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Hydrogel delivery of erythropoietin to the brain for endogenous stem cell stimulation after stroke injury

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ABSTRACT

Drug delivery to the brain is challenging because systemic delivery requires high doses to achieve diffusion across the blood-brain barrier and often results in systemic toxicity. Intracerebroventricular implantation of a minipump/catheter system provides local delivery, yet results in brain tissue damage and can be prone to infection. An alternate local delivery strategy, epi-cortical delivery, releases the biomolecule directly to the brain while causing minimal tissue disruption. We pursued this strategy with a hyaluronan/methyl cellulose (HAMC) hydrogel for the local release of erythropoietin to induce endogenous neural stem and progenitor cells of the subventricular zone to promote repair after stroke injury in the mouse brain. Erythropoeitin promotes neurogenesis when delivered intraventricularly, thereby making it an ideal biomolecule with which to test this new epi-cortical delivery strategy. We investigated HAMC in terms of the host tissue response and the diffusion of erythropoeitin therefrom in the stroke-injured brain for neural repair. Erythropoeitin delivered from HAMC at 4 and 11 days post-stroke resulted in attenuated inflammatory response, reduced stroke cavity size, increased number of both neurons in the peri-infarct region and migratory neuroblasts in the subventricular zone, and decreased apoptosis in both the subventricular zone and the injured cortex. We demonstrate that HAMC-mediated epi-cortical administration is promising for minimally invasive delivery of erythropoeitin to the brain.

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1. Introduction

Stroke is the fourth leading cause of death in the world and causes 15 million debilitating injuries each year [1]. Stroke is caused by a disruption in blood supply to the brain, and is classified as either ischemic or hemorrhagic. In ischemic stroke, decreased blood flow results in an insufficient supply of nutrients to cells in the core, which leads to necrotic cell death. A secondary phase of injury subsequently occurs in the tissue surrounding the core (the penumbra), resulting in apoptosis [1].

There is currently no cure for stroke and the only clinically proven drug is tissue plasminogen activator, an anti-thrombolytic agent used to reduce the extent of injury [2]. However, this treatment does not afford tissue regeneration. A number of neuroregenerative strategies have shown improved functional recovery in animal models of stroke, including stem cell transplantation and endogenous stem cell stimulation. For the latter, exogenous factors are delivered to the brain to stimulate endogenous neural stem/progenitor cells (NSPCs) in the subventricular zone (SVZ) to promote tissue repair after injury [3]. Endogenous NSPC stimulation with erythropoietin (EPO) has resulted in NSPC proliferation, migration, and maturation, as well as promoting regeneration and replacement of cells and tissues lost following stroke injury [4,5].

EPO is a 30.4 kDa glycoprotein that has been shown to be both neuroprotective and neuroregenerative after injury to the central nervous system (CNS) [6,7], and it has been shown to reduce stroke cavity size clinically [8]. In the brain, EPO binds with the erythropoietin receptor (EPOr), which is expressed on multiple cell types including neurons, astrocytes, and NSPCs in the SVZ [7]. When bound to EPOr on NSPCs, EPO promotes their survival and differentiation into mature neurons [9].

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One limitation in using EPO, as well as a number of other growth factors, for stimulating endogenous brain repair after stroke is the lack of appropriate delivery systems. Systemic delivery of proteins, by intravenous or intranasal delivery, results in less than 1% crossing the blood-brain barrier BBB [10]. Moreover, intravenous delivery can lead to systemic toxicity at high concentrations [11,12] while chronic intranasal delivery is associated with systemic toxicity, patient discomfort, low patient compliance and thus sub-optimal therapeutic benefit [13,14]. Local drug delivery strategies typically involve the insertion of a cannula or drug delivery scaffold into the brain tissue, both of which are highly invasive [15,16]. The optimum paradigm involves delivering drugs from the cortical surface using a minimally invasive strategy, controlling the release and transport of the drugs such that they reach the SVZ, and maintaining the bioactivity of the drugs in order that they stimulate NSPCs upon reaching the SVZ.

With a view toward circumventing the blood-brain barrier and achieving delivery to the brain, we asked whether EPO could be delivered locally to the stroke-injured brain using an injectable hydrogel and what the brain host tissue response would be to this hydrogel. The physically cross-linked blend of hyaluronan (HA) and methyl cellulose (MC) (HAMC) is bioresorbable, injectable through a fine needle, and gels rapidly at physiological temperature [17]. HAMC spatially localizes the drug of interest at the site of delivery and facilitates short-term controlled release to the CNS [18,19].

One of the major limitations of delivering protein drugs to the brain is the fast rate of protein elimination and the consequent short penetration distance. Unmodified proteins often do not penetrate more than 1 mm in the uninjured brain, and penetration distance decreases significantly after brain injury because stroke injury results in the upregulation of many protein receptors in the brain [18,20]. Modification of proteins with poly(ethylene glycol), PEG, has been used to increase protein penetration distance [18,21–23].

Here we studied the diffusion of EPO from the HAMC hydrogel, delivered directly to the cortex, in the uninjured as well as stroke-injured mouse brain. In order to understand the kinetics of EPO penetration in the brain, EPOr expression was examined at the time points of EPO delivery: 4 and 11 days post-stroke. These two time points were selected because previous reports have delivered EPO at these time points, albeit using a highly invasive cannula/mini-pump system, and shown that in mice models of ischemia, cortical EPOr upregulation increases between 1 and 7 days post ischemia [24], and persists up to 28 days [25]. The host tissue response of EPO delivered from HAMC was examined in terms of: NSPC stimulation/migration and neurogenesis; and the inflammatory response by immunohistochemical staining for macrophages/microglia and astrocytes.

2. Materials and methods

2.1. Materials

Recombinant human erythropoietin (EPREX) was supplied by Ortho Biotech Canada (Toronto, ON, Canada). Sodium hyaluronan (HA, $1.4–1.8 \times 10^6$ g/mol) was purchased from NovaMatrix (Sandvika, Norway). Methyl cellulose (MC, 3.4×10^5 g/mol) was obtained from Shin Etsu (Chiyoda-ku, Tokyo, Japan). Mouse anti-human Ki-67 was purchased from BD biosciences (Mississauga, ON, Canada), mouse anti-rat NeuN and GFAP were obtained from Millipore Inc. (Billerica, MA, USA), rat anti-mouse CD68^{+} and rabbit anti-mouse double-cortin were obtained from Abcam (Cambridge, MA, USA), and Vectashield with DAPI stain was purchased from Vectorlabs (Burlington, ON, Canada). Alexa 488 goat-anti-rat, Alexa 488 and 586 goat-anti-rabbit IgG, and Alexa 568 goat-anti-mouse IgG were obtained from Invitrogen Inc. (Burlington, ON, Canada). Sodium cyanoborohydride (NaCNBH$_3$), NaCl, MgCl$_2$, CaCl$_2$, BaCl$_2$, Na$_2$HPO$_3$, NaH$_2$PO$_3$, trehalose, and cresyl violet acetate were supplied by Sigma Aldrich (Oakville, ON, Canada). Triton X-100 was supplied by ACROS (NJ, U.S.A.). Artificial cerebrospinal fluid (aCSF [23]) and all buffers were prepared with distilled and deionized water prepared from a Millipore Milli-RO 10 Plus and Milli-Q

UF Plus at 18 MΩ m resistivity (Millipore, Bedford, USA). Recombinant EPO ELISA kit was purchased from BD biosciences (Mississauga, ON, Canada).

2.2. Preparation of sterile HAMC hydrogel

HA and MC were dissolved separately in dH$_2$O at 4 °C overnight, sterile-filtered and lyophilized. The resulting sterile powders were kept at 4 °C until use. HAMC was prepared with 1.1% HA and 2.2% MC in sterile aCSF and mixed using a SpeedMixer (DAC 150 FVZ, Siemens). Immediately prior to injection, 100 μl of sterile EPO solution (10,000 U/ml) was added to 900 μL HAMC (yielding a final concentration of 1% HA and 2% MC), mixed and centrifuged to eliminate air bubbles.

2.3. In vitro release of EPO from HAMC

The time required for EPO to diffuse out of HAMC was determined in vitro. EPO was mixed into HAMC to yield a final concentration of 1000 U/ml, and 100 μl was injected into the bottom of a 2 ml eppendorf tube and gelled at 37 °C for 20 min. 900 μl of aCSF at 37 °C was added. The supernatant was completely replaced at each of the following time points and EPO concentrations determined using ELISA: 0, 30 min, 1, 2, 4, 6, 8, 12, 24 h.

2.4. Stroke surgeries and injection of drug delivery device

All animal work was carried out in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care) and approved by the Animal Care Committee at the University of Toronto. A total of 54 C57BL/6 mice (aged 9–11 weeks) were used in this study (Charles River, QC, Canada).

Stroke surgeries were carried out as described previously [26]. Mice were anesthetised with isoflurane, shaved and placed into a Kopf stereotaxic instrument. Scalps were cleaned and a midline incision made next, a small burr hole was made in the skull at the coordinates 2.25 lateral to the midline and 0.6 anterior to Bregema. Using a 26G needle, 1.0 μl of the vasoconstrictor endothelin-1 (400 pM, Calbiochem, Gibbstown, NJ, USA.) was injected 1.0 ventral to the brain surface at 0.1 μl/min. The needle was left in place for 10 min prior to removal. The incision was sutured, antibiotic ointment applied and animal recovered under a heat lamp.

