potential adverse effects and limitations of other cell-based or pharmaceutical treatments, our results have significant implications for treatment of retinal vascular diseases. In effect, the delivery of conditioned medium from autologous hypoxic BMSCs capitalizes upon the previously demonstrated phenomena of both hypoxic preconditioning and ischemic postconditioning, without the necessity to deliver another (brief) ischemic stimulus as required for these methods of inducing endogenous ischemic tolerance.^{21,34}

We have demonstrated that conditioned medium from BMSCs protects against ischemic retinal injury, and we showed

that attenuation of apoptosis is one of the underlying mechanisms.¹² Correspondingly, in this study, the decrease in number of cells in the RGC layer undergoing apoptosis, as evident with TUNEL, also points to a similar mechanism of protection by the BMSC hypoxic-conditioned medium. We chose to study apoptosis 48 hours post ischemia based on multiple studies of apoptosis at 24 to 48 hours post ischemia.^{12,24,35} We have also previously shown that the RGCs undergo apoptosis after ischemia.²⁴ In the present study, direct comparison of medium from hypoxic BMSCs to a group treated with conditioned medium from normoxic BMSCs demonstrated a significant improvement in return of retinal function and histology after ischemia. Accordingly, we compared the composition of the secretome, that is, the proteins released from stem cells into the conditioned medium under either normoxic or hypoxic conditions, to determine differences that may underlie the improved postischemic recovery.

We discovered clues to the mechanism of action of the hypoxic medium. In particular, that treatment 24 hours after ischemia robustly returns retinal function nearly to normal suggests either an interruption or reversal of the cell death process or repair of already damaged, but not dead, neurons. Postischemic apoptosis peaks at 24 to 48 hours after ischemia,^{12,24,35} rendering it susceptible to delayed treatment. However, because other processes, including necrosis, autoph-

PANTHER GO-Slim Biological Process

Total # Genes: 21 Total # process hits: 79

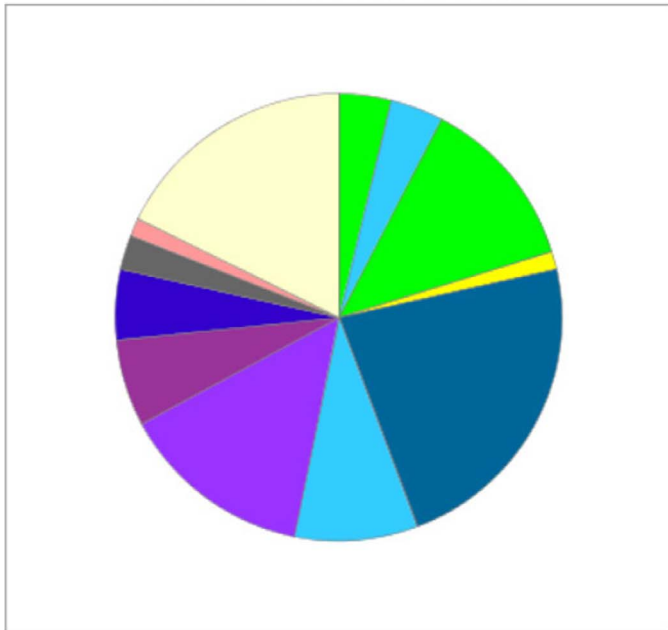


FIGURE 5. The biological functions of proteins in the array that were significantly increased in hypoxic versus normoxic medium are shown using PANTHER.

agy, and necroptosis, also participate in cell death after ischemia,³⁶ it is likely that multiple means to enhance retinal survival are necessary. Notably, nine proteins in the medium, or approximately 40% of those showing significant increase, are negative regulators of apoptosis or cell death, while others regulate transcription, immune function, JAK-STAT, and MAPK signaling, all of which have been implicated in damage from ischemia-reperfusion in the retina,³⁷ suggesting that hypoxic BMSC medium may provide broad coverage against the deleterious mechanisms set in motion by ischemia-reperfusion injury.

The effects of cytokines on cell survival *in vivo* are complex. A number of the proteins identified in this study associated with cell survival have also been associated with proinflammatory and/or triggering of apoptosis, for example, TNF α ,³⁸ GM-CSF,³¹ IL-2,³⁹ IL-4,³⁹ IL-1 β ,⁴⁰ and IL-6, while IL-10 is neuroprotective⁴¹; the role of NGF in retinal cell survival in particular has been well described.⁴² Factors influencing the impact of inflammatory cytokines in ischemia depend upon the levels of the cytokines, underlying systemic conditions such as atherosclerosis that might evoke a chronic inflammatory state, and downstream effectors.⁴³ Interleukin-1, for example, can induce neurogenesis; at low levels it influences synaptic plasticity.⁴¹ In our study, we cannot identify yet the mechanisms by which these cytokines influence postischemic recovery. Other mechanisms such as altered gene transcription, cellular signaling via phosphokinases, and effects upon the cell cycle are other known effects of cytokines increased in the hypoxic medium that could additionally influence cell survival.

Our study cannot precisely define the specific molecules that are responsible for delayed neuroprotection by hypoxic stem cell medium. Such experiments would require adding in “cocktails” of proteins, or blocking specific proteins using, for example, blocking antibodies or interfering RNA. The assay we used is not quantitative; it cannot tell us the relative or absolute concentrations of proteins present in a sample of the medium.

