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Differential Activation of Microglia in an in vitro Model of Intracerebral Hemorrhage

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Abstract

An *in vitro* model of intracerebral hemorrhage was established to examine the protective versus cytotoxic roles of microglia in the context of mild versus severe injury. Co-cultures of microglia, astrocytes, and granule neurons were prepared from the cerebellar cortex of neonatal rats, and grown in standard medium containing fetal bovine serum or, in some cases, a serum-free chemically defined medium. To mimic hemorrhagic stroke, co-cultures grown for 7-8 days *in vitro* were challenged with two different concentrations of the toxic blood product hemin, corresponding to a mild versus a severe brain bleed. Immunocyto-chemical, real-time RT-PCR, iron deposition, and cell survival assays were performed to assess the effects of hemin, with emphasis on the functional effector states of microglia. In cultures grown in serum-containing medium, the lower concentration of hemin (20 μM) induced significant expression of the protective enzyme, heme oxygenase 1 (HO-1). Consistent with this, iron deposition was localized to the microglia in the cultures. Only a modest induction of the inflammatory molecule TNF-α, and no significant cell death was observed 24 hrs after hemin addition. In contrast, the higher concentration of hemin (100 μM) induced greater expression of HO-1, and iron deposition was detected in all three cell types in the cultures. Moreover, significant and robust induction of the inflammatory molecules TNF-α, COX-2, iNOS, and significant neuronal death were observed. These results suggest that microglia are protective and serve to effectively catabolize hemin when exposed to 20 μM hemin. However, increasing the hemin concentration to 100 μM appears to exceed the capacity of the microglia to effectively catabolize the hemin load, resulting in an inflammatory and cytotoxic environment. Serum proteins, in particular albumin and hemopexin, can bind blood products such as hemin, and slowly release them to be processed by microglia after a brain bleed. To test the possible protective effect of albumin, co-cultures grown in a chemically defined medium lacking serum were exposed to hemin as described above, with or without co-addition of an equivalent amount of albumin. In these cultures, significant neuronal death was observed in response to 20 μM hemin, with essentially total cell death in response to 100 μM hemin. Addition of albumin to the cultures significantly decreased the amount of neuronal death in response to both concentrations of hemin. These data suggest that addition of agents that bind toxic blood products may serve as protective agents after hemorrhagic stroke. In future studies, this *in vitro* model should prove valuable in characterizing the signