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Bioengineered sequential growth factor delivery stimulates brain tissue regeneration after stroke

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ABSTRACT

Stroke is a leading cause of disability with no effective regenerative treatment. One promising strategy for achieving tissue repair involves the stimulation of endogenous neural stem/progenitor cells through sequential delivery of epidermal growth factor (EGF) followed by erythropoietin (EPO). Yet currently available delivery strategies such as intracerebroventricular (ICV) infusion cause significant tissue damage. We designed a novel delivery system that circumvents the blood brain barrier and directly releases growth factors to the brain. Sequential release of the two growth factors is a key in eliciting tissue repair. To control release, we encapsulate pegylated EGF (EGF-PEG) in poly(lactic-co-glycolic acid) (PLGA) nanoparticles and EPO in biphasic microparticles comprised of a PLGA core and a poly(sebacic acid) coating. EGF-PEG and EPO polymeric particles are dispersed in a hyaluronan methylcellulose (HAMC) hydrogel which spatially confines the particles and attenuates the inflammatory response of brain tissue. Our composite-mediated, sequential delivery of EGF-PEG and EPO leads to tissue repair in a mouse stroke model and minimizes damage compared to ICV infusion.

1. Introduction

Stroke is a devastating neurological disorder and a leading cause of disability in the world [1]. Pathologically there are two stages in stroke, the primary and secondary injuries [2]. The primary injury refers to the initial insult and the affected area, the core region, is characterized by necrosis. The secondary injury follows the primary injury, and is caused by the depletion of oxygen and ATP in the tissue surrounding the core. This leads to apoptosis and forms an area of partial cell death, which is termed the penumbra [2]. The inflammatory response associated with stroke further devastates the injury [3] and impedes tissue repair [4].

Given limited treatment options [5], regenerative strategies such as stimulation of the endogenous neural stem/progenitor cells (NSPCs) in the subventricular zone (SVZ) [6] of the adult brain have been pursued. NSPCs proliferate at constitutive levels in the healthy adult brain and migrate to the olfactory bulbs. After injury, the level of NSPC proliferation in the SVZ increases, and some precursor cells migrate towards the injured cortex [7,8]. However, the level of proliferation and migration induced by stroke is insufficient to promote repair, as a large number of these newborn cells die before they can mature and integrate into the neuronal network [9]. The goal of regenerative strategies is to stimulate NSPC proliferation and migration to the injury site, thereby replenishing the tissue lost during stroke [10]. A number of growth factors, including erythropoietin (EPO) [11], vascular endothelial growth factor (VEGF) [12] and fibroblast growth factor-2 (FGF-2) [13] have been shown to stimulate neurogenesis. Moreover, treatments where two or more factors are delivered in a controlled manner have demonstrated enhanced efficacy compared to single-factor treatments [14].

Delivering growth factors to the brain is not trivial, as the blood brain barrier (BBB) isolates the brain from systemic circulation both physically and chemically [15]. Conventional systemic delivery strategies, such as intravenous infusion, are relatively simple and non-invasive, but many growth factors cannot efficiently penetrate the BBB [16], and thus high doses are required, which can result in systemic toxicity. Systemic delivery combined with localized disruption of the BBB, such as with ultrasound, provides an alternative strategy, but opens the BBB non-selectively and may permit harmful substances to enter the brain [17]. Local delivery strategies such as intracerebroventricular (ICV) infusion through the catheter/osmotic minipump system bypass the BBB, but require invasive surgeries [18]. Notwithstanding the invasiveness of this procedure, sequential infusion using a catheter/minipump system of EGF and EPO (but neither individually) led to tissue repair in a pial vessel disruption (PVD) rat model of stroke [19]. EGF stimulates the proliferation of NSPCs to expand the pool, while EPO is neuroprotective and reduces apoptosis of the newly generated cells. The temporally controlled infusion of EGF for the first 7 days followed by EPO for the subsequent

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7 days is a key to tissue repair, which was achieved with neither simultaneous EGF and EPO delivery nor each factor alone. Here we are interested in: (1) engineering a new, less invasive strategy for local delivery of each of EGF and EPO while maintaining temporal controlled release; and (2) testing this strategy relative to the conventional catheter/minipump system in a different animal model of stroke — the endothelin-1 mouse model of focal ischemic stroke.

Using an epicortical delivery strategy, our challenge was to achieve both controlled temporal growth factor release and sufficient penetration in brain tissue of the released factors for endogenous stem cell stimulation. EGF modified with poly(ethylene glycol) (EGF-PEG) [20,21] and EPO [22] were previously shown to penetrate through the ischemic cortex and reach the SVZ when delivered epicortically from a physical blend of hyaluronan and methylcellulose, HAMC [23]. However, protein delivery from a hydrogel scaffold is governed by Fickian diffusion and tends to occur rapidly. The sustained release over a minimum of 2 weeks, which is required for tissue repair, cannot be easily achieved from a hydrogel alone [24]. Here we developed a novel composite system that allows for sustained release of EGF-PEG followed by EPO. This was achieved by encapsulating each of EGF-PEG and EPO in polymeric particles and incorporating the particles into the HAMC hydrogel.

We confirm the validity of the endothelin-1 model of stroke in the mouse by showing that sequential infusion of EGF-PEG and EPO using the catheter/minipump system promotes tissue repair in this model, similar to that observed previously with the PVD model. Importantly, we demonstrate a new method for local and sustained drug release to the brain with temporal control, comprised of a composite of HAMC hydrogel and polymeric particles encapsulating EGF-PEG and EPO. We show that this delivery vehicle achieves sequential release of each of EGF-PEG and EPO *in vivo* and stimulates endogenous NSPCs in the adult mouse brain, resulting in tissue repair in the endothelin-1 model of stroke. This new delivery strategy promotes greater tissue repair than the traditional catheter/minipump system and avoids the consequent tissue damage observed with the latter.

2. Materials and methods

2.1. Materials

Recombinant human epidermal growth factor (EGF) and the EGF ELISA detection kit were purchased from PeproTech Inc. (Rocky Hill, NJ, USA). Recombinant human erythropoietin (EPREX) was supplied by Ortho Biotech Canada (Toronto, ON, Canada). Recombinant EPO ELISA kit was purchased from BD biosciences (Mississauga, ON, Canada). Methoxy-poly(ethylene glycol, 5 kDa) activated with propionaldehyde (mPEG-PPA) was purchased from NOF Corp. (Tokyo, Japan). Sodium hyaluronan (HA, 1.4–1.8 × 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_2HPO_3, NaH_2PO_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 [21]) 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).

2.2. Encapsulation of EGF-PEG in PLGA nanoparticles

EGF is modified with 5 kDa poly(ethylene glycol) (PEG) using a N-terminus specific mono-PEGylation chemistry as previously described [21]. A 16 mM EGF solution was prepared in sodium acetate (pH 5.5), and 3 M excess of methoxy-PEG-aldehyde and 140 M excess of $NaCNBH_3$ was added to the reaction mixture. The reaction was allowed to proceed at room temperature overnight. The product is dialyzed against TES buffer (pH 8.2) and purified using size-exclusion FPLC and lyophilized for storage.

EGF-PEG was encapsulated in poly(lactic-co-glycolic acid) (PLGA) nanoparticles using a double-emulsion process. 6.3 mg EGF-PEG was reconstituted in 100 μl PBS (pH 7.4) to form the inner aqueous phase. Either 60 mg or 120 mg of PLGA was dissolved in 900 μl dichloromethane (DCM) to form the organic phase. The two phases were sonicated for 10 min over ice. The primary emulsion was transferred to an outer aqueous phase of 2.5% PVA and 10% NaCl in ddH_2O, and sonicated for 10 min over ice. The final emulsion was stirred overnight in a 2.5% PVA, and either 0% or 10% NaCl hardening bath. The particles were centrifuge-washed five times and lyophilized for storage. Blank nanoparticles were similarly prepared by using 100 μl of 10× PBS (pH 7.4) without protein as the inner aqueous phase.

Particle size was measured on a Malvern zeta-sizer (Mastersizer 2000, Worcestershire, UK). To determine protein encapsulation, EGF-PEG loaded particles were dissolved in 1 ml DCM. The protein was extracted into 9 ml of aCSF and an EGF ELISA was used to determine protein encapsulation as per manufacturer's instructions. The encapsulation efficiency was calculated as (Eq. 1):

$$\text{Encapsulation efficiency } (EE\%) = \frac{\text{Measured protein concentration}}{\text{Initial protein concentration in encapsulation}} \times 100\% \quad (1)$$

PLGA nanoparticles loaded with EGF-PEG have an average diameter of 800 nm (polydispersity index = 0.622) and are formed by the double emulsion solvent evaporation method [25]. The particles have an EGF-PEG encapsulation efficiency of 54 ± 3.3% (Supplementary Fig. S1a).

2.3. Encapsulation of EPO in biphasic microparticles

EPO was encapsulated first in PLGA nanoparticles similar to EGF-PEG. To optimize encapsulation, various formulations were tested (Supplementary Fig. S1b). 500 U of EPO was encapsulated in test formulations. The optimal formulation with 3.5 wt.% BSA (compared to weight of PLGA used) and 400 mM trehalose coencapsulated with 500 U EPO (A) was defined as that which yielded the highest encapsulation with least amount of additives required. This formulation was used to encapsulate 40,000 U of EPO for all subsequent batches.

PLGA nanoparticles containing EPO were subsequently coated with poly(sebacic acid) (PSA) in a single emulsion process. 120 mg of EPO-loaded nanoparticles were dispersed in an organic phase containing 120 mg PSA in 900 μl THF. The primary emulsion was sonicated for 15 s and transferred to an outer aqueous phase of 1% PVA and 10% NaCl. The secondary emulsion was homogenized for 1 min and stirred for 4 h at room temperature in a 0.1% PVA, 10% NaCl hardening bath. The microparticles were centrifuge washed and lyophilized for storage. Blank biphasic particles were similarly prepared by using 100 μl of PBS (pH 7.4) with no proteins as the inner aqueous phase of the PLGA nanoparticles.

Particle size was determined using a Malvern mastersizer 2000: particle diameter was 137 ± 48 μm (mean ± standard deviation). Surface morphology was examined using SEM and surface chemical composition was determined using XPS (Supplementary Fig. S2). Particles were dissolved as described above and encapsulation efficiency was measured using an EPO ELISA kit as per manufacturer's instructions. EPO encapsulation efficiency was determined to be 23 ± 4.8%, and

the particles have an average diameter of 18 ± 4.7 μm (Supplementary Fig. S2).

2.4. Preparation of HAMC/particle composite drug delivery system (DDS)

EGF-PEG was encapsulated in PLGA nanoparticles, and EPO was encapsulated in PLGA nanoparticles which were embedded into poly(sebacic acid) (PSA) microparticles. HAMC was prepared as described previously [23]. HA and MC were used as received and dissolved in ddH_2O, sterile filtered and lyophilized under sterile conditions. A solution of 1% sterile HA and 2% sterile MC was prepared in sterile aCSF [21]. 120 mg EGF-PEG-loaded PLGA nanoparticles and 50 mg EPO-loaded PSA/PLGA biphasic microparticles were added to 1 ml of HAMC, mixed using SpeedMixer (DAC150FVZ, Landrum, SC, USA.), and kept at 4 °C overnight to remove air bubbles.

2.5. In vitro degradation of the composite DDS

100 μl of the blank composite DDS was injected into the bottom of a 2 ml microcentrifuge tube and allowed to gel at 37 °C. Terminal samples were prepared for the same time points as the *in vitro* release study. Tubes were weighed before and after composite injection. Each tube was then filled with 900 μl of warm aCSF and incubated at 37 °C. The aCSF was removed at each time point and the tube was weighed to obtain the wet composite mass. The composite degradation was calculated as (Eq. 2):

$$\%\ remaining\ composite\ mass = \frac{Wet\ mass\ at\ time\ t}{Initial\ wet\ mass} \times 100\%$$

2.6. In vitro release profiles of EGF-PEG and EPO from composite DDS

100 μl of composite was injected through a 26G needle into the bottom of 2 ml microcentrifuge tubes and allowed to gel at 37 °C for 10 min. 900 μl of aCSF equilibrated to 37 °C was added to each tube as the release medium. The buffer was completely removed at t = 0, 1, 2, 3, 4, 7, 10, 14, 21, 28, 35 days and replaced with fresh buffer. Samples were analyzed for protein concentration using EGF and EPO ELISA kits as per manufacturer's instructions. All samples were digested after the final time point to determine the amount of protein remaining in the composite.

2.7. Preparation of drug delivery device

The drug delivery casing was prepared as described previously [20]. The device consisted of two disks (0.5 mm height × 5.9 mm diameter) and was used to confine the composite at the site of injection. The bottom disk contained a 2 mm central opening, and both disks were molded to the shape of the mouse skull. Sterilization with ethylene oxide gas was carried out prior to use.

2.8. Surgeries

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. 9–11-week-old male C57BL/6 mice were used in this study (Charles River, QC, Canada). A total of 60 animals were used in these studies.

2.9. Stroke surgeries and implantation of drug delivery device

Stroke surgeries were carried out as described previously [26]. Mice were anesthetized with isoflurane, shaved and placed into a Kopf stereotaxic instrument. A midline incision in the scalp was made and a small burr hole was made in the skull at the coordinates 2.25 lateral to the midline and 0.6 anterior to the Bregma. 1 μl of endothelin-1 (400 pmol, Calbiochem, Gibbstown, NJ, USA.) was injected 1.0 mm ventral to the brain surface at 0.1 μl/min. The needle was left in place for 10 min prior to removal and the incision was sutured. Uninjured mice were similarly treated, but no injection was carried out.

The drug delivery system was injected 4 days post stroke to ensure that the majority of neuronal death has occurred, and any neurons found in the peri-infarct site at the completion of treatment are due to neurogenesis. The burr hole was exposed and any tissue debris was removed. The disk with 2 mm central opening was fixed over the burr hole with bone glue. 3 μl of the composite was injected into the hole such that it is in direct contact with the brain's cortical surface. A second disk with no opening was fixed over the first disk and the skin was sutured over the system (Fig. S3).

2.10. Implantation of catheter/osmotic minipump system

Stroke surgeries were carried out as described above. A cannula (Alzet Brain Infusion Kit 3, Durect Inc., Cupertino, CA, USA.) was stereotactically implanted at the coordinates −0.8 mm lateral to the midline and 0.2 mm anterior to the Bregma on the day of stroke, as adapted from Kolb et al. [19]. On day 4 after stroke, an osmotic minipump (Alzet model 1007D, 0.5 μl/h flow rate) containing 45 μg/ml EGF-PEG in sterile aCSF was inserted subcutaneously and attached to the cannula. On day 11 after stroke (i.e. 7 days later), the first pump was replaced with a pump containing 100 μl of 3000 U/ml EPO. The pump was removed on day 18.

2.11. Analysis of in vivo protein penetration

Protein penetration analysis was carried out as described previously [20]. Animals that received EGF-PEG and EPO treatment from the composite were sacrificed at 1, 4, 8, 11, 14, and 21 days post implantation and the drug delivery device containing the composite was removed and protein extracted into aCSF overnight at 4 °C. Animals that received EGF-PEG and EPO through catheter/osmotic minipumps were sacrificed at 7 and 14 days post implant and treated similarly.

Brains were snap frozen in $CO_{2(s)}$-cooled isopentane and three 1 mm coronal sections around the implant site was prepared using the McIlwain tissue chopper. Dorsal–ventral sections (0.5 mm each, 6 section totally 3 mm depth, with the deepest 3 sections corresponding to the depth of the SVZ in mice) were then prepared from each coronal slice using Leica CM3050S cryomicrotone. Each subsection was homogenized in 400 μl lysis buffer (40 mM trehalose, 1% Triton X-100), and the supernatant was removed after centrifuging at 15,000 RPM for 15 min at 4 °C.

The amount of protein remaining in the composite, as well as that in each brain section at each time point was determined using both an EGF-PEG and an EPO ELISA kit as per the manufacturers' instructions. The level of EGF-PEG and EPO in the brain of sham-treated animals were subtracted as background.

2.12. Immunohistochemistry

Brain tissue from sacrificed animals was prepared for immunohistochemical analysis as previously described [22]. Animals were sacrificed and transcardially perfused with saline followed by 4% paraformaldehyde (PFA). The brains were fixed in 4% PFA at 4 °C overnight, followed by cryoprotection in 30% sucrose. Brains were snap frozen and cryosectioned at 10 μm. Primary antibodies for Ki-67, GFAP, CD68, Mash-1, and NeuN were used at 1:400 dilutions. Secondary antibodies (Alexa 488 or Alexa 568) were used at 1:200 dilutions. Sections were mounted using Vectashield with DAPI and sealed. Immunohistochemical analysis for each marker was performed on 10 tissue sections, 100 μm apart. The peri-infarct tissue surrounding the cavity was

examined for NeuN, GFAP, and CD68, while the subependymal layer of the ipsilateral SVZ was examined for Ki-67 and Mash-1 (Supplementary Fig. S4).

2.13. Analysis of stroke cavity size

Stroke cavity size analysis was carried out as previously described [20]. Sections were stained with cresyl violet, covered and sealed. The area of the cavity on each section was obtained using ImageJ analysis software and the cavity volume obtained by summing the cavity size of each section, multiplying by the total thickness of the 10 sections examined.

2.14. Analysis of apoptosis using TUNEL

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

2.15. Statistics

All data are shown as mean ± standard deviation. One-way ANOVA with Bonferroni correction was used to compare between multiple groups. Significance levels are indicated as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

3. Results

3.1. Controlled release of EGF-PEG and EPO

To achieve sequential release of EGF-PEG followed by EPO, EGF-PEG is encapsulated in poly(lactic-co-glycolic acid) (PLGA) nanoparticles and EPO is encapsulated in PLGA nanoparticles that are coated with poly(sebacic acid) (Fig. 1a). The EGF-PEG and EPO particles are incorporated into a HAMC hydrogel comprised of 1% HA and 2% MC and the release profile is characterized both *in vitro* and *in vivo*. The composite retains at least 50% of its initial wet mass over 35 days (Supplementary Fig. S5a). EGF-PEG is completely released within the first week and EPO is released linearly between 4 days and 21 days *in vitro* (Fig. 1b). This linear profile demonstrates temporally-controlled sequential release of bioactive factors (Fig. 1, Supplementary Fig. S5b), similar to that obtained with the catheter/osmotic minipump system. Notably, the *in vivo* release profiles of EGF-PEG and EPO from the composite system (Fig. 1c, Supplementary Fig. S6) mirror those observed *in vitro*. EGF-PEG is observed in brain tissue only in the first two weeks, reaching a maximum at 7 days whereas EPO is not detected until after the first week and peaks at 21 days (Fig. 1d). This demonstrates that sequential release and accumulation of EGF-PEG followed by EPO is achieved. Importantly, by examining the accumulation of EGF-PEG and EPO at various depths in the brain tissue, we confirm that both proteins are able to penetrate through the injured cortex to the SVZ (Fig. 1e).

One key difference between epicortical delivery and ICV infusion is the amount of protein that accumulates in the tissue following delivery (Supplementary Fig. S6, S7). The maximum tissue accumulation of EGF-PEG (detected at days 7–8) and EPO (detected at day 14) are 134 ± 12-fold and 552 ± 48.6-fold higher, respectively, following epicortical delivery compared to catheter/minipump ICV infusion. Proteins delivered ICV are likely dispersed by the cerebrospinal fluid throughout the central nervous system (CNS), thereby accounting for the small amount of EGF-PEG and EPO detected in the brain.

Notwithstanding the differences in amount and region of protein accumulation achieved with epicortical composite vs. catheter/mini pump delivery systems, the latter is the best comparative group due to its widespread use and previous success in a rat PVD stroke model. The epicortical composite delivery represents a less invasive strategy than catheter/minipump for controlled temporal release of EGF-PEG and EPO enabling us to compare the tissue benefits in the endothelin-1 mouse model of stroke. We hypothesize that transiently higher concentrations of EGF-PEG and EPO in the brain will lead to enhanced NSPC stimulation and increased tissue benefit.

3.2. SVZ cellular response to growth factors delivered from epicortical composite vs. ICV pump at 18 days after stroke

The rationale for sequential delivery of EGF-PEG followed by EPO is based on previous studies demonstrating that EGF promotes NSPC proliferation [27] and expands the cell population, while EPO promotes neurogenesis from SVZ NSPCs [11]. We therefore analyze NSPC proliferation and neural progenitors in the SVZ ipsilateral to injury at 18 days after stroke to capture the cellular response following growth factor delivery.

Compared to vehicle controls, growth factor treatment using either pump or composite significantly increases the number of $Ki67^+$ proliferating cells (Fig. 2a–e) and $Mash\text{-}1^+$ neural precursors (Fig. 2f–j) in the SVZ. When aCSF is infused using catheter/minipumps following stroke, a reduction is observed for both $Ki67^+$ and $Mash\text{-}1^+$ compared to stroke alone, suggesting a negative tissue benefit with CSF flow alone in the ventricles (the rate = 0.5 μl/h). Stroke + growth factor (G/F) composite delivery also significantly reduces the number of $TUNEL^+$ apoptotic cells in the SVZ (Fig. 2k–o) relative to both Stroke + vehicle composite delivery ($p = 0.043$) and Stroke + G/F pump infusion ($p = 0.027$). These data suggest that the composite delivery system promotes tissue repair beyond that resulting from temporally controlled release of EGF-PEG and EPO. Thus the epicortical HAMC composite delivery of EGF-PEG and EPO shows greater tissue repair than that of ICV infusion, suggesting an additional tissue benefit of the composite delivery vehicle itself.