The drug delivery system was injected at either day 4 or day 11 post-stroke (Supplementary Fig. S1). The sutures were removed to expose the stroke site and any tissue debris was removed. The drug delivery device was prepared as described previously [18] to spatially localize HAMC at the cortical surface. A disk with 2 mm opening was fixed over the burr hole with bone glue. 3 μl of either HAMC containing EPO or HAMC alone was injected into the hole such that the gel is in direct contact with the brain cortical surface. A second disk with no opening was fixed over the first spacer. The skin was sutured over the spacer system. For uninjured animals, the surgery was identical (without the stroke injury itself) to that of stroke-injured mice.

2.5. Analysis of in vivo protein penetration

Animals were sacrificed at 4 h, 1 day, and 2 days post injection and the drug delivery device containing HAMC was retrieved. The extracted device was placed into 0.5 ml 0.1% Tween 20 in PBS and agitated overnight at 4 °C overnight to extract any remaining EPO.

Brains were snap frozen with CO$_{2(s)}$ cooled isopentane and stored at −80 °C. A 3 mm coronal section around the injection site was prepared using the McIlwain tissue chopper (790744-11, Mickle laboratory engineering company, Surrey, UK). Dorsal-ventral sections (0.5 mm) were then cryosectioned from each coronal slice using a Leica CM3050S cryostat system. Each section was homogenized in 400 μl lysis buffer (40 mM trehalose, 1% Triton X-100 in dH$_2$O), and the homogenate supernatant was removed after centrifugation at 15,000 RPM for 15 min at 4 °C.

The amount of protein remaining in HAMC and in the brain homogenate at each time point was determined using a recombinant EPO ELISA kit (BD Biosciences) as per the manufacturer's instructions. The difference between the amounts of protein that remain in HAMC at each time was used to calculate the amount of protein released. We assume that no protein is lost from the entire system during the period of release and that all protein released has diffused into the brain tissue. The amount of EPO in the homogenate was used to generate tissue penetration profiles as well as the protein mass balance at each time point.

2.6. Brain tissue preparation for morphological analysis

At the appropriate time points animals were transcardially perfused with saline followed by 4% paraformaldehyde (PFA). Brains were extracted and fixed in 4% PFA at 4 °C overnight, followed by cryoprotection in 30% sucrose. Cryoprotected brains were snap frozen and cryosectioned to 10 μm.

2.7. Analysis of stroke cavity size

Sections were defatted in 50:50 chloroform:ethanol solution overnight, and rehydrated in 100%, 95%, and dH$_2$O for 2 min each. Cresyl violet acetate was dissolved at 0.1 w/v % in dH$_2$O and 0.3 ml of glacial acetic acid was added to 100 ml of

solution. Brain sections were incubated in cresyl violet solution for 1 h at 37 °C, washed in H_2O and serially dehydrated in 95%, 100% ethanol, and xylene. Sections were mounted and sealed.

Sections were examined for the start and end points of stroke cavity. Every 10th section was imaged from the start to the end of the cavity. The cavity size on each section was measured using ImageJ, summed and multiplied by 10 to obtain the cavity size.

2.8. Immunohistochemistry

From each brain 16 sections were analyzed using immunohistochemistry. Sections were permeabilized for 30 min with 1% Triton X-100 in PBS and blocked for 30 min with a solution of 0.1% Triton X-100 and 5% BSA in PBS. Sections were incubated with primary antibodies at 4 °C overnight. Mouse anti-human Ki-67, mouse anti-rat GFAP, rat anti-mouse CD68$^+$ and rabbit anti-mouse double-cortin (DCX), and mouse anti-rat NeuN were used at 1:200 dilution. Sections were then washed 3 times in PBS and incubated in secondary antibodies for 1 h at room temperature. Alexa 488 goat-anti-rat IgG, Alexa 488 and 586 goat-anti-rabbit IgG, and Alexa 568 goat-anti-mouse IgG were used at 1:200 dilutions. Sections were washed 3 times in PBS, mounted with Vectashield and sealed.

2.9. Analysis of apoptosis using TUNEL

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was used to determine apoptosis after stroke. The ApopTag Fluorescein In Situ Apoptosis kit was purchased from Millipore (Danvers, MA, U.S.A.) and the TUNEL assay performed as per the manufacturer's instructions. A total of 8 sections were analyzed from each brain.

2.10. Statistics

All experiments were conducted with three replicates ($n = 3$), and data are shown as mean ± standard deviation. One-way ANOVA with Bonferonni correction was used to compare between multiple groups. Significance levels were indicated as $p < 0.05$ (*), 0.01 (**), and 0.001 (***).

3. Results

3.1. Cortical EPO Receptor (EPOr) expression in uninjured and stroke-injured brains

The rate of protein elimination in the brain is an important determinant of penetration distance, which can be significantly increased by reducing elimination kinetics. Receptor mediated endocytosis is a key route of EPO elimination from the brain [27] and since many cell types in the brain express EPO receptors (EPOr) [27,28], it was important to quantify the number of

EPOr$^+$cells in both uninjured and stroke-injured brain tissues. By quantifying the number of EPOr$^+$ cells in two regions along the diffusion pathway and both ipsilateral and contralateral to the injury (Fig. 1A), we found that the total number of EPOr$^+$ cells in our two selected volumes of cortical tissue was similar between uninjured and stroke-injured animals (Fig. 1B). The similarity in brain EPOr expression before and after stroke injury suggests that EPO penetration in brain tissue will be unaffected by stroke injury, and EPO may penetrate significantly in the tissue without requiring further modification with, for example, PEG, as has been required for other proteins [21,22].

3.2. Release and penetration of EPO in uninjured and stroke-injured brains

Prior to studying EPO release from HAMC in vivo, we tested the in vitro release profile of EPO from HAMC and found that approximately 80% of EPO was released in the first 24 h, and all of the encapsulated protein was released within 48 h (Supplementary Fig. S2A). Plotting the cumulative fractional release against the square root of time ($t^{1/2}$) yields a linear relationship, indicating Fickian diffusion kinetics (Supplementary Fig. S2B).

The in vivo release and penetration of EPO from HAMC in both uninjured and stroke-injured brains was quantified at 4 h, 1 d, and 2 d after injection at two different injection times after stroke injury: 4 and 11 days (Fig. 2A,B). For each set of time points and in all uninjured, 4 d injured and 11 d injured brain tissues, the amount of EPO remaining in HAMC was similar as quantified by ELISA. This demonstrates that the tissue type did not affect the rate of release of EPO from HAMC (Fig. 2C). In order to calculate the percent of EPO released from HAMC and in the brain tissue, we compared the amount of EPO detected to a theoretical amount. The theoretical amount of EPO was estimated by assuming that the entire quantity of EPO released from HAMC diffused into the brain. The difference between theoretical and measured EPO was used to estimate the rate at which protein is eliminated from the tissue (Fig. 2D).

The penetration depth of EPO in the uninjured brain and 4 and 11 day-stroke-injured brains were determined at 4 h, 1 d, and 2 d post injection (Fig. 2E–G). Similar penetration profiles were found between all groups at the three time points examined. Importantly, a significant amount of EPO was found between 2 and 3 mm ventral
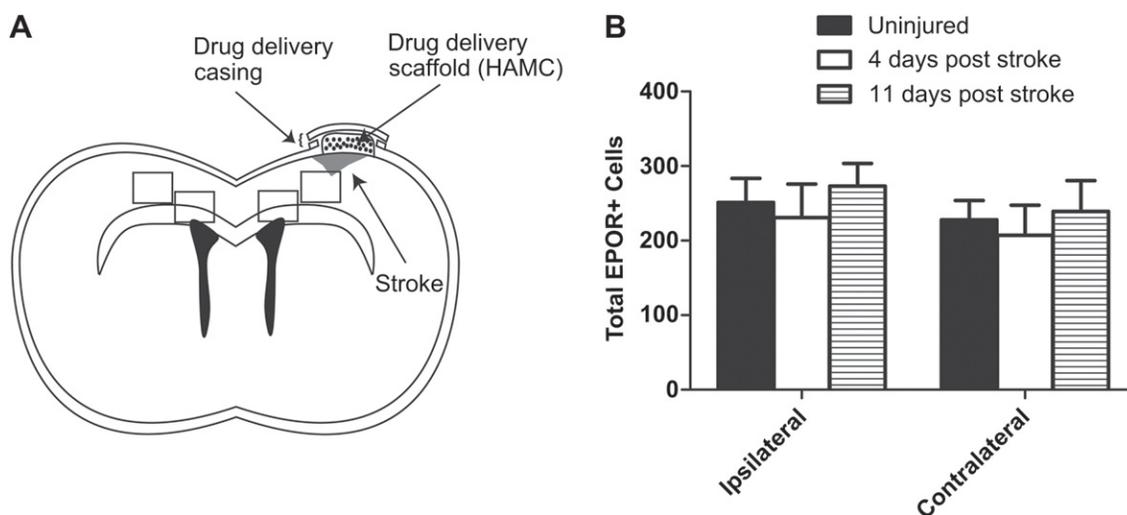
Fig. 1. EPOr expression is similar between uninjured and stroke-injured brain tissues. (A) Diagrammatic representation of the stroke-injured brain and the regions of interest for EPOr quantification. Rectangles indicate regions examined for EPOr expression. (B) Total numbers of EPOr$^+$ cells are similar in all three conditions and both ipsilateral and contralateral to injury (mean ± standard deviation, $n = 3$ animals, 16 tissue slices were analyzed per animal).
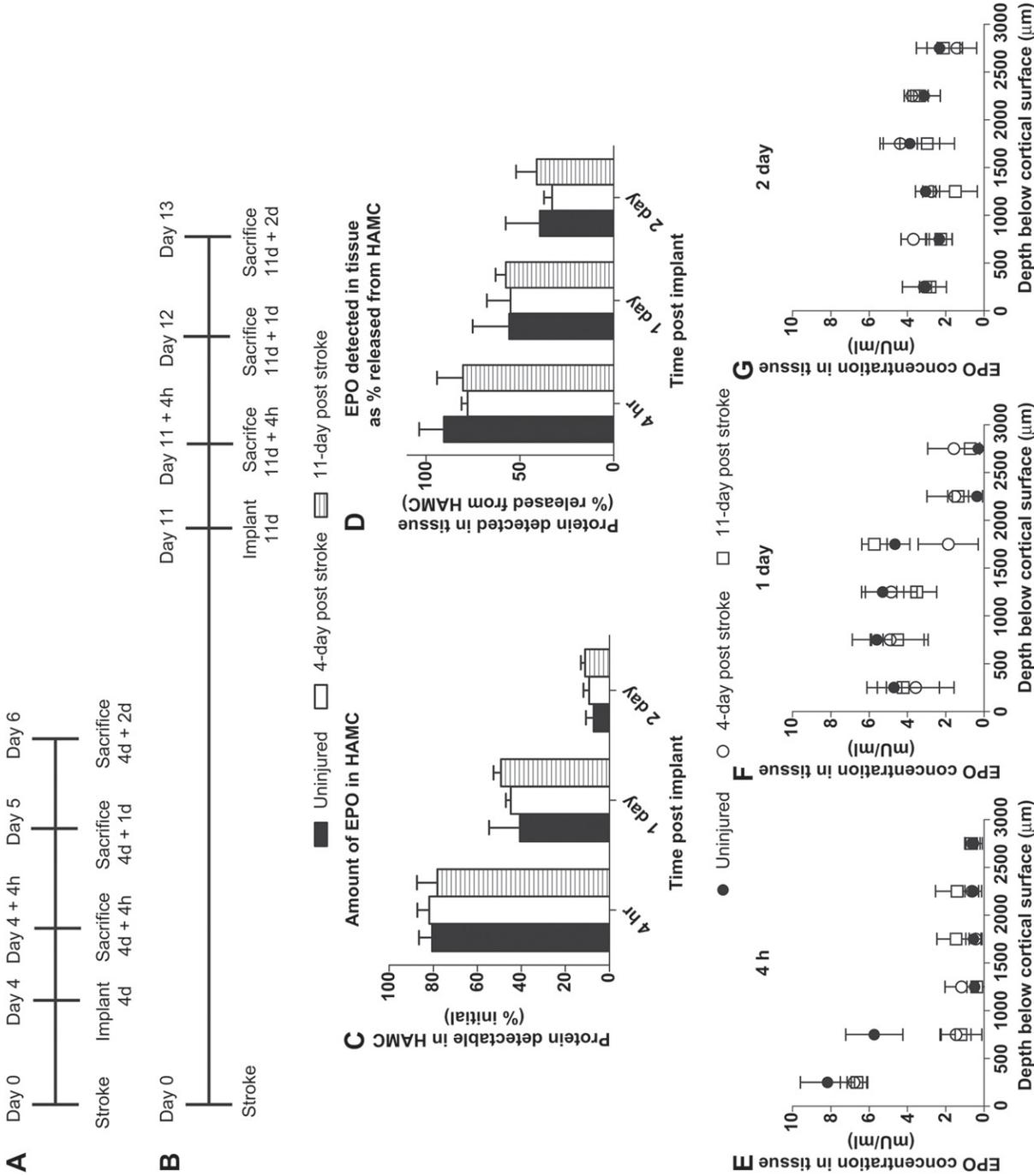
Fig. 2. Penetration of EPO in stroke-injured brains. (A–B) Paradigm for surgical induction of stroke and injection of drug delivery device. (C) Mass of EPO released from HAMC at 4 h, 1 d, and 2 d after injection indicate similar rates of release when injected onto uninjured tissue, stroke-injured tissue 4 days post-stroke, and stroke-injured tissue 11 days post-stroke. (D) EPO detected in brain tissue as percentage released from HAMC at 4 h, 1 d, and 2 d after injection indicate similar rates of EPO elimination due to stroke alone, stroke with HAMC, and stroke with EPO in HAMC. Penetration profiles of EPO in uninjured and stroke-injured brain tissues were examined by ELISA. EPO penetration in brain tissue examined at (E) 4 h, (F) 1 d, and (G) 2 d after injection. Penetration was similar between the three types of brain tissue: uninjured, 4 days after stroke injury, and 11 days after stroke injury (mean ± standard deviation, n = 3 animals, 16 tissue slices were analyzed per animal).

to the cortical surface, which corresponds to the location of the SVZ in the mouse brain. The lack of EPOr upregulation after stroke supports these data and enables significant EPO penetration in the brain. These promising results led us to investigate the effects of HAMC and EPO on cortical tissue and endogenous brain NSPCs.

3.3. Effects of EPO delivered from HAMC on stroke cavity size

To investigate the biological effect of EPO delivered epi-cortically from HAMC, stroke was induced on day 0 by injection of endothelin-1 into the mouse brain, and either HAMC alone or HAMC with EPO was injected on either day 4 or day 11 post-injury (Supplementary Fig S1). The drug delivery system was injected ipsilateral to the stroke. The contralateral hemisphere served as an internal control in all groups examined.

Injection of endothelin-1 into the mouse brain led to the formation of a cavity at the injection site (Fig. 3A). We measured the cavity volume in stroke-injured animals, and compared this to that of stroke-injured animals treated with HAMC alone or HAMC with EPO (Fig. 3B). To establish a time course on the effects of EPO, we examined two time points: 4 d and 11 d after stroke. When injected 4 d post-stroke, the vehicle alone (HAMC) appeared to reduce cavity volume relative to stroke alone; however, when EPO was delivered in HAMC this reduction in cavity size was significant ($p = 0.031$).

When injected 11 d post-stroke, HAMC alone significantly decreased the cavity volume compared to injury alone ($p = 0.033$). HAMC with EPO further reduced cavity size compared to both HAMC treated ($p = 0.045$) and untreated ($p = 0.006$) animals. The reduction in cavity size observed with HAMC alone was unexpected and led us to investigate the possible mechanism further.

3.4. Effect of HAMC on the inflammatory response after stroke

When stroke-injured animals received injections of HAMC at both 4 d and 11 d post-injury, the number of reactive astrocytes, as indicated by GFAP$^+$ immunostained cells with ramified morphology (Fig. 4A–E), decreased in the ipsilateral cortex ventral

to the lesion site ($p = 0.003$ at 4 d and $p = 0.001$ at 11 d; Fig. 4F). Interestingly, there was no difference observed between injections of HAMC vs. HAMC with EPO, suggesting that the reduced gliotic response was due to the biomaterial and not EPO.

The number of CD68$^+$ activated microglia and macrophages (Fig. 5A–E) also decreased around the lesion site in response to injection of HAMC at 4 d and 11 d post-stroke ($p = 0.01$ at 4 d and $p = 0.004$ at 11 d, Fig. 5F). Similar to the astrogliotic response, there was no difference observed between injections of HAMC vs. HAMC with EPO in terms of the number of CD68+ cells, suggesting that the attenuated inflammatory response was due to the biomaterial and not EPO. Taken together, the reduced stroke cavity can be predominantly attributed to the biomaterial, HAMC.

3.5. Effect of EPO on Ki-67$^+$ and double cortin$^+$ (DCX$^+$) Ki-67$^+$ cells in the SVZ

Compared to controls of either stroke alone (Fig. 6A–E) or stroke with HAMC, injection of HAMC with EPO onto stroke-injured mouse brains significantly increased the number of Ki-67$^+$ and DCX$^+$ Ki-67$^+$ double positive cells in the ipsilateral SVZ (Fig. 6F–J). Ki-67 is a marker for proliferating cells while DCX is a marker associated with migratory neuroblasts [29]. Similarly, when injected 4 d (Fig. 6K) and 11 d (Fig. 6L) post-stroke, HAMC with EPO treatment led to an approximate 2-fold increase in Ki-67$^+$ cells compared to HAMC treatment alone and stroke alone. Only the EPO-treated groups had significantly more Ki-67$^+$ cells in the ipsilateral SVZ than the contralateral SVZ at both time points ($p = 0.031$ at 4 d and $p = 0.005$ at 11 d). This shows that the hydrogel alone does not induce cell proliferation, and that exogenous EPO is required to stimulate cell proliferation in the SVZ. Furthermore, there was no difference between any groups in the contralateral SVZ, suggesting that EPO diffusion was specific to the ipsilateral cortex, where it was injected.

Interestingly, the absolute numbers of Ki-67$^+$ cells in both the ipsilateral and contralateral SVZ were lower when EPO was delivered 11 d post-stroke compared to 4 d post-stroke ($p = 0.031$). This
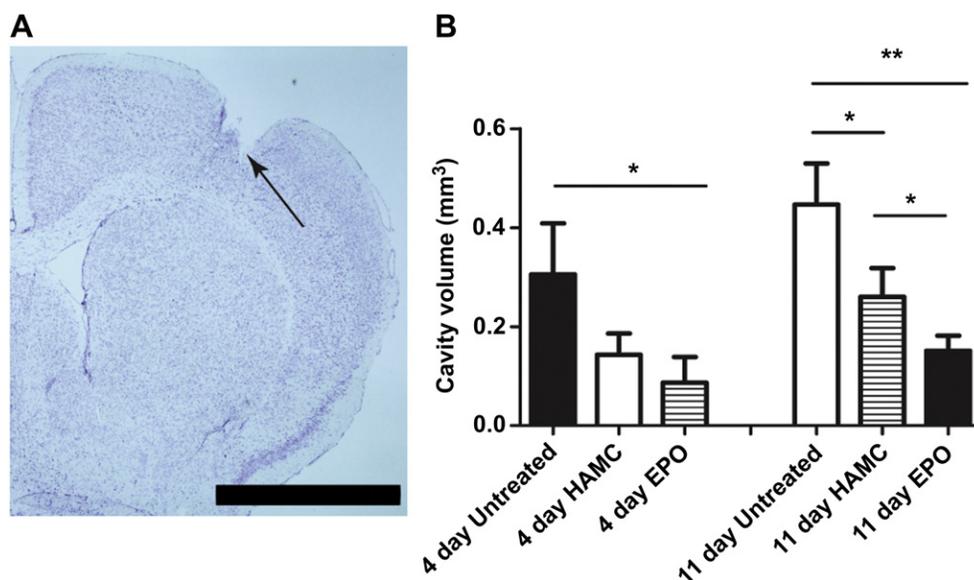
Fig. 3. EPO delivered epi-cortically from HAMC decreases the stroke cavity size at 4 and 11 days after stroke. (A) Image of a brain section with stroke cavity (indicated by arrow) shown at 1.25x (scale: 2 mm). (B) Quantification of the cavity sizes. Stroke-injured animals received treatments at either 4 or 11 d post-stroke. Treatment with EPO in HAMC significantly reduced cavity size at 4 d, while both HAMC and EPO in HAMC significantly reduced cavity size at 11d (mean ± standard deviation, $n = 3$ animals, 16 tissue slices were analyzed per animal).
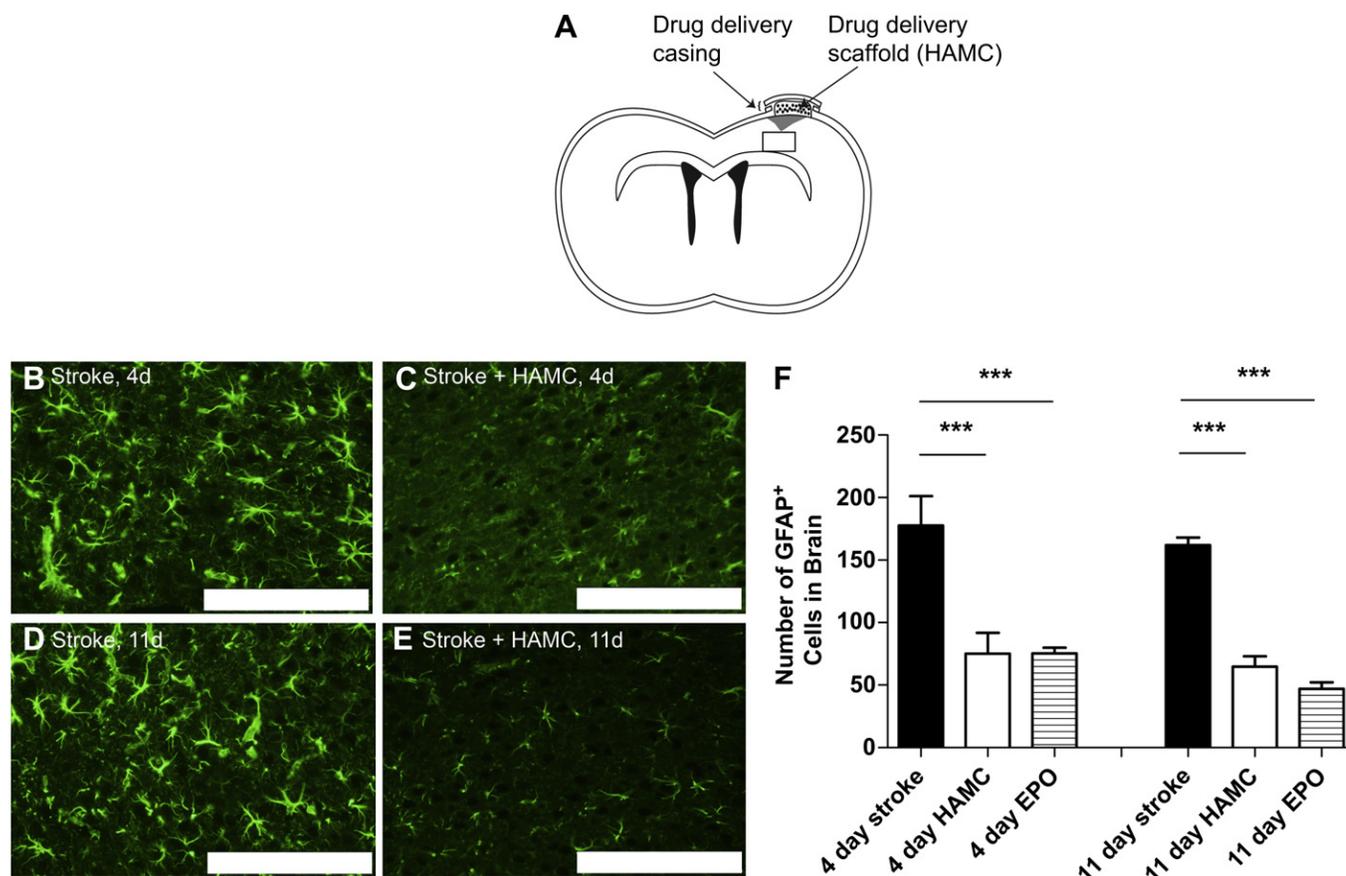
Fig. 4. Epi-cortical delivery of EPO from HAMC attenuates the reactive astrocyte response at 4 and 11 days after stroke injury. (A) Diagram of the stroke-injured brain with drug delivery device. Rectangle indicates the region of interest. Representative images of GFAP$^+$ reactive astrocytes in the ipsilateral cortex ventral-lateral to the lesion site are shown for animals that received (B) stroke alone and (C) stroke followed by HAMC injection at 4 d, as well as (D) stroke alone and (E) stroke with HAMC injection at 11 d. Scale: 100 μm. (F) Quantitative comparison of average number of GFAP$^+$ cells are shown for the ipsilateral cortex per field of view. Treatment with both HAMC and EPO in HAMC significantly reduced the total number of GFAP$^+$ cells at both 4 and 11 days while the total number of DAPI$^+$ cells remain the same (mean $\pm$ standard deviation, $n = 3$ animals, 16 tissue slices were analyzed per animal).

suggests that proliferation of cells in the SVZ decreased with time after stroke injury, but may be partially restored with EPO treatment.

The number of double positive DCX$^+$ Ki-67$^+$ cells in the SVZ was close to 2-fold greater with EPO delivery in the ipsilateral SVZ at both 4 d (Fig. 6M) and 11 d (Fig. 6N) post-stroke. This increase was not observed following treatment with HAMC alone. No difference was found between any groups in the contralateral SVZ.

When delivered 11 d post-stroke, the number of double positive DCX$^+$ Ki-67$^+$ cells was also enhanced compared to 4 d post-stroke ($p = 0.033$). Moreover, only EPO delivery at 11 d post-stroke resulted in significantly higher DCX$^+$ Ki-67$^+$ cells in the ipsilateral SVZ compared to the contralateral SVZ ($p = 0.03$). These results indicate that when given 11 d (vs. 4 d) after stroke, EPO enhanced the migratory potential of proliferating cells in the ipsilateral SVZ. Since DCX is an immature marker for precursor cells but not found in mature neurons [30], these findings suggest that EPO promotes neuroregeneration.

3.6. Effect of EPO on NeuN$^+$ mature neurons in the injured cortex

We examined neuronal responses in stroke animals that received EPO. Stroke injuries cause neuronal death in the ipsilateral cortex (Fig. 7A–D). EPO delivery both 4 d and 11 d post-stroke significantly increased the number of NeuN$^+$ mature neurons in the injured cortex relative to stroke alone ($p = 0.001$ at 4 d, $p = 0.032$ at 11 d, Fig. 7E–G), although the total number of mature

neurons in the injured cortex was still more than ten-fold lower than that in the uninjured contralateral cortex.

When stroke injured animals received no treatment or HAMC alone, the number of NeuN$^+$ cells found in the ipsilateral cortex was higher at 4 days post-stroke compared to 11 days post-stroke, although the difference was not statistically significant. Cell death likely continues to occur around the lesion site between 4 and 11 days after stroke injury. Unexpectedly, the number of NeuN$^+$ cells in the ipsilateral cortex was higher when EPO was delivered 11 d post-stroke than 4 d post-stroke ($p = 0.017$). This result mirrors the increase of DCX$^+$ Ki-67$^+$ double positive cells in the SVZ at 11 d compared to 4 d, and indicates that epi-cortically delivered EPO has a direct benefit on increasing the number of neurons around the infarct site.

