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COMBINING ERYTHROPOEITIN AND BONE MARROW STROMAL CELL THERAPY AFTER STROKE

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Abstract

Both Erythropoietin (EPO) and bone marrow stromal cells (BMSCs) have been shown to improve outcome after stroke. EPO may improve outcome after stroke through its actions on blood progenitor cells, angiogenesis, or direct action in the CNS. BMSCs may improve outcome after stroke by regeneration, altering plasticity of viable cells, or prevention of cell death. Sorting out these potential modes of actions for EPO and BMSCs has been difficult using *in vivo* models of stroke. This study investigated neuroprotection afforded by EPO, BMSCs and the combination of these modalities in mouse hippocampal slice cultures after oxygen glucose deprivation (OGD). Significant neuroprotection was observed following post-injury treatment of slice cultures with BMSCs and neuroprotection was augmented by treating BMSCs with EPO. EPO alone did not protect neurons from OGD when given after injury, but was effective when given prior to OGD. The failure of EPO to protect when given after injury did not appear to result from its inability to activate EPO signaling pathways involving phosphorylation of Akt. This study supports the implication that BMSCs may rescue dying neurons after ischemia by providing trophic support. The data also show that EPO's actions as a neuroprotective agent following stroke may be mediated by its actions on BMSCs.

Keywords

• Stroke • Erythropoeitin • Bone marrow stromal cells • Slice culture • Neuroprotection

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1. Introduction

In recent years, blood derived factors have been targeted as potential therapeutic agents for treatment of stroke. Among these are erythropoietin and progenitor cells from bone marrow. Erythropoietin (EPO) is a 34kDa glycoprotein produced by the kidney and CNS tissue [1] in response to hypoxic stresses such as high altitude, cardiac arrest, emphysema, asthma and stroke. Clinically, EPO has been used for more than a decade to treat anemia, bone marrow decomposition in association with HIV, chemotherapy related depletion of erythrocytes subsequent to treatment of cancer, chronic renal failure and was examined in a multi-center clinical trial for stroke [2,3]. EPO exerts a direct effect on erythrocytic precursor cells in endogenous bone marrow resulting in erythropoiesis, angiogenesis, a decrease in apoptosis, and increased delivery of oxygen to depleted tissue as a result of hypoxic stress [2,4,5]. In addition to the erythrocytic precursors, EPO receptor expression and the effects of EPO are observed in other cell types including neural cells [6].

The downstream mechanisms of the EPO initiated response after ischemia are well defined [7,8]. EPO exerts its influence primarily through the recruitment and differentiation of bone marrow precursor cells to become hematocytes. Bone marrow-derived cells, including bone marrow stromal cells (BMSCs) are also confirmed contributors of CNS neuroprotection after ischemia in animals [9,10]. Exogenous cultured BMSCs express the EPO receptor (EPOr) and EPO is known to increase the viability of the BMSC cultures [5]. It has been proposed that BMSCs increase cell survival after injury by providing increased trophic support to the injured CNS [11]. EPO increases oxygenation of the tissue by erythropoeisis and enhances proliferation and survival of BMSCs, potentially influencing a relatively hostile environment to become a conducive milieu for survival [4,12]. Although studies of treatment with EPO or BMSCs independently results in significant neuroprotection in various injury models, the analysis of the combination of these therapies after injury is novel. The aim of this research was to directly compare neuroprotection provided by EPO, BMSCs, and the combination of BMSCs with EPO using the organotypic slice culture model of in-vitro ischemia.

2. Materials and Methods

All procedures involving animals were conducted with approval of the University of Miami Animal Care and Use Committee according to established federal guidelines.