3.3. Tissue repair in the peri-infarct brain

To gain greater insight into the tissue response following growth factor treatment, we assess the number of $TUNEL^+$ apoptotic cells and $NeuN^+$ mature neurons in the peri-infarct region, as well as the size of cavity that forms after endothelin-1 stroke. These outcomes are assessed at 18 days and 32 days post stroke, two time points that were selected to both correspond to the completion of ICV growth factor delivery (18 days), and allow examination of tissue at longer time points (32 days).

At 18 days, treatment with growth factors decreases the extent of apoptosis (Fig. 3a–d) and increases the number of $NeuN^+$ mature neurons (Fig. 3e–h) in the peri-infarct region compared to stroke alone or Stroke + vehicle controls. Importantly, even though composite-mediated delivery of growth factors does not return the number of NeuN + cells to the pre-injury level, the number of cells is significantly higher compared to that achieved using catheter/minipump infusion. Additionally, all animals except the Stroke + aCSF pump group show a significant reduction in the cavity size relative to stroke alone (Fig. 3i).

The HAMC delivery vehicle is anti-inflammatory in the CNS [23]. As stroke causes inflammation in the peri-infarct area [28], which is detrimental to recovery [3], we examine the peri-infarct tissue at 18 days to assess whether the composite attenuates inflammation in the stroke injured brain. We observe that only those animals treated with HAMC – either HAMC alone or vehicle composite – have significantly lower inflammatory response in terms of the number of $GFAP^+$ reactive astrocytes (Fig. 4a–d) and $CD68^+$ activated microglia and macrophages (Fig. 4e–h). This confirms the anti-inflammatory effect of HAMC previously observed in other CNS injuries [23].

At the 32 days terminal time point, stroke injured animals that received composite-mediated treatments (both vehicle alone and G/F) show a reduced number of $GFAP^+$ reactive astrocytes (Fig. 5a) and

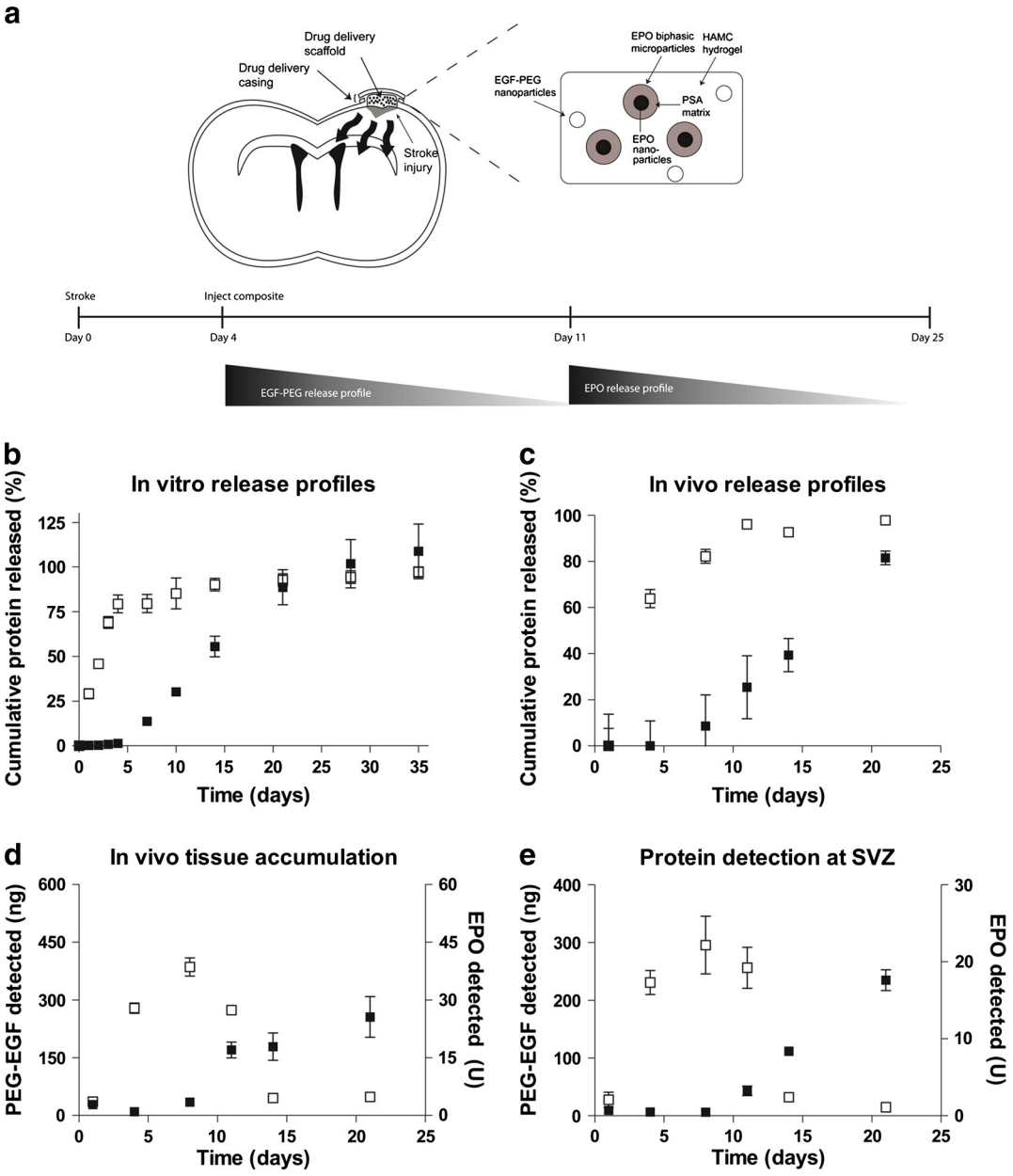

Fig. 1. Sequential delivery of EGF-PEG followed by EPO can be achieved using composite drug delivery system (DDS). (a) Schematic of the drug delivery system and *in vivo* EGF-PEG and EPO release from the composite on a microscopic level. Time course of the desired release profile is a sequential release of EGF-PEG for one week, followed by EPO. (b) *In vitro* release profiles of (□)EGF-PEG and (■) EPO engineered to yield the desired release profiles, with the majority of EGF-PEG released within the first week, followed by EPO released linearly for two weeks. (c) *In vivo* the release profiles of EGF-PEG and EPO from composite shows that EGF-PEG is released first followed by EPO release. (d) EGF-PEG can be observed in the stroke-injured brain tissue within days of injection, with the peak concentration detected at 1 week. EPO is not detected in the tissue in the first week, but accumulation is observed after the initial delay. (e) Endogenous stem cells reside in the SVZ. Accumulation of protein in the tissue corresponding to this region of the brain was examined and found to occur over the desired time course. All data are based on ELISA analysis (mean ± standard deviation, n = 3).

$CD68^+$ macrophages and microglia (Fig. 5b) compared to osmotic catheter/minipump-treated animals, again demonstrating the anti-inflammatory effect of HAMC in the CNS. Relative to controls, G/Fs delivered by either epicortical composite or ICV infusion also show reduced cavity volumes that are not significantly different from each other at 32 days (Fig. 5c). Apoptosis in the peri-infarct area continues to be evident at 32 days, with the greatest reduction in TUNEL + cells observed in the G/F composite delivery group (Fig. 5d). Additionally, the level of apoptosis in animals that received G/F from the epicortical composite is not significantly different from that of uninjured controls. This enhanced brain tissue repair observed after epicortical composite delivery may be attributed to either the greater concentration and longevity of EPO in the brain tissue of animals receiving G/Fs by epicortical composite and/or a combination of HAMC's anti-inflammatory effect and the reduced invasiveness of epicortical delivery compared to intraventricular infusion.