3.7. Effect of EPO on TUNEL$^+$ apoptotic cells in the injured cortex and SVZ

We studied the extent of apoptotic cell death by TUNEL-immunostaining in both the cortex and the SVZ after stroke and injection of either HAMC alone or HAMC with EPO. In the SVZ (Fig. 8A,B), injection of HAMC alone, at either 4 d or 11 d, did not change the TUNEL$^+$ response compared to stroke-injured groups. However, when EPO was delivered from HAMC, a significant decrease in the TUNEL$^+$ signal was found at both 4 d ($p = 0.007$) and 11 d ($p = 0.049$) compared to HAMC alone. This reduction in the
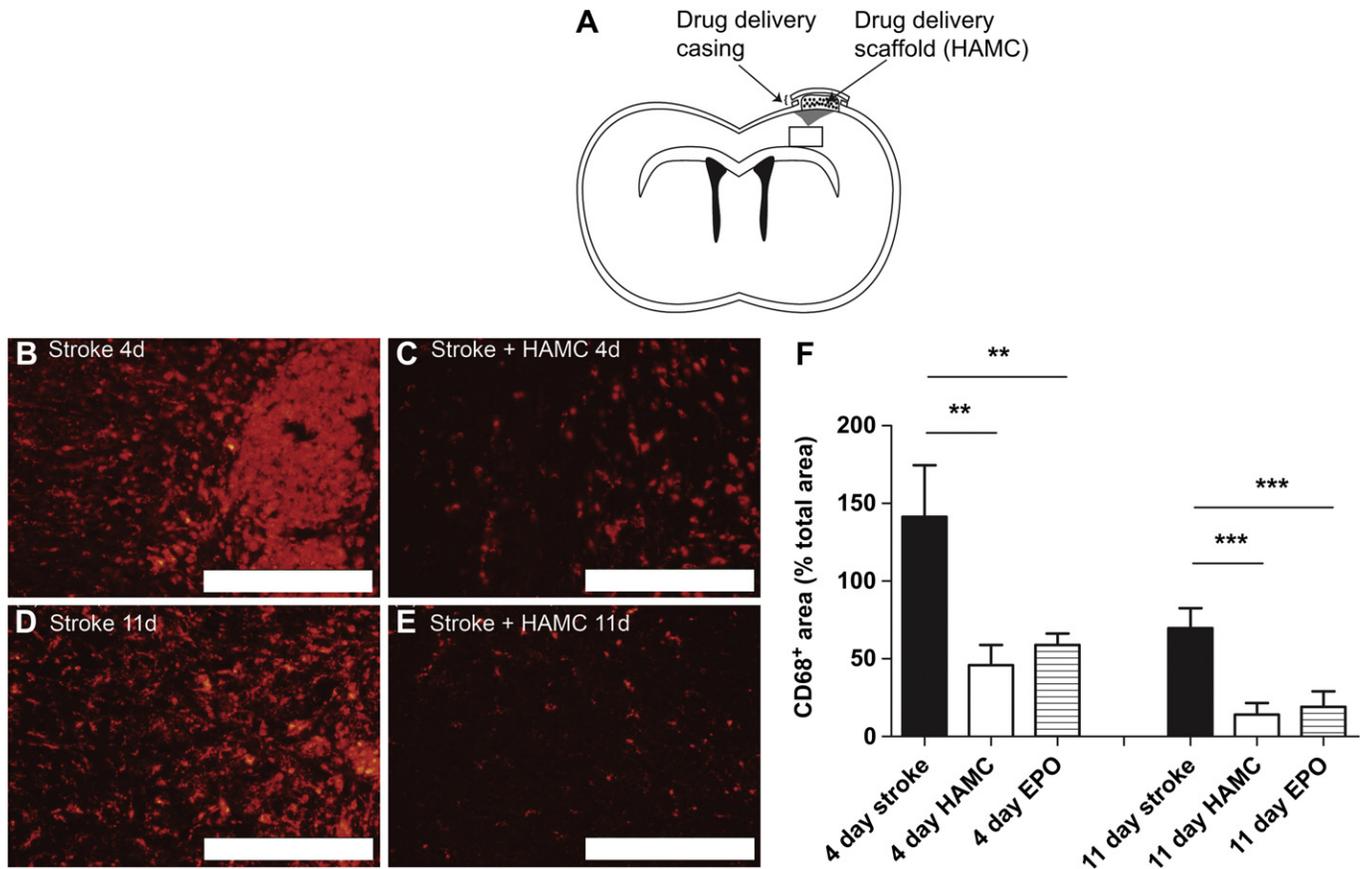
Fig. 5. Epi-cortical delivery of EPO from HAMC attenuates the activated microglia response at 4 days after stroke injury. A) Diagram of the stroke-injured brain with drug delivery device. Rectangle indicates the region of interest. Representative images of CD68+ cells in the ipsilateral cortex are shown for animals that received (B) stroke alone and (C) stroke followed by HAMC injection at 4d, as well as (D) stroke alone and (E) stroke with HAMC at 11d. Scale: 100 μm. (F) Quantitative comparison of average number of CD68+ cells are shown for the ipsilateral cortex per field of view. Treatment with both HAMC and HAMC with EPO significantly reduced the level of CD68+ signal at both 4 and 11 days while the total number of DAPI+ cells remain the same (mean ± standard deviation, $n = 3$ animals, 16 tissue slices were analyzed per animal).

number of apoptotic cells in the SVZ may be correlated to the increase in Ki67+ proliferating cells in the SVZ and suggests a neuroprotective role for EPO. This effect is further supported by the data that show the number of TUNEL+ cells in the ipsilateral SVZ being similar to that found in the contralateral SVZ only after EPO delivery at 4 d. Thus EPO delivery appeared to decrease cell death to the basal level in the SVZ. The data at 11 d follows similar trends to those at 4 d; however, the differences in ipsilateral and contralateral SVZ are not significant at 11 d, suggesting that neuroprotective effects associated with EPO delivery are more significant when it is present at earlier time points after stroke.

In the ipsilateral cortex, we also observed a decrease in the number of TUNEL+ cells after EPO delivery relative to injection of HAMC alone or stroke alone (Fig. 8C,D). HAMC treatment did not lead to changes in cortical TUNEL stain relative to stroke alone. The decrease at 4 d was significantly different whereas that at 11 d post-stroke was not statistically significant. The lack of statistically significant improvement when EPO was delivered 11 d post-stroke further suggests the necessity of early EPO administration for neuroprotection.

4. Discussion

The HAMC-mediated epi-cortical delivery of EPO allowed us to observe tissue benefit resulting from both EPO and HAMC. Injection of HAMC itself led to an attenuated inflammatory response after

stroke, as indicated by fewer reactive astrocytes and activated microglia around the lesion site. HAMC has been shown to attenuate the inflammatory response after spinal cord injury [17]. HA itself has been reported to be anti-inflammatory [31] through reducing white cell migration to the site of injury [32], and through inhibiting proinflammatory molecules of the prostaglandin pathway [33]. It also enhances wound healing and angiogenesis after traumatic brain injury [34]. The attenuated inflammatory response may account for the reduced cavity size observed [35] with injection of HAMC following stroke injury and likely reflects the wound healing properties of HA [36].

We found that the penetration profiles and in vivo elimination rate of EPO are similar between the uninjured and stroke injured brains at two times after stroke. This was interesting given that epidermal growth factor (EGF) has been previously reported to be eliminated significantly faster in the infarct brain compared to the uninjured brain due to EGF receptor overexpression [18,23]. The transport of epidermal growth factor (EGF) in the brain decreased more than three-fold after stroke injury [18,23], and PEG-modification of EGF was required to enhance the amount of EGF reaching the SVZ. Conversely, EPO penetration was independent of injury and EPO was able to penetrate the brain tissue sufficiently to reach the SVZ after stroke.