2.1 Hippocampal Organotypic Slice Cultures

Organotypic slice cultures of the hippocampus were prepared according to the methods described previously [13] with modifications to meet the current experimental paradigm. C57Bl mice pups (7-9 days old) were anesthetized by intraperitoneal injections of ketamine (.5 mg/pup). The pups were decapitated and

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the hippocampi were dissected free from the forebrain and transversely sliced (400 µm) on a McIlwain tissue chopper. Slices were placed in Gey's Balanced Salt Solution (Gibco/Life Technologies) supplemented with 6.5 mg/ml glucose (Sigma) and 10mM $MgSO_4$ for 1 h at 4°C. They were then transferred onto 30 mm diameter membrane inserts (Millicell-CM, Millipore) and placed into 6-well-culture-trays with 1.0 ml of slice culture medium per well [13]. The slice culture medium consisted of 50% Basal Medium Eagle (Gibco/Life Technologies), 25% Earl's balanced salt solution (Gibco/Life Technologies), 25% heat-inactivated horse serum (Gibco/Life Technologies), 2% B27 and 6.5 mg/ml glucose (Neurobasal/B27, Invitrogen) for 1 week, then medium excluding B27 was used for the remainder of the experiments. The cultures were maintained at 37 °C in an atmosphere of humidified air and 5% CO_2. The slice culture medium was changed three times a week commencing 24 hours after slices were obtained. No antibiotics were used. Slices were kept in culture for 3 weeks before experiments to allow organotypic hippocampal slice maturation and stabilization. Slices were viable in culture for up to 8 weeks utilizing this protocol.

2.2Oxygen/Glucose Deprivation (OGD) in organotypic slice cultures

OGD was achieved by combining severe hypoxia with aglycemia [13]. The slices were washed three times with glucose free artificial cerebrospinal fluid containing $CaCl_2$ 1.26 mM; KCl, 5.37 mM; KH_2PO_4, 0.44 mM; $MgCl_2$, 0.49 mM; $MgSO_4$, 0.41 mM; NaCl, 136.9 mM, $NaHCO_3$, 4.17 mM; Na_2HPO_4, 0.34 mM; sucrose, 15 mM (all from Sigma) pH 7.4. The slices were then placed into an airtight incubation chamber (Biospherix, Ltd.) housed inside an incubator (VWR CO_2 water-jacketed incubator). Nitrogen gas (95% N_2, 5% CO_2) was flushed through the chamber and decreases in oxygen were measured with an oximeter (Pro-Ox110, Biospherix) until there was no further detectable oxygen in the chamber. Temperature was monitored by an external digital temperature gauge and the Nitrogen gas flushing through the chamber was warmed to maintain the internal chamber temperature between 35-37°C. The aglycemic slices were exposed the hypoxic gas mixture for 20 minutes. Following OGD, the slices were returned to their original culture medium conditions except when they were exposed to experimental treatments.

2.3Assessment of Cell Death in the Hippocampus of Slice Cultures by Image Analysis using Propidium Iodide

Propidium iodide (PI, Sigma) was used for identifying dead cells [13]. Prior to experimental treatment (OGD), slices were incubated in culture medium supplemented with 2 µg/ml propidium iodide for 1h. Propidium iodide fluorescence was examined with an inverted epi-fluorescence microscope (Olympus CK-40) and digital images were acquired with a CCD camera (Photometrics CH250 and Optronix). Fluorescence was quantified using Scion Image (Scion Image is a Windows version of the Macintosh program, NIH Image, written at the National Institutes of Health) software. Experimental slices were imaged prior to the experimental condition (eg. OGD), 1 day after the experimental condition (eg. 20 min OGD), and 1 day after lethal treatment of the culture with 100 µM NMDA. The intensity of PI fluorescence in the CA1, CA3 and dentate gyrus subfields of the hippocampal slices was used as an index of cell death per area. For quantification, the region of interest (ROI) corresponding to each hippocampal subfield was selected from bright field images of each slice. The Cell Death Index after OGD was calculated from each ROI as follows: Cell Death Index (% of NMDA induced PI Fluorescence)= $(F_{OGD} - F_{initial})/(F_{NMDA} - F_{initial}) \times 100$, where $F_{initial}$ is the fluorescence of the slice prior to OGD, F_{OGD} is maximum fluorescence after OGD, and F_{NMDA} is fluorescence after NMDA treatment. The resulting value represents OGD-induced damage as a percent of NMDA-induced cell death.