There are also significantly more NeuN+ mature neurons in the peri-infarct area of G/F composite treated animals compared to the stroke alone and vehicle composite controls at 32 days (Fig. 5e). While no significant difference in NeuN+ cells is observed between G/F delivery by either composite or catheter/minipumps, no significant difference is observed in G/F treated and uninjured groups, suggesting tissue repair to pre-injury levels and a neuroregenerative effect of G/F treatment. Since we wait 4 days after stroke injury prior to G/F delivery, and most effective neuro-protective treatments need to be delivered within 1–4 days post injury [29], the tissue repair observed is likely a

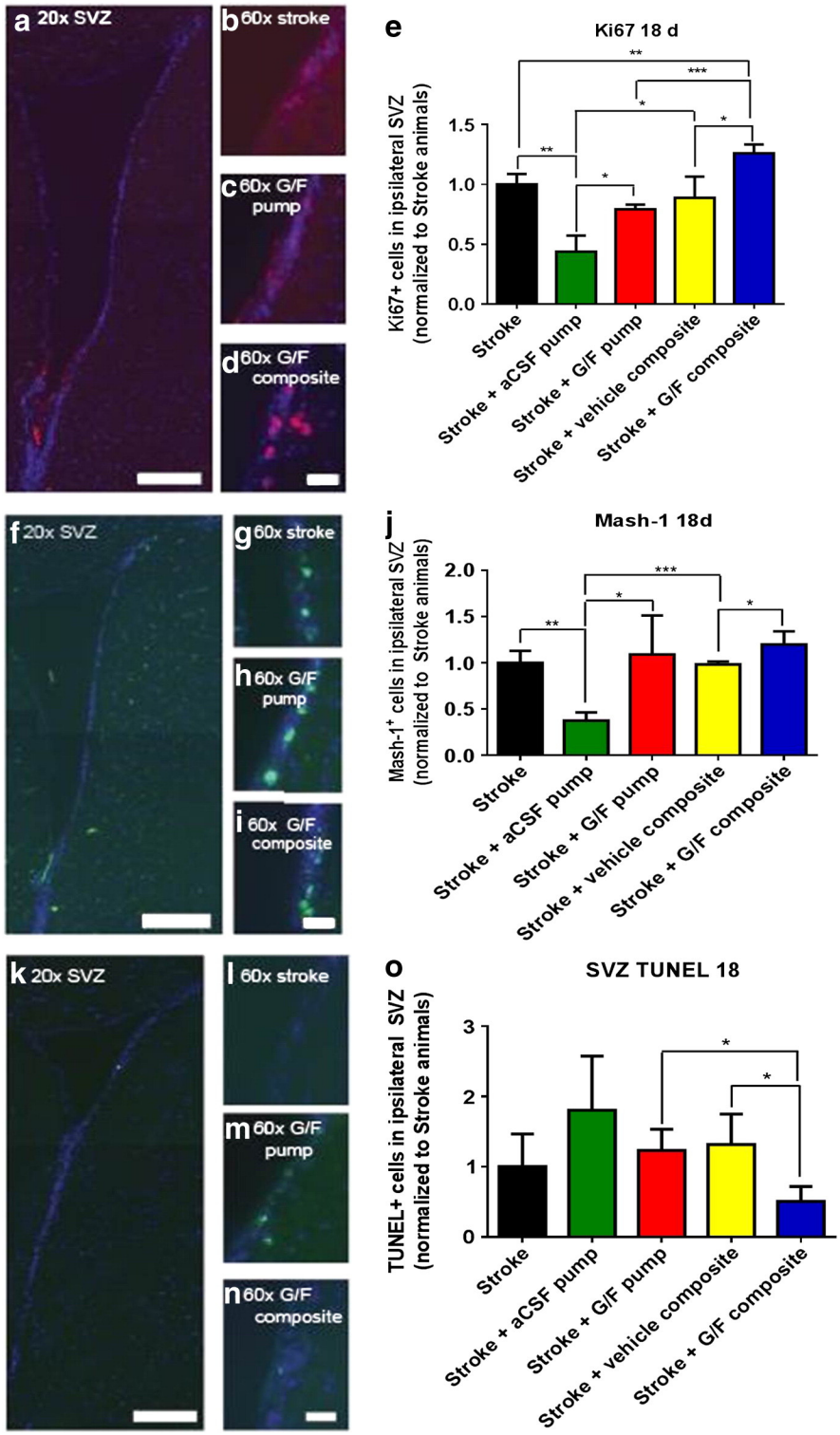

Fig. 2. EGF-PEG and EPO delivered from composite increase proliferation of NSPCs and decrease cell death in the SVZ 18 days after stroke. (a–d) Representative images and (e) quantification of Ki67$^+$ proliferating cells in the ipsilateral SVZ at 18 days. Compared to vehicle controls, growth factor treatment using either pump or composite significantly increase the number of Ki67$^+$ proliferating cells in the ipsilateral SVZ. Stroke + G/F composite delivery animals showed significantly more Ki67+ cells than Stroke + G/F pump delivery animals ($p = 0.001$), and only G/F composite delivery showed significantly more Ki67+ cells than stroke alone ($p = 0.008$). When aCSF is infused through osmotic minipumps to the ventricles, the level of proliferation decreases significantly compared to stroke alone controls ($p = 0.004$). (f–i) Representative image and (j) quantification of Mash-1$^+$ neuroprecursors in the ipsilateral SVZ on day 18. Stroke + G/F pump infusion increases the number of Mash-1+ neural precursor cells in the SVZ compared to Stroke + aCSF pump infusion controls ($p = 0.045$); and Stroke + G/F composite increases the number of Mash-1$^+$ cells compared to Stroke + vehicle composite ($p = 0.033$). Stroke + aCSF pump intraventricular infusion resulted in a significant reduction in Mash-1$^+$ cells compared to Stroke + vehicle composite ($p = 0.001$) and stroke alone ($p = 0.002$). No significant differences in Mash-1$^+$ cells were found between stroke alone and Stroke + vehicle composite or between stroke alone and Stroke + G/F composite. (k–n) Representative image and (o) quantification of TUNEL$^+$ apoptotic cells in the ipsilateral SVZ on day 18. At 18 days after stroke, the number of apoptotic cells in the SVZ was characterized by TUNEL + cell staining following stroke vs. the other interventions. Stroke + G/F composite delivery significantly reduced the number of apoptotic TUNEL + cells relative to both Stroke + vehicle composite delivery ($p = 0.043$) and Stroke + GF pump infusion ($p = 0.027$). (Mean ± standard deviation, n = 3. Results are normalized to Stroke animals. Scale: a, f, k: 100 μm; b–d, g–i, l–n: 50 μm.).