Receptor-mediated endocytosis is one of the major routes for EPO clearance from the brain [28]. We found that the extent of EPOr expression was similar between uninjured and stroke-injured
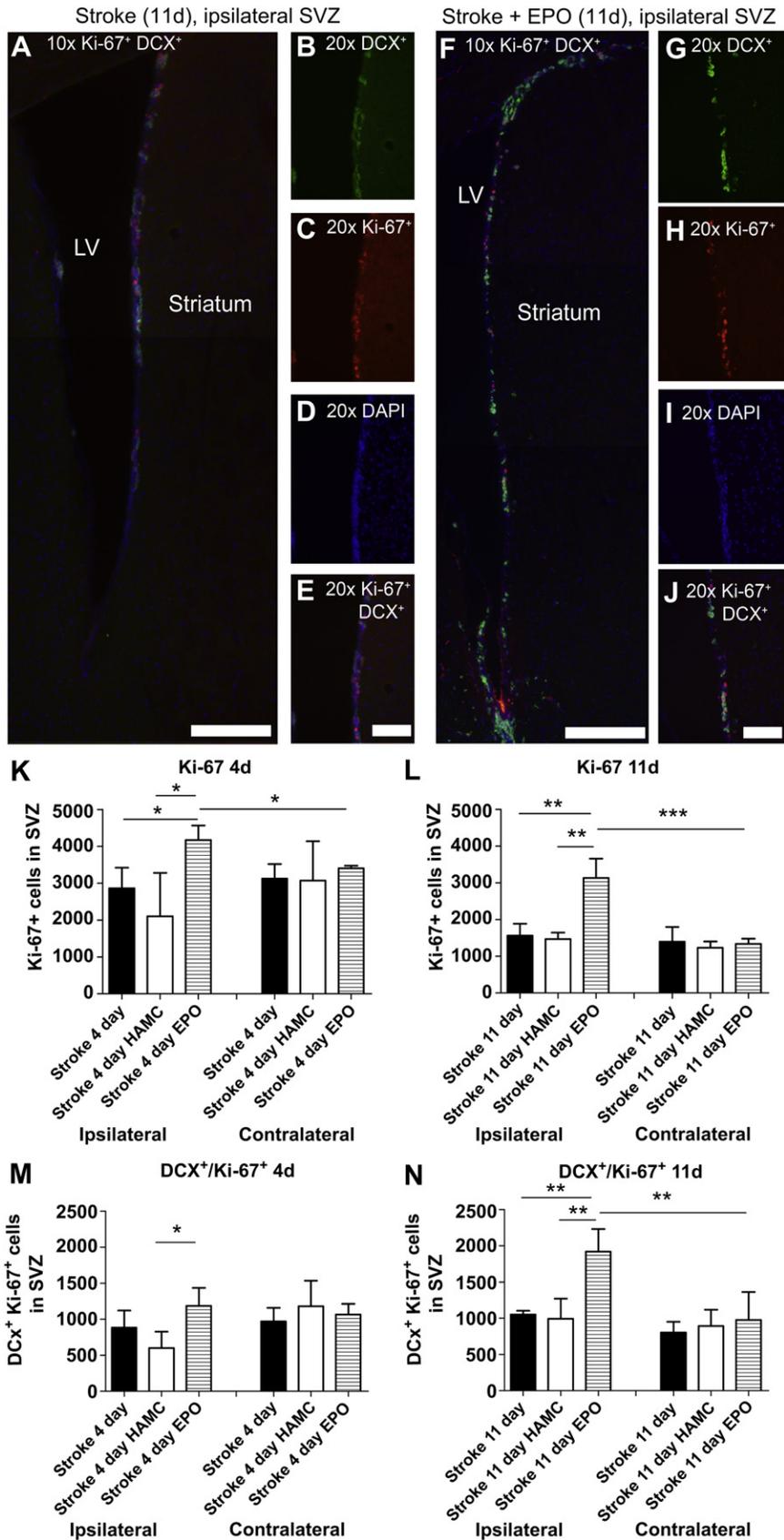
Fig. 6. Epi-cortical delivery of EPO in HAMC increases the number of Ki-67$^+$ proliferating cells and DCX$^+$ migratory neuroblasts in the SVZ at 4 and 11 days post-injury. Representative images are shown for Ki-67$^+$ cells and DCX$^+$ Ki-67$^+$ double-labeled cells in the SVZ ipsilateral to the stroke injury following (A–E) stroke injury and (F–J) EPO injected with HAMC 11 days after stroke. The lateral ventricle (LV) and the striatum are labeled. Scale: (A, F) 500 μm at 10x, (B − E, G − J) 100 μm at 20×. The number of Ki-67$^+$ cells in the ipsilateral SVZ at (K) 4 d and (L) 11 d is significantly increased by HAMC-mediated EPO treatment while the number of Ki-67$^+$ cells in the contralateral SVZ remain similar between treatments. DCX$^+$ Ki-67$^+$ double positive cells in the ipsilateral SVZ at (M) 4 d and (N) 11 d post-stroke also increased following HAMC-mediated EPO treatment (mean ± standard deviation, $n = 3$ animals, 16 tissue slices were analyzed per animal).
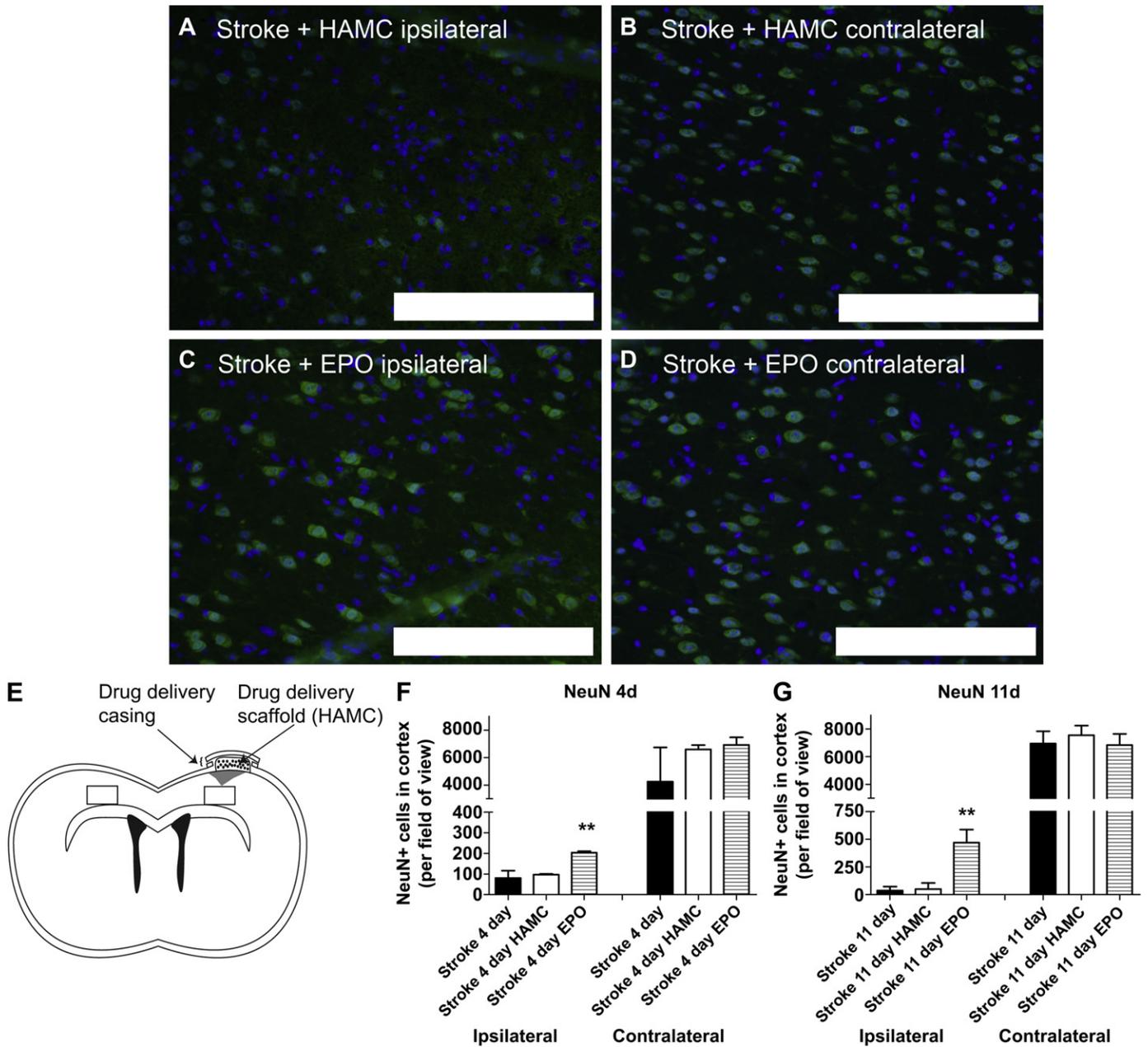
Fig. 7. EPO delivered from HAMC enhances the number of NeuN+ mature neurons in the injured cortex at 4 and 11 days post-injury. Representative images are shown for NeuN+ cells in the cortex of stroke-injured, sham treated animals (A) ipsilateral and (B) contralateral to the injury, as well as stroke-injured, EPO treated animals (C) ipsilateral and (D) contralateral to the injury at 4 days post-stroke. Scale: 100 μm. (E) Diagram of the brain with rectangles showing the regions analyzed. Quantification of NeuN+ cells are shown for (F) 4 d and (G) 11 d after stroke (mean ± standard deviation, $n = 3$ animals, 16 tissue slices were analyzed per animal).

brains in regions of the cortex and striatum through which it would have to diffuse to reach the SVZ, and which helps to explain the similar diffusion profiles of EPO in the brain before and after stroke injury. While EPOr has been previously reported to be upregulated in the penumbra of stroke-injured brains, the specific regions where EPOr overexpression was observed were not reported [7,37,38], which may explain our different results. Interestingly, since neurons and astrocytes in the brain both express EPOr [39], and the number of reactive astrocytes, are reduced in the presence of HAMC, EPOr expression may have been further attenuated. It is clear that the in vivo environment has a profound impact on protein transport in the brain, as the rate of protein elimination is directly influenced by the number of cell surface receptors and

binding moieties on the extracellular matrix (ECM) [40]. Since protein penetration is dictated by both the rates of diffusion and elimination, a good understanding of the in vivo environment allows protein penetration distance to be predicted and whether minimally invasive drug delivery from the cortical surface is feasible.