2.4Treatment in-vitro with Erythropoietin

Erythropoietin-α (EPO- α) was delivered in culture medium either 24 hrs before OGD or immediately after OGD for 24 hours in the following concentrations: 0.1U/ml, 1 U/ml, 10 U/ml, and 100 U/ml. Data are described here for treatment with 1.0 U/ml as no differences were observed among dosing groups. For combination EPO-BMSC treatments, EPO was added to BMSC cultures 24 hr prior to hippocampal slice OGD. Images of PI treated hippocampal slice cultures were acquired for data analysis as previously described.

2.5Western Analysis of total Akt, phosphorylated Akt, and EPO

Western analysis was used for detection of total Akt, phosphorylated Akt, and EPO receptor in single organotypic slice cultures. The following antibodies were used: EPOR (M-20; Santa Cruz Biotechnology); phospho-Akt (pAkt, Ser 473; Cell Signaling Technology); Akt (New England Biolabs, Beverly, MA); and b-Actin (Santa Cruz Biotechnology). Single hippocampal culture slice homogenates were obtained by direct SDS digestion. The single samples were incubated at 95°C, centrifuged, and allowed to cool for loading into gel (4% stacking gel and 10% SDS PAGE). The isolated protein ran on the gel for qualification at 20mA for stacking gel and 25mA for separating gel. The subsequent experimental protocol followed standard Western Blot protocol . Protein quantifications were obtained with the use of Kodak ID 3.6 software and apparatus. Bands were quantified by measuring the optical density and expressing each experimental band as a ratio of the mean optical density of the controls.

2.6Bone Marrow Stromal Cell Cultures

BMSCs were isolated from individual GFP {C57BL/6-TgN(ACTbEGFP)1Osb Jackson Laboratory} mice as described by Song and Sanchez-Ramos [14]. GPF mice were used to facilitate visualization of BMSCs in culture and to determine whether BMSCs migrated into slice cultures during treatment. Mice were anesthetized as described above and placed in a supine position and fur was disinfected with 70% isopropyl alcohol. Fur and skin were removed and the femurs and tibias of the mice were dissected free of ligaments and excess tissue. Bones were placed in a sterile petri dish with 70% isopropyl alcohol and placed on ice. The ends of the long bones were trimmed to expose the interior marrow shaft. Using a 3 cc syringe with a 21 gauge needle, 1-3 ml of heparinized cold saline solution was used to flush bone

marrow into heparinized blood collection tubes. Collection tubes were centrifuged at 800 rpm for 10 min to pellet the bone marrow cells. Careful removal of the supernatant was performed and cells were resuspended into culture medium as previously defined with 100 U/ml strepdamycin/gentamyocin and 1000U/ml of Fungizone (Invitrogen). Bone marrow cell counts were performed utilizing a hemocytometer and methylene blue at a dilution of 1/50. The average cell count per square (x) dilution factor (x) 10^4 = cell count per ml resulted in approximately $12.5x10^6$ cells/ml with two animal femoral and tibial isolations. Bone marrow cells were resuspended in 12ml of medium. Aliquots (2ml) of these cells were placed into 6 well culture dishes (NUNC Brand Products Nunclon Surface) for expansion (Brazelton, Rossi *et al.* 2000). Each well plate contained 25 x 10^6 GFP cells of whole bone marrow explants. On day 3 debris and non-adherent cells were removed by manually shaking trays followed with 3 washes of medium and replacement with new media. Bone Marrow Stromal Cells (BMSCs) were cultured to confluency and expanded for 7-14 days with medium changes every other day.

2.7 Post OGD Treatment in-vitro with BMSCs or BMSC/EPO

Hippocampal slice cultures were treated with BMSCs after OGD by immediately placing the culture well inserts into wells containing BMSCs in oxygenated, glucose containing medium. For combination EPO/BMSC treatment, the slices were transferred into culture wells containing BMSCs and 1.0 U/ml EPO. EPO was applied to BMSCs 24 hr earlier and remained in the culture medium. Slices remained in BMSC or BMSC/EPO culture wells for 24 hr and then were transferred to control culture medium.