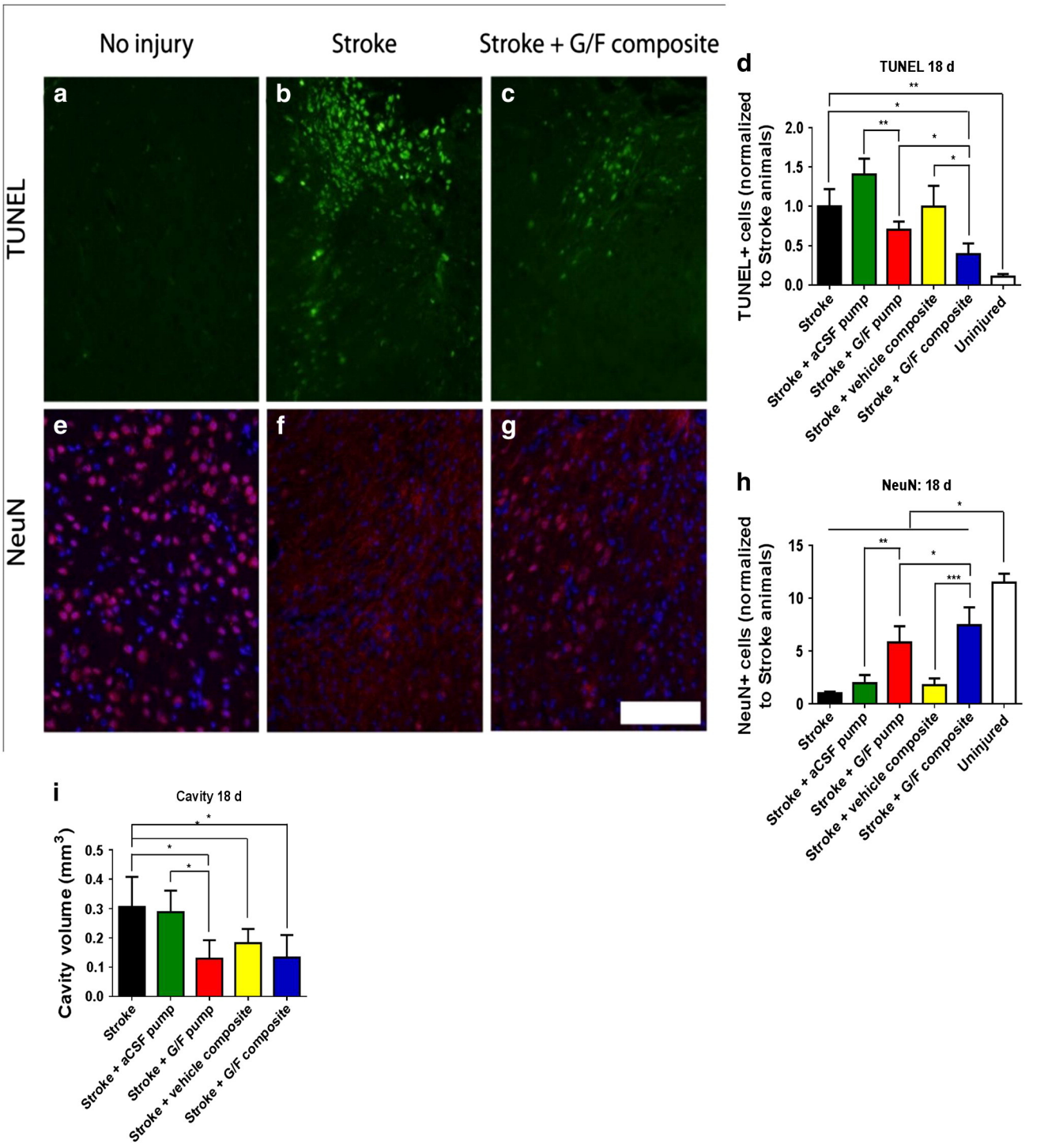

Fig. 3. Sequential composite-mediated delivery EGF-PEG and EPO attenuates the injury response and cell death, and increases NeuN$^+$ mature neurons in the penumbra 18 days after stroke. At 18 days post stroke, (a–d) the level of TUNEL$^+$ cells decreases significantly in the injured cortex following growth factor treatment compared to stroke + vehicle controls. Importantly, stroke + G/F composite leads to a significant decrease in TUNEL$^+$ cells compared to stroke alone ($p = 0.015$) and stroke + G/F pump ($p = 0.038$). (e–h) Both stroke + G/F composite and stroke + G/F pump animals have significantly more NeuN$^+$ cells in the peri-infarct region compared to vehicle controls ($p = 0.001$). The level of NeuN + neurons is significantly higher after stroke + G/F composite vs. stroke + G/F pump ($p = 0.044$), yet still significantly less than uninjured tissue controls. (i) All animals showed a significant reduction in cavity size relative to stroke alone except for the stroke + aCSF pump group. (All results normalized to Stroke animals, mean + standard deviation, n = 5). Scale: 100 μm.

neuroregenerative (vs. neuro-protective) effect. The increased number of NeuN$^+$ cells and reduced cavity size is correlated with increased NSPC proliferation in the SVZ at 18 days.

In addition to the tissue regeneration observed, the composite is capable of delivering growth factors without causing the tissue damage associated with cannula insertion through the brain parenchyma into the contralateral ventricles. TUNEL + apoptotic cells in the contralateral SVZ and cortex significantly exceed that in the uninjured, stroke alone, or Stroke + composite groups (Supplementary Fig. S8a, b), demonstrating the significant tissue damage associated with cannula insertion. At both 18 and 32 days after stroke, the injury response as measured by GFAP and CD68 upregulation is also magnified by cannula insertion (Supplementary Fig. S8c–f) relative to epicortical composite delivery or stroke alone.