EPO delivered from HAMC shows significant benefits in the post-stroke brain. The effect of EPO on reducing the stroke cavity size agrees with results reported previously [8]. The numbers of SVZ neuroblasts as well as mature neurons following EPO delivery at 11 d are enhanced compared to those following EPO delivery at 4 d. This may be due to the fact that the environment in the brain 4 d post-stroke is more inflammatory than at 11 d post-stroke. We
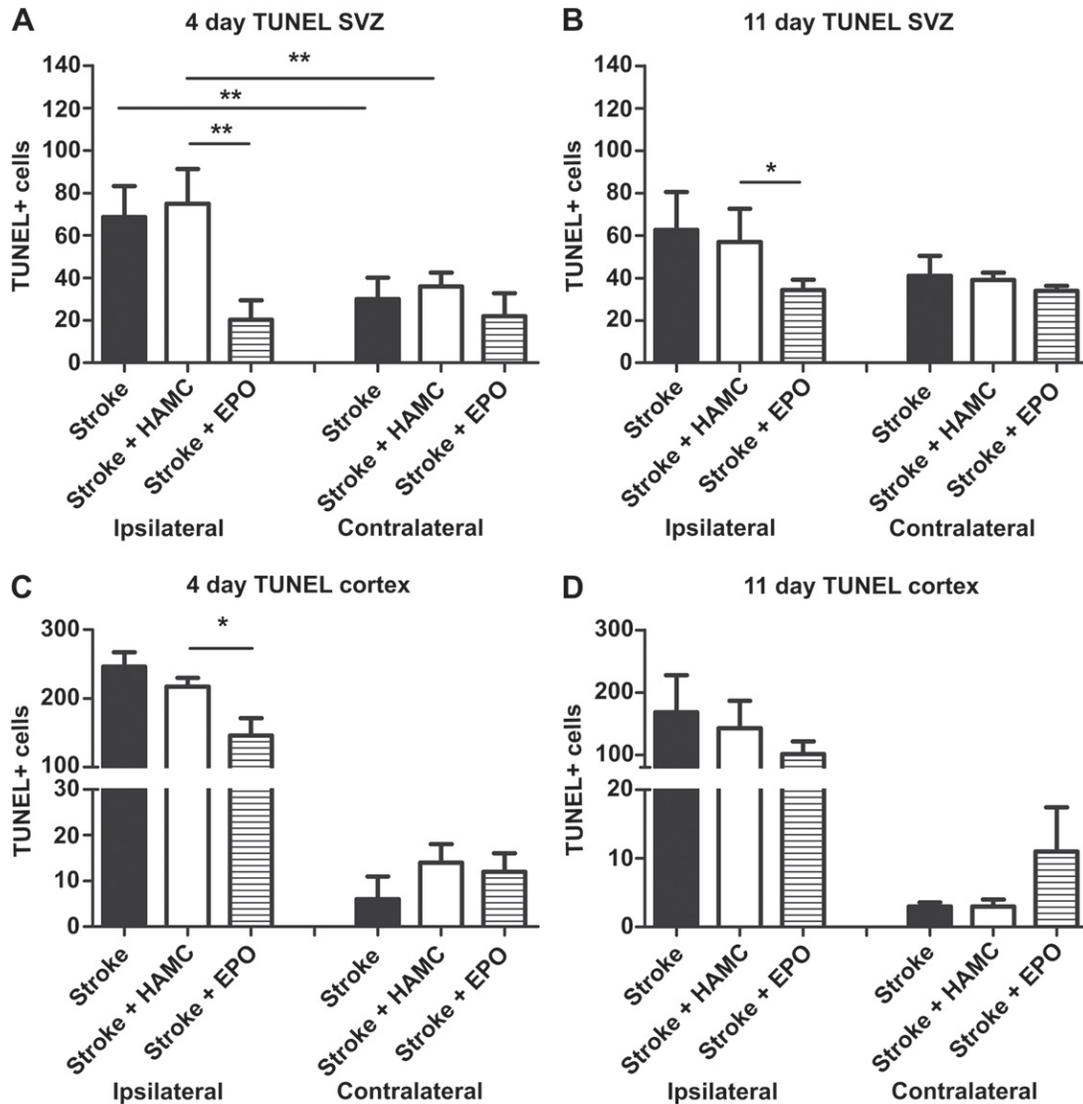
Fig. 8. Treatment with EPO delivery led to decreased number of TUNEL+ cells in the ipsilateral SVZ and cortex. When stroke-injured animals were treated with EPO, the number of TUNEL+ apoptotic cells decreased both in the (A, B) ipsilateral SVZ and the (C, D) ipsilateral cortex at 4 d or 11 d post-stroke. While significant decrease was observed at 4 d, only a non-significant decrease in TUNEL+ cells was observed at 11 d. HAMC alone had no effect relative to stroke alone. (mean ± standard deviation, $n = 3$ animals, 16 tissue slices were analyzed per animal).

showed that at 11 d, the CD68+ activated microglia/macrophages in the brain decreased compared to 4 d, which is consistent with others' findings that in the acute period after stroke, there is a higher degree of proteases, inflammatory cytokines, and scavenging inflammatory and immune cells in the brain [41,42]. Thus the beneficial effect of EPO may be masked by these deleterious factors at 4 d [43].

EPO has been shown to have both neuroprotective and neuroregenerative properties in the CNS [44]. EPO delivered 4 d post-stroke decreased apoptosis in the SVZ and the injured cortex, and maintained higher numbers of mature neurons in the injured cortex. Since 4 d after stroke is unlikely sufficient time for newly generated neuronal precursors to migrate to the stroke site and mature [45], the observed benefit is likely attributable to the neuroprotective role of EPO. EPO delivery 11 d post-stroke also led to qualitatively fewer apoptotic cells in the injured cortex, although the lack of statistically significant difference at 11 d suggests that acute EPO treatment post-injury is required to achieve neuroprotection. We also observed a neuroregenerative effect associated

with epi-cortical EPO delivery. EPO delivered at 4 d and 11 d post-stroke increased the number of both proliferating cells and migratory neuroblasts in the SVZ. The decrease in TUNEL+ cells in combination with the increase in proliferative cells in the SVZ following EPO delivery at both 4 d and 11 d also suggests that EPO mediates neuroregeneration by promoting the survival of NSPCs in the brain as has been also reported by others [46,47]. EPO delivery also increased the number of mature neurons 11 d post-stroke at the lesion site. This further suggests enhanced neuroregeneration following EPO treatment, and that delayed treatment provides a tissue benefit.

The benefits of epi-cortical EPO delivery observed here demonstrate that HAMC is a promising vehicle for enabling local delivery of therapeutics to the brain. The potential advantages of the system may be enhanced by increasing the length of delivery or by delivering a panel of factors. Many groups have shown that prolonged delivery of drugs to the brain, including EPO [48], significantly increases the benefits observed [15,49]. HAMC is a hydrogel that itself will not facilitate prolonged delivery;

however, tunable and controlled delivery of factors from HAMC may be achieved by incorporating polymeric particles that encapsulate drugs into the hydrogel [49]. Importantly, local epi-cortical delivery of drugs, such as EPO with HAMC, should result in a lower overall dose delivered and thus fewer side effects. For example, with intracerebroventricular delivery, cerebrospinal fluid is continually replenished resulting in the drug being distributed throughout the central nervous system. In fact, only 1–2% of drugs delivered to the ventricles can be found in the tissue 1 mm away from the ependyma [16] whereas in our epicortical HAMC delivery strategy, EPO was found 3 mm from the injection site. This illustrates that HAMC-mediated epi-cortical delivery is a true local delivery strategy, and promising for eliciting tissue benefit with reduced side effects relative to both systemic and intraventricular delivery. Co-delivering a panel of factors often facilitates synergistic benefits. EPO has been delivered with EGF [50], brain derived neurotrophic factor [10], ciliary neurotrophic factor [10], and insulin-like growth factor [51], among others, to elicit neuro-regeneration or neuroprotection after CNS injuries. HAMC has previously been used to deliver a multitude of proteins and small molecule drugs to the CNS to achieve tissue and functional benefits [17,19,49,52–54]. The findings here illustrate that HAMC is a versatile drug delivery platform to improve the treatment of CNS disorders of the brain.

5. Conclusions

HAMC is an injectable hydrogel that attenuates the inflammatory response in the brain and can be used to achieve controlled short-term delivery of EPO, which is a versatile glycoprotein that induces both neuroprotection and neuroregeneration in the CNS. EPO penetrated deep into the brain, reaching the cells in the subventricular zone and had neuroprotective and neuroregenerative effects after stroke. The benefits observed from epi-cortical EPO delivery from HAMC suggest that this hydrogel can be used as a platform technology to enable minimally invasive CNS drug delivery.

Author contributions

Y.W. – concept and design, performed surgeries, collection and assembly of data, data analysis and interpretation; M.J.C. – concept and design, performed surgeries, data analysis and interpretation; C.M.M – concept and design, data interpretation; M.S.S. – concept and design, data analysis and interpretation. All authors contributed towards preparation and final approval of manuscript.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgements

Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2011.12.031.