2.8 Statistical Analysis of Data

All fluorescent intensities are expressed as mean ±SEM percentage of the hippocampal area of interest given the described condition. Statistical significance was assessed using ANOVA. Differences were considered statistically significant at $P<0.05$. Post hoc analysis to determine significant difference between groups was obtained using Tukey's Post Hoc and Dunnett Two Sided Test. Statistical significance was accepted at $P<0.05$.

3. Results

3.1 Neuroprotective effect of BMSCs coupled with EPO-a in-vitro

In the following experiments, we compared neuroprotection by BMSCs and EPO using hippocampal slice cultures and OGD. We compared neuroprotection provided by BMSCs alone, BMSCs in the presence of EPO, and EPO alone given both before and after ODG.

Slice cultures exposed to OGD for 20 min showed extensive damage in all hippocampal subfields within 24 hr after injury. Examples of propidium iodide fluorescence images showing hippocampal damage are shown in Figure 1 (A-C). Average cell death in control slice cultures after OGD was 96.9 ± 0.6 % (mean ± SEM) in subfield CA1, 90.7 ± 3.8% in subfield CA3, and 75.9 ± 6.1% in the dentate gyrus (Figure 2). Greater neuronal death in subfield CA1 than in CA3 or dentate gyrus was observed consistently throughout this study and confirmed that selective neuronal vulnerability to OGD was a feature of organotypic slice cultures.

Neuroprotection was observed after OGD in hippocampal slices exposed to BMSCs alone in the CA1 (40.14+17.06 %), CA3 (33.72+12.83%), and Dentate Gyrus (15.70+10.24%) (Figure 1D-F, Figure 2). Neuroprotection associated with BMSCs was not due to direct interactions of BMSCs with the slice cultures since the GFP-

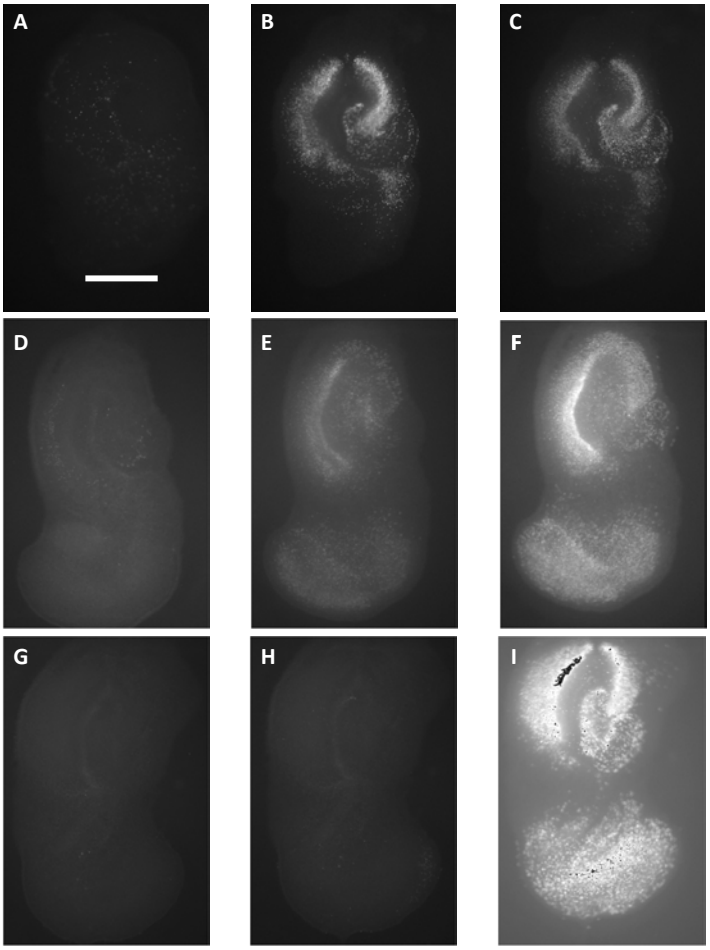

Figure 1. Bone Marrow Stromal Cells in combination with EPO provide enhanced neuroprotection. A-C) OGD Control Images: PI before treatment; PI after OGD; PI after terminal NMDA exposure. D-F) OGD/BMSC Images: PI before OGD; PI after OGD/BMSC treatment; PI after NMDA. G-I) OGD/BM-EPO Images: PI before treatment; PI after OGD/BM-EPO treatment; PI after NMDA. Calbration: 1 mm