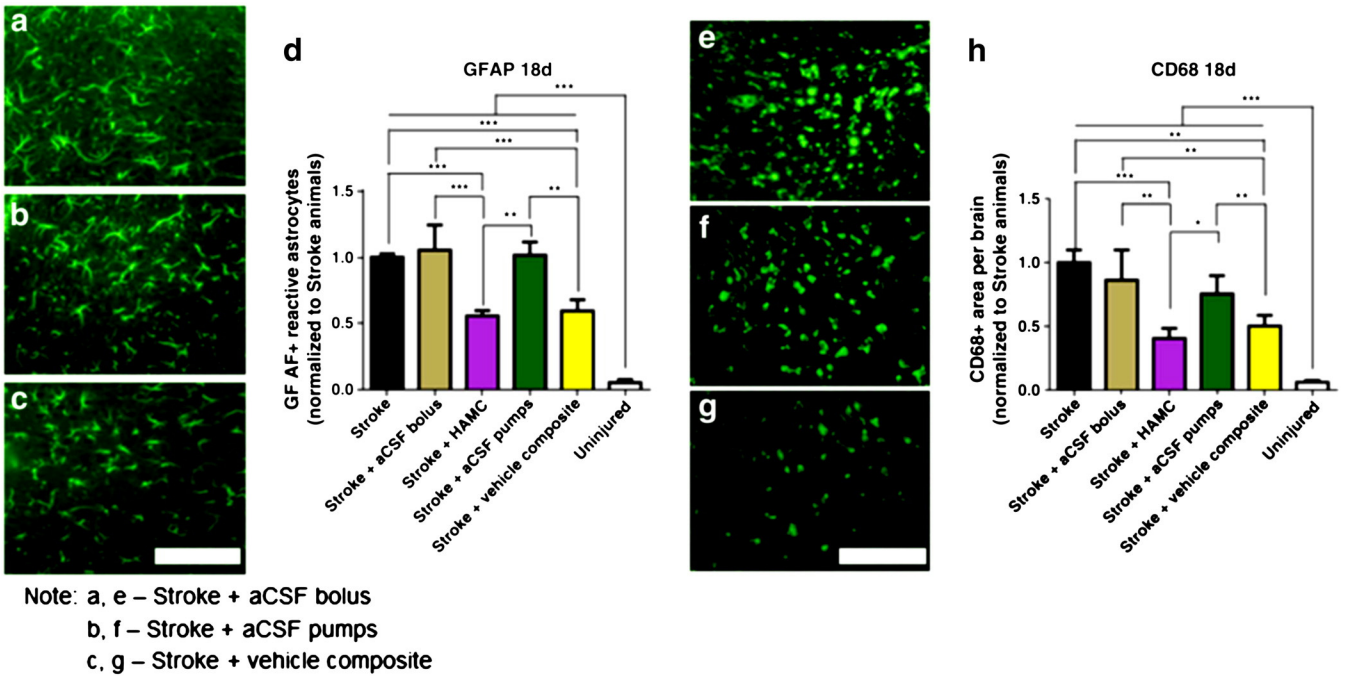

Fig. 4. Epicortical delivery of the composite vehicle attenuates inflammatory response in the peri-infarct region 18 days after stroke. Given that HAMC demonstrates anti-inflammatory effects in the CNS, we examined the peri-infarct cortical tissue for inflammation. Only those animals that were treated with HAMC – either HAMC alone or vehicle composite comprised of HAMC and polymeric particles – showed significantly reduced (a–d) GFAP$^+$ reactive astrocytes and (e–h) CD68$^+$ macrophages/microglia around the lesion. For stroke + aCSF bolus, stroke-injured animals had aCSF injected in an identical manner to HAMC, thereby providing a control for epicortical delivery of HAMC. (All results normalized to Stroke animals, mean + standard deviation, n = 3, *p < 0.05; **p < 0.01; ***p < 0.001). Scale: 100 μm.

3.4. Cellular response in the SVZ at 32 days after stroke

The regeneration in the peri-infarct region at 32 days after stroke is likely correlated to the increased NSPC proliferation observed in the SVZ at 18 days after stroke. We further examined the SVZ at 32 days after stroke to assess if epicortical delivered EGF-PEG and EPO is likely to elicit tissue benefits in the peri-infarct area beyond the treatment period.

Stroke-injured animals treated with the epicortical G/F composite show significantly greater numbers of Ki67+ cells in the SVZ relative to all other groups studied, including animals that received G/F treatment through ICV infusion (Supplementary Fig. S9a). The number of Mash-1$^+$ neural precursors is significantly greater in G/F delivery animals relative to their appropriate controls yet not different from each other and not from stroke alone (Supplementary Fig. S9b). The extent of apoptosis in the SVZ is lowest in stroke-injured animals that receive EGF-PEG and EPO from the composite (Supplementary Fig. S9c). Use of catheter/minipumps leads to increased apoptosis in the SVZ, although growth factor delivery attenuates the number of TUNEL + cells relative to aCSF delivery alone. The reduction in apoptotic cells observed in the composite vs. pump delivery strategies may reflect the greater concentration and duration of the neuroprotective EPO in those animals having composite vs. pump delivery. Alternatively, the reduced apoptosis in the stroke + G/F composite and stroke + vehicle composite vs. the relevant pump infusion animals may reflect the greater invasiveness of the pump strategy relative to the epicortical HAMC strategy.

4. Discussion

Sequential delivery of EGF and EPO was originally shown to promote tissue repair after stroke injury in the rat PVD model [19]. From those studies, it was evident that repair resulted from tissue regeneration rather than neuroprotection, as EGF and EPO were not delivered until 4 and 11 days after stroke, respectively. It was also clear that the sequential delivery paradigm of EGF for 7 days followed by EPO for 7 days was a key to the regeneration because simultaneous delivery did not show the same success. We mimic this ICV catheter/minipump delivery strategy in the endothelin-1 mouse model of stroke, and compare it to our new epicortical injectable hydrogel composite system. Neural tissue repair is observed in both delivery strategies; however, overall tissue repair is enhanced by cortical delivery and the tissue damage observed with cannula insertion is obviated by using the epicortical strategy.

Compared to either single protein delivery or simultaneous delivery of multiple proteins, the benefits of sequential delivery of multiple proteins have been observed in other systems [30]. For example, sequential delivery of basic fibroblast growth factor (bFGF) and neurotrophin-3 (NT-3) improved neuronal survival after CNS injury compared to either factor alone [31]. When EPO alone is delivered epicortically [32], the number of mature neurons in the injured cortex is less than half of that achieved here by the sequential delivery of EGF-PEG and EPO from the composite.

In this study, we engineer the sequential release of EGF-PEG followed by EPO by encapsulating EGF-PEG in PLGA particles and EPO in PLGA-PSA biphasic microparticles, both of which are dispersed in HAMC. While the hydrogel alone is insufficient to achieve prolonged sustained release [33], the inclusion of polymeric particles, which encapsulate growth factors and are dispersed in the hydrogel, results in sustained release that is localized to the site of injection by the hydrogel [34]. The temporal profile of drug released from the composite mimics that achieved using conventional osmotic minipump systems. To the best of our knowledge, this is the first time that a polymer composite has been used to achieve sequential delivery of growth factors to the brain without multiple surgeries. Other strategies have been used to deliver two proteins at varying rates, but the release profiles are not truly sequential. Typically, one protein is released significantly slower than the other, but with no initial delay period [25].

Furthermore, the epicortical delivery strategy overcomes some of the challenges associated with drug delivery to the brain. By delivering growth factors directly to the brain tissue, this strategy circumvents the blood–brain barrier while localizing release. Unlike the