References

[1] Foulkes MA, Wolf PA, Price TR, Mohr JP, Hier DB. The stroke data-bank - design, methods, and baseline characteristics. Stroke 1988;19:547–54.
[2] Ridker PM, Hennekens CH, Stampfer MJ, Manson JE, Vaughan DE. Prospective-study of endogenous-tissue plasminogen-activator and risk of stroke. Lancet 1994;343:940–3.
[3] Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. Sub-ventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 1999;97:703–16.
[4] Ehrenreich H, Aust C, Krampe H, Jahn H, Jacob S, Herrmann M, et al. Eryth-ropoietin: novel approaches to neuroprotection in human brain disease. Metab Brain Dis 2004;19:195–206.
[5] Wang L, Zhang ZG, Wang Y, Zhang RL, Chopp M. Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. Stroke 2004;35:1732–7.
[6] Ehrenreich H, Falkai P. Recombinant human erythropoietin: approaches to neuroprotection and neuroregeneration in schizophrenia. Schizophrenia Bull 2009;35. 358–358.
[7] Tsai PT, Ohab JJ, Kertesz N, Groszer M, Matter C, Gao J, et al. A critical role of erythropoietin receptor in neurogenesis and post-stroke recovery. J Neurosci 2006;26:1269–74.
[8] Ehrenreich H, Hasselblatt M, Dembowski C, Cepek L, Lewczuk P, Stiefel M, et al. Erythropoietin therapy for acute stroke is both safe and beneficial. Mol Med 2002;8:495–505.
[9] Shingo T, Sorokan ST, Shimazaki T, Weiss S. Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian fore-brain neural stem cells. J Neurosci 2001;21:9733–43.
[10] McLoon LK, Alcala-Barraza SR, Lee MS, Hanson LR, McDonald AA, Frey WH. Intranasal delivery of neurotrophic factors BDNF, CNTF, EPO, and NT-4 to the CNS. J Drug Target 2010;18:179–90.
[11] Giannouli S, Voulgarelis M, Ziakas PD, Tzioufas AG. Anaemia in systemic lupus erythematosus: from pathophysiology to clinical assessment. Ann Rheum Dis 2006;65:144–8.
[12] Gomezfernandez P, Almaraz M, Ramos M, Perezmijares R, Castro A, Machuca JCV. Blood-pressure, erythropoietin and autonomic function. Nefrologia 1993;13:291–8.
[13] Meltzer EO, Orgel HA, Bush RK, Haltom JR, Metzger J, Moss BA, et al. Evaluation of symptom relief, nasal airflow, nasal cytology, and acceptability of two formulations of flunisolide nasal spray in patients with perennial allergic rhinitis. Ann Allergy; 1990:536–40.
[14] Genc S, Zadeoglulari Z, Oner MG, Genc K, Digicaylioglu M. Intranasal eryth-ropoietin therapy in nervous system disorders. Expert Opin Drug Del 2011;8: 19–32.
[15] Misra A, Ganesh S, Shahiwala A, Shah SP. Drug delivery to the central nervous system: a review. J Pharm Pharm Sci 2003;6:252–73.
[16] Pardridge WM. Strategies for drug delivery through the blood-brain-barrier. Neurobiol Aging 1989;10:636–7.
[17] Gupta D, Tator CH, Shoichet MS. Fast-gelling injectable blend of hyaluronan and methylcellulose for intrathecal, localized delivery to the injured spinal cord. Biomaterials 2006;27:2370–9.
[18] Cooke MJ, Wang Y, Morshead CM, Shoichet MS. Controlled epi-cortical delivery of epidermal growth factor for the stimulation of endogenous neural stem cell proliferation in stroke-injured brain. Biomaterials 2011;32: 5688–97.
[19] Kang CE, Poon PC, Tator CH, Shoichet MS. A new paradigm for local and sustained release of therapeutic molecules to the injured spinal cord for neuroprotection and tissue repair. Tissue Eng Pt A 2009;15: 595–604.
[20] Saltzman WM, Radomsky ML. Drugs released from polymers - diffusion and elimination in brain-tissue. Chem Eng Sci 1991;46:2429–44.
[21] Saltzman WM, Stroh M, Zipfel WR, Williams RM, Ma SC, Webb WW. Multi-photon microscopy guides neurotrophin modification with poly(ethylene glycol) to enhance interstitial diffusion. Nat Mater 2004;3:489–94.
[22] Thorne RG, Hrabetova S, Nicholson C. Diffusion of epidermal growth factor in rat brain extracellular space measured by integrative optical imaging. J Neurophysiol 2004;92:3471–81.
[23] Wang Y, Cooke MJ, Lapitsky Y, Wylie RG, Sachewsky N, Corbett D, et al. Transport of epidermal growth factor in the stroke-injured brain. J Control Release 2011;149:225–35.
[24] Bernaudin M, Marti HH, Roussel S, Divoux D, Nouvelot A, MacKenzie E, et al. A potential role for erythropoietin in focal permanent cerebral ischemia in mice. J Cerebr Blood F Met 1999;19:643–51.
[25] Siren AL, Knerlich F, Poser W, Gleiter CH, Bruck W, Ehrenreich H. Erythro-poietin and erythropoietin receptor in human ischemic/hypoxic brain. Acta Neuropathol 2001;101:271–6.
[26] Tennant KA, Jones TA. Sensorimotor behavioral effects of endothelin-1 induced small cortical infarcts in C57BL/6 mice. J Neurosci Meth 2009;181: 18–26.
[27] Juul SE, Anderson DK, Li Y, Christensen RD. Erythropoietin and erythropoietin receptor in the developing human central nervous system. Pediatr Res 1998; 43:40–9.
[28] Nalbant D, Saleh M, Goldman FD, Widness JA, Veng-Pedersen P. Evidence of receptor-mediated elimination of erythropoietin by analysis of erythropoietin

receptor mRNA expression in bone marrow and erythropoietin clearance during anemia. J Pharmacol Exp Ther 2010;333:528–32.

[29] Kuhn HG, Brown JP, Couillard-Despres S, Cooper-Kuhn CM, Winkler J, Aigner L. Transient expression of doublecortin during adult neurogenesis. J Comp Neurol 2003;467:1–10.

[30] Bartlett PF, Walker TL, Yasuda T, Adams DJ. The doublecortin-expressing population in the developing and adult brain contains multipotential precursors in addition to neuronal-lineage cells. J Neurosci 2007;27:3734–42.

[31] Cooper CA, Brown KK, Meletis CD, Zabriskie N. Inflammation and hyaluronic acid. Altern Complement Therapies 2008;14:78–84.

[32] Forrester JV, Wilkinson PC. Inhibition of leukocyte locomotion by hyaluronic-acid. J Cell Sci 1981;48:315–31.

[33] Yasui T, Akatsuka M, Tobetto K, Hayaishi M, Ando T. The effect of hyaluronan on interleukin-1-alpha-induced prostaglandin-E2 production in human osteoarthritic synovial cells. Agents Actions 1992;37:155–6.

[34] Hou SP, Xu QY, Tian WM, Cui FZ, Cai Q, Ma J, et al. The repair of brain lesion by implantation of hyaluronic acid hydrogels modified with laminin. J Neurosci Meth 2005;148:60–70.

[35] Li GH, Jin R, Yang GJ. Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. J Leukoc Biol 2010;87:779–89.

[36] Stylli SS, Kaye AH, Novak U. Induction of CD44 expression in stab wounds of the brain: long term persistence of CD44 expression. J Clin Neurosci 2000;7:137–40.

[37] Wei L, Li Y, Lu ZY, Keogh CL, Yu SP. Erythropoietin-induced neurovascular protection, angiogenesis, and cerebral blood flow restoration after focal ischemia in mice. J Cerebr Blood F Met 2007;27:1043–54.

[38] Banks WA, Jumbe NL, Farrell CL, Niehoff ML, Heatherington AC. Passage of erythropoietic agents across the blood-brain barrier: a comparison of human and murine erythropoietin and the analog darbepoetin alfa. Eur J Pharmacol 2004;505:93–101.

[39] Marti HH, Wenger RH, Rivas LA, Straumann U, Digicaylioglu M, Henn V, et al. Erythropoietin gene expression in human, monkey and murine brain. Eur J Neurosci 1996;8:666–76.

[40] Tang L, Persky AM, Hochhaus G, Meibohm B. Pharmacokinetic aspects of biotechnology products. J Pharm Sci-Us 2004;93:2184–204.

[41] Becker KJ. Modulation of the postischemic immune response to improve stroke outcome. Stroke 2010;41:S75–8.

[42] Zealley B, de Grey ADNJ. Timing the microenvironment: the effect of inflammatory cues on neural stem cell properties and cell-mediated potential for recovery and repair after spinal cord injury. Rejuv Res 2011;14:92–3.

[43] Fernandez C, Buyse M, German-Fattal M, Gimenez F. Influence of the pro-inflammatory cytokines on P-glycoprotein expression and functionality. J Pharm Pharm Sci 2004;7:359–71.

[44] Sargin D, Friedrichs H, El-Kordi A, Ehrenreich H. Erythropoietin as neuro-protective and neuroregenerative treatment strategy: comprehensive over-view of 12 years of preclinical and clinical research. Best Pract Res Cl 2010;24:573–94.

[45] Jacquet BV, Patel M, Iyengar M, Liang H, Therit B, Salinas-Mondragon R, et al. Analysis of neuronal proliferation, migration and differentiation in the post-natal brain using equine infectious anemia virus-based lentiviral vectors. Gene Ther 2009;16:1021–33.

[46] Wang Y, Yao M, Zhou C, Dong D, Jiang Y, Wei G, et al. Erythropoietin promotes spinal cord-derived neural progenitor cell proliferation by regulating cell cycle. Neuroscience 2010;167:750–7.

[47] Wang L, Zhang ZG, Zhang RL, Jiao ZX, Wang Y, Pourabdollah-Nejad S, et al. Neurogenin 1 mediates erythropoietin enhanced differentiation of adult neural progenitor cells. J Cerebr Blood F Met 2006;26:556–64.

[48] Rasmussen P, Foged EM, Krogh-Madsen R, Nielsen J, Nielsen TR, Olsen NV, et al. Effects of erythropoietin administration on cerebral metabolism and exercise capacity in men. J Appl Physiol 2010;109:476–83.

[49] Baumann MD, Kang CE, Stanwick JC, Wang Y, Kim H, Lapitsky Y, et al. An injectable drug delivery platform for sustained combination therapy. J Control Release 2009;138:205–13.

[50] Kolb B, Morshead C, Gonzalez C, Kim M, Gregg C, Shingo T, et al. Growth factor-stimulated generation of new cortical tissue and functional recovery after stroke damage to the motor cortex of rats. J Cerebr Blood F Met 2007;27:983–97.

[51] Digicaylioglu M, Fletcher L, Kohli S, Sprague SM, Scranton RA, Lipton SA, et al. Intranasal delivery of erythropoietin plus insulin-like growth factor-I for acute neuroprotection in stroke. J Neurosurg 2009;111:164–70.

[52] Kang CE, Tator CH, Shoichet MS. Poly(ethylene glycol) modification enhances penetration of fibroblast growth factor 2 to injured spinal cord tissue from an intrathecal delivery system. J Control Release 2010;144:25–31.

[53] Kang C, Baumann MD, Poon P, Tator CH, Shoichet MS. Intrathecal drug delivery strategy is safe and efficacious for localized delivery to the spinal cord. Prog Brain Res 2007;161:385–92.

[54] Wang Y, Lapitsky Y, Kang CE, Shoichet MS. Accelerated release of a sparingly soluble drug from an injectable hyaluronan-methylcellulose hydrogel. J Control Release 2009;140:218–23.