BMSCs used in this study did not migrate into the hippocampal cultures (data not shown).

To determine whether the neuroprotective action of BMSCs could be augmented by EPO, BMSC cultures were pretreated with 1.0 U/ml EPO prior to OGD of hippocampal slice cultures. Slice cultures exposed to both EPO and BMSCs after injury exhibited significant neuroprotection beyond post-treatment with BMSCs alone, in all subfields of the hippocampus (e.g. Figure 1G-I). Average cell death in CA1, CA3, and DG respectively of slices treated with EPO plus BMSCs was 12.74 + 3.54%, 4.55 + 1.49 %, and 1.82 + 0.58% (Figure 2).

Paradoxically, some cell death was observed when hippocampal slice preparations were exposed to BMSCs (Figure 2) without OGD or EPO. It appeared that this BMSC-induced cell death, was due to glutamtate excitotoxicity. We found a 7 fold higher glutamate concentration (138 ± 0.85 mM) in medium exposed to BMSCs than in medium exposed to slice cultures (20 ± 0.75uM). Moreover, combined treatment of slice cultures with the glutamate receptor antagonists CNQX and APV (100 mM each) reduced BMSC-induced cell death by 72%, 66% and 74% in subfields CA1, CA2 and dentate respectively.

3.2 Neuroprotection by EPO-a pre- and post-injury.

In contrast to the enhancement of neuroprotection observed when BMSCs were cultured with EPO, EPO (1.0 U/ml) alone did not protect the hippocampus from OGD when applied post-injury (Figure 3D-F; Figure 4). In fact, cell death observed 24 hr after OGD in the presence of EPO was often as great or greater than that found 24 hr later with NMDA suggesting that EPO might have accelerated cell death after OGD. The failure of post-injury treatment with EPO to protect hippocampus after OGD was not dose related as similar results were obtained with 0.1 U/ml, 10.0 U/ml and 100 U/ml EPO (data not shown). However, pre-treatment of slice cultures with EPO protected neurons after OGD (Figure 3H-I). Following pre-treatment of slice cultures with 1.0 U/ml EPO, cell death after OGD averaged 11.67 ±7.49%; 13.20 ± 6.14%; and 6.49 ± 5.26% respectively in subfields CA1, CA3 and Dentate gyrus.

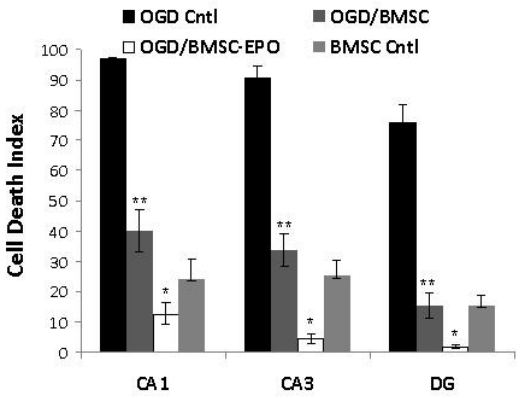

Figure 2. Quantification of the neuroprotective effects of Bone Marrow Stromal Cells in combination with EPO-α in-vitro. A) Cell death measured after OGD, BMSCs, OGD/BMSC, and OGD/BMSC-EPO in CA1, CA3, Dentate Gyrus (DG). OGD injured slice preparations were treated with BMSCs alone and in combination with EPO-α. Significant neuroprotection was observed in all hippocampal sub-fields with BMSCs alone. However, increasingly significant cell survival was observed in the CA1, CA3, and DG regions of the hippocampus treated with both BMSCs and EPO. p<0.0001*/p<0.005** n=12