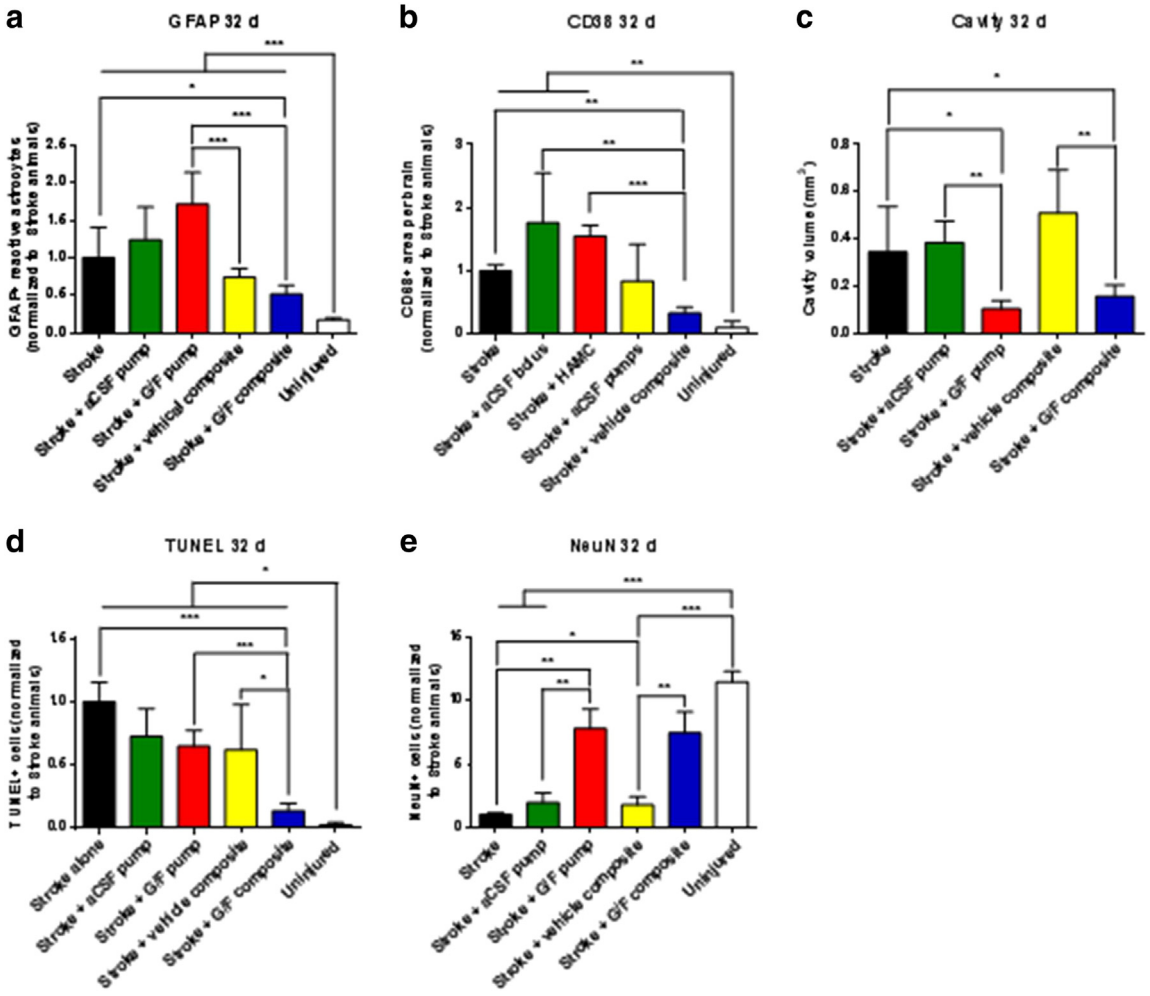

Fig. 5. Epicortical delivery of composite vehicle attenuates peri-infarct inflammatory response at 32 days post stroke, and sequential composite-mediated delivery of EGF-PEG and EPO attenuates the injury response and cell death, and increases NeuN$^+$ mature neurons at the same time point. Injection of vehicle composite post stroke reduces the number of (a) GFAP$^+$ reactive astrocytes and (b) CD68$^+$ activated microglia at 32 days compared to stroke alone and stroke with osmotic minipump treatments. This is similar to the effects seen at 18 days post stroke. (c) Growth factor treatment significantly reduces the stroke cavity size compared to vehicle controls. Quantification of (d) TUNEL$^+$ and (e) NeuN$^+$ stains at 32 days indicates G/F composite attenuates apoptosis and increases the number of mature neurons in the peri-infarct region compared to vehicle control. No significant difference was observed for NeuN + cells at 32 days between stroke + G/F composite and uninjured tissue, suggesting tissue repair to pre-injury levels (All results normalized to Stroke animals, mean + standard deviation, n = 5, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

ICV catheter/minipump, which causes significant tissue damage [35], the epicortical composite provides a minimal invasiveness and no tissue damage. Thus the epicortical composite that comprised polymeric particles dispersed in HAMC provides a versatile strategy for local, sustained release to the brain while obviating the need for invasive or systemic delivery strategies.

The epicortical composite outperforms the ICV catheter/minipump in terms of tissue benefit. Relative to ICV catheter/minipump infusion, epicortical composite-treated animals show greater tissue repair, with reduced apoptosis and increased proliferation in the ipsilateral SVZ. Given that growth factors were delivered to the SVZ in both systems, this result is unexpected but may be explained by greater tissue concentrations of EGF-PEG and EPO when delivered from the cortical surface vs. into the ventricle. When delivered through ICV infusion, the majority of the proteins are dispersed by the cerebrospinal fluid throughout the central nervous system [36,37], resulting in low tissue accumulation. It has been shown that a population of quiescent cortical neural precursor cells is activated by brain injury [38]. The composite allows higher quantities of EGF-PEG to accumulate in the cortex compared to the osmotic minipumps, and since EGF stimulates NSPC proliferation, it is possible that these cortical precursors, in addition to the NSPCs from the SVZ, may be involved in tissue repair. EPO is neuroprotective, and higher concentrations of neuroprotective factors in the brain following delivery from the composite may decrease cortical apoptosis and enhance the effect of regeneration.

We observe additional evidence of tissue repair in terms of an attenuated inflammatory response, with epicortical delivered HAMC at both 18 and 32 dayss after stroke, thereby minimizing detrimental effects of inflammation to adult brain neurogenesis [4]. Hyaluronan promotes wound healing, and is anti-inflammatory in the CNS [39], suggesting that the HA of HAMC is responsible for the reduced inflammatory response. The difference in inflammatory response between epicortical composite and intraventricular catheter/pump is heightened by the significant damage and inflammation in the tissue surrounding the ventricle resulting from cannula insertion. This is a common problem associated with metallic implants in the brain parenchyma [40,41], and can be avoided by using an alternate delivery strategy, such as the epicortical composite.

One challenge in epicortical delivery is the short penetration distance of proteins in the brain, which limits the volume of tissue that can be targeted. Typical protein penetration in the uninjured brain tissue is 1 mm [42]. Since the SVZ is approximately 3 mm ventral to the cortical surface in a mouse brain [43], the protein must be engineered to achieve adequate penetration. PEG-modification increases the penetration of EGF in the ischemic brain [20] while EPO requires no modification to penetrate to the SVZ [32]. This difference in EGF and EPO tissue

penetration can be largely ascribed to differences in their relevant receptors in the stroke injured brain where EGFr is upregulated [21] and EPOr is not [32]. Here we demonstrate that by using the composite system, sequential accumulation of EGF-PEG and EPO at the approximate depth of the SVZ can be achieved over a 2–3-week period. Translation from the mouse to larger animal models and ultimately to humans will require additional innovations in order to achieve the penetration distances required. For example, the mouse SVZ resides 2–3 mm ventral to the cortical surface [43] whereas the human SVZ is situated at a depth between 5 and 10 cm ventral to the cortical surface [44]. The differences in brain size, cell composition, and cell density will impact protein transport in the brain, which is governed by both diffusivity and the rate of protein removal [42]. These parameters can be used to our advantage: modifying proteins with a polymer such as PEG can significantly increase tissue penetration [45,46]. For example, conjugating a single PEG molecule to EGF can increase penetration in a stroke-injured brain by up to 27-fold [19]. In addition, a constant growth factor source, such as that provided by the composite system, results in a further 2–3 fold increase in penetration distance [21]. This distance can be further enhanced by varying the molecular weight and structure of PEG, by using different conjugation polymers [47], or reducing protein elimination through protein caging [48] and point mutations [49].

5. Conclusions

The epicortical delivery system, that comprised HAMC hydrogel and polymeric particles, provides temporally-controlled release of therapeutically relevant factors to achieve neural tissue repair without the concomitant tissue damage of catheter/minipump system. While clinical translation of this system will be non-trivial, the polymer composite is less invasive than other local delivery strategies to the brain. The application of this composite DDS is not limited to the stimulation of endogenous NSPCs for the treatment of stroke. A number of CNS disorders, including Alzheimer's disease [50] and spinal cord lesions [51] result from loss of healthy neurons and glia. The epicortical local, minimally-invasive delivery strategy may have therapeutic use in these disorders as well.

Author contributions

Y.W. and M.J.C. performed all the experiments. All authors contributed to the experimental design, data analyses and interpretations, and the preparation of the manuscript.

Competing financial statement

The authors declare no competing financial interests, yet acknowledge that a patent has been submitted based on the epicortical delivery strategy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2013.07.032.

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