- [apoptotic process \(GO:0006915\)](#)
- [biological adhesion \(GO:0022610\)](#)
- [biological regulation \(GO:0065007\)](#)
- [cellular component organization or biogenesis \(GO:0071840\)](#)
- [cellular process \(GO:0009987\)](#)
- [developmental process \(GO:0032502\)](#)
- [immune system process \(GO:0002376\)](#)
- [localization \(GO:0051179\)](#)
- [locomotion \(GO:0040011\)](#)
- [metabolic process \(GO:0008152\)](#)
- [multicellular organismal process \(GO:0032501\)](#)
- [response to stimulus \(GO:0050896\)](#)

Both specific functions and concentrations of proteins in the secretome, as well as their interactions, likely play a role in the neuroprotective function.

There are limitations to our methods. We did not determine the long-term effects of introducing medium from hypoxic BMSCs; rather, we studied the retinae at 7 days after injection. This was done in order allow comparison to previous studies. We do not know to what extent the medium entered the retina, that is, the penetration to specific retinal layers or specific retinal cells.

As with our results with delayed administration of BMSC-conditioned medium after retinal ischemia, our current results show a more robust and possibly more clinically important approach to prevent ischemic retinal damage. The present study shows the possibility of “direct delivery” of neuroprotection by the simple maneuver of administering medium from hypoxic-preconditioned BMSCs. Future endeavors may center on the analysis of individual factors and/or a combination of several important factors that can efficiently protect against retinal ischemic damage.

Acknowledgments

Supported by National Institutes of Health (Rockville, MD) Grants RO1EY10343 and EY10343-16S1 (American Recovery and Reinvestment Act) to SR; RO1CA122930 to MSL; AG029795-02 for the Medical Student Summer Research Program at the Pritzker School of Medicine; UL1RR024999 to the University of Chicago Institute for Translational Medicine; National Eye Institute Visual Sciences Core Grant P30 001792 to the Department of Ophthalmology and Visual Sciences of the University of Illinois-Chicago; and a Center-Style Grant from the Dean's Research Advisory Committee of the Division of Biological Sciences of the University of Chicago (MSL, SR).

Disclosure: **S. Roth**, None; **J.C. Dreixler**, None; **B. Mathew**, None; **I. Balyasnikova**, None; **J.R. Mann**, None; **V. Boddapati**, None; **L. Xue**, None; **M.S. Lesniak**, None

TABLE 4. DAVID Bioinformatics Analysis (Levels of 21 Proteins Were Increased in Hypoxic-Conditioned Medium Compared to Normoxic Medium; DAVID Categorized the Proteins by Enrichment Score)

Term	N	%	P	Genes	Fold Enrichment	Bonferroni	FDR
Enrichment score: 6.333							
GO:0043066~negative regulation of apoptosis	9	43	2.87E-08	<i>IL10, IL1b, GM-SCF, IL2, IL4, bNGF, VEGFA, IL6, TNFa</i>	16	2.74E-05	4.51E-05
GO:0043069~negative regulation of programmed cell death			3.22E-08		15	3.08E-05	5.06E-05
GO:0060548~negative regulation of cell death			3.30E-08		15	3.15E-05	5.18E-05
GO:0042981~regulation of apoptosis			6.40E-06		8	0.00608885	0.01
GO:0043067~regulation of programmed cell death			7.05E-06		8	0.006713	0.011
GO:0010941~regulation of cell death			7.28E-06		8	0.00693276	0.011
Enrichment score: 5.38							
GO:0001934~positive regulation of protein amino acid phosphorylation	6	29	3.80E-07	<i>GM-CSF, IL1b, IL2, IL4, IL6, TNFa</i>	36	3.63E-04	5.97E-04
GO:0042327~positive regulation of phosphorylation			6.27E-07		33	5.99E-04	9.85E-04
GO:0010562~positive regulation of phosphorus metabolic process			7.22E-07		32	6.89E-04	0.001
GO:0045937~positive regulation of phosphate metabolic process			7.22E-07		32	6.89E-04	0.001
GO:0001932~regulation of protein amino acid phosphorylation			6.96E-06		20	0.00662803	0.011
GO:0031401~positive regulation of protein modification process			8.47E-06		19	0.00805456	0.013
GO:0032270~positive regulation of cellular protein metabolic process			2.47E-05		16	0.02329904	0.039
GO:0051247~positive regulation of protein metabolic process			3.11E-05		15	0.02930682	0.049
GO:0031399~regulation of protein modification process			7.36E-05		12	0.06787632	0.116
Enrichment score: 5.08							
GO:0051091~positive regulation of transcription factor activity	5	24	1.29E-06	<i>ICAM1, IL1b, IL10, IL4, TNFa</i>	56	0.00123479	0.002
GO:0043388~positive regulation of DNA binding			2.67E-06		47	0.00254966	0.004
GO:0051099~positive regulation of binding			4.65E-06		41	0.00443298	0.007
GO:0051090~regulation of transcription factor activity			1.21E-05		32	0.01152784	0.019
GO:0051101~regulation of DNA binding			2.52E-05		27	0.02375619	0.04
GO:0051098~regulation of binding			7.02E-05		21	0.06486222	0.11

N indicates number of proteins and % of the 21 proteins. P, Bonferroni, and FDR (false discovery rate) assess the categorization into GO (gene ontology) terms. P > 0.05 for FDR indicates a weak association with the GO term. See Supplementary Table S1 for categorization of additional groups.

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