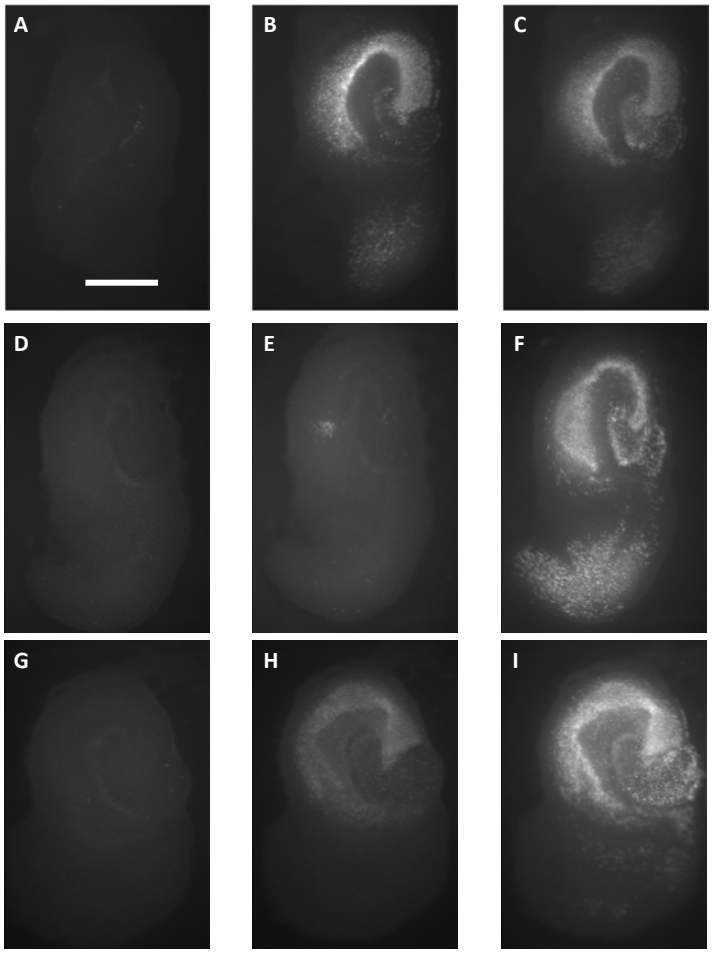

Figure 3. Epo-α Pre-Treatment provides significant neuroprotection in organotypic hippocampal slice cultures prior to Oxygen Glucose Deprivation (OGD). A) Propidium Iodide (PI) fluorescence in control section. B) PI fluorescence after OGD. C,F,I) PI fluorescence after OGD and terminal NMDA treatment. D,E) PI fluorescence after EPO Pre-Treatment and OGD. G,H) PI fluorescence after EPO Post-Treatment and OGD. Calibration: 1 mm

In untreated slices, cell death averaged 93.6 ± 4.8%, 86.7 ± 6.1%, and 80.9 ± 7.1% (Figure 4) in the same hippocampal subfields.

We postulated that failure of EPO to provide neuroprotection when given post-injury might be due to the inability of EPO to stimulate signaling pathways in the post-ischemic hippocampus. To test this hypothesis, we compared expression of EPO-receptor, total Akt and phosphorylation of Akt (p-Akt) by Western Blot analysis in slice cultures treated with EPO before and after OGD. These data are summarized in Figure 5. While there were no differences in total Akt among groups we found that OGD resulted in increased levels of the 66 Kd EPO receptor subunit and increased levels of phospho-Akt. There were no differences between EPO pre-treatment and post-treatment groups that could explain the lack of neuroprotection by EPO when given after injury. In fact, levels of EPO receptor and phospho-Akt were higher in post-treated slices, but likely due to the effects of OGD rather than enhanced activation by EPO.

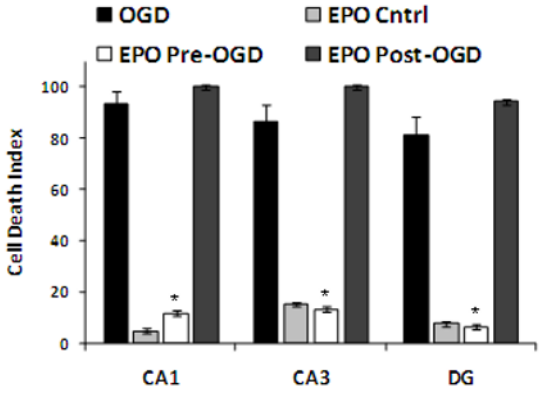

Figure 4. Quantification of neuroprotection after treatment with EPO before or after OGD in slice cultures. Pre-Treatment with EPO 24 hours pior to OGD provided a significant decrease in cell death in CA1, CA3, and Dentate Gyrus of the hippocampal slice culture preparations in comparison to treatment with EPO after injury. No significant difference between Post-Treatment and OGD. (* p<0.0001, n=12 per condition).

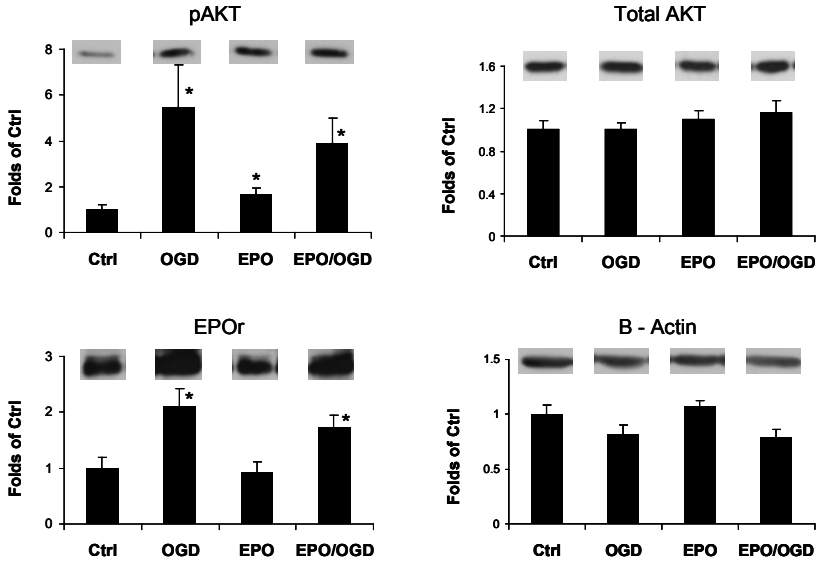

Figure 5. Western Blot analysis of total phospho-Akt (pAkt) , total Akt, and EPO receptor in Control, EPO, OGD, and EPO/OGD treatment from hippocampal slice culture preparations. A) pAKT was significantly increased after treatment with EPO alone, OGD alone, and EPO/OGD treatment in comparison to Control; B) Total Akt between conditions was conserved. There was no significant difference between controls, OGD, EPO alone, or OGD/EPO; C) EPO receptor was increased after OGD and Post-Treatment with EPO after OGD coinciding with activation of Akt; D) Beta-Actin as Western Blot loading controls. (* p<.05)

4. Discussion

Experiments were performed to examine the direct neuroprotective actions of erythropoeitin (EPO) and Bone Marrow Stromal Cells (BMSCs) using an organotypic slice culture model of ischemia. Our experiments showed that hippocampal organotypic slice cultures showed less neuronal damage after oxygen-glucose deprivation when allowed to recover in the presence of BMSCs. Moreover, neuronal damage was further minimized when slice cultures recovered in BMSC containing medium treated with EPO. EPO alone provided no neuroprotection when administered after OGD in the absence of BMSCs. However, EPO was neuroprotective when administered prior to the onset of OGD. The data demonstrate direct paracrine neuroprotection of BMSCs and suggest that previously described neuroprotection by EPO after stroke might be mediated at least in part BMSCs.

BMSC transplantation therapy has been shown to improve outcome in a variety of CNS injury models including stroke [10,15-17], brain trauma [18,19] and spinal cord injury [20-23]. The mechanisms by which BMSCs improve outcome after injury remains poorly understood. The possibilities include: 1) replacement of lost neurons and glia through differentiation of BMSCs into these cell types; 2) plastic changes in surviving neurons through trophic actions of BMSCs at the injury site; and 3) BMSC-mediated neuroprotection of injured cells in CNS [9,10,12,24-26]. Our data provide direct evidence that BMSCs can provide neuroprotection after oxygen glucose deprivation and suggest that BMSCs may do so by releasing neuroprotective factors. The data reported here confirm an earlier study with BMSCs and organotypic slice cultures [27].

BMSCs or subpopulations of these cells are known to produce and release neurotrophins or growth factors [28] and a number of these factors have neuroprotective actions after CNS injury [12,16,17]. The neuroprotective action of BMSCs may not simply involve the release of trophic factors that protect dying neurons. There may be complex interactions between BMSCs and various cell types in injured CNS. For example, BMSCs may interact with injured astrocytes [26] which in turn protect neurons. It is also possible that factors released from injured neurons or glia promote changes in BMSCs to render them more neuroprotective. Using a model of chemically induced apoptosis in neurons, Isele *et al* [29] found that BMSCs exposed to medium derived from injured neurons provided better neuroprotection than BMSCs cultured in the absence on damaged cells.

Like BMSC therapy, EPO has been shown to improve outcome after stroke [30,31]. The mechanisms by which EPO improves outcome is largely unknown but may involve improved brain oxygenation through hematopoeisis [31] or angiogenesis [4,31], or its direct actions on neurons and glia [6]. Both neurons and glia express receptors for EPO [7], and activate downstream signaling pathways in response to EPO exposure [32]. EPO exerts its function through the activation of the downstream cascade commencing with the EPOr signaling through the IP3 pathway, subsequently activating AKT, resulting in the expression of many anti-apoptotic, anti-oxidant, and anti-inflammatory factors [32]. Akt is one of the downstream regulators of anti-apoptotic factors and transcription factors associated with neuroprotection in-vivo after ischemia by EPO implementation or endogenous upregulation [4,8,32-34]. Exposing the hippocampal slice culture preparations to EPO prior to injury influences the tissue to initiate this pathway in the absence of injury resulting in increased cell survival [7]. Previously published data suggested that in-vivo EPO mediated neuroprotection is a result of paracrine production of EPO by astrocytes and upregulation of EPOr on both astrocytes and neurons of the CNS [7,35]. Treatment with EPO prior to ischemia (pre-conditioning) protects neurons both *in vitro* [36] and *in vivo* [30,33], possibly by activating these pathways. We confirmed here that pretreatment of organotypic slice cultures with EPO provided neuroprotection when the slices subsequently were deprived of oxygen and glucose.

In contrast, our finding that treatment with EPO after OGD was ineffective is at odds with numerous studies that show improved outcome when EPO was given after ischemia *in vivo* [2,4,6]. We speculated that perhaps the failure of EPO to provide protection when given after injury might result from down regulation of the EPO receptor or the inability of the injured hippocampus to activate EPO-mediated signaling pathways after injury. This did not appear to be the case since both EPO receptor and phosphorylation of AKT were increased in slices after OGD. In our study post injury treatment with EPO was only effective when we combined EPO with BMSCs. This finding raises the possibility that the presence of BMSCs *in vivo* may contribute to the post injury ischemic neuroprotective actions previously reported for EPO in intact animals.

In conclusion our results directly confirm that BMSCs can promote neuroprotection of hippocampal neurons following simulation of ischemia *in vitro*. We further show that EPO can provide neuroprotection but only when given in the presence of BMSCs after injury. These data suggest that therapeutic strategies directed at blood elements such as BMSCs, possibly in addition to therapies directed at the CNS, may be beneficial for preventing the debilitating neurological consequences of stroke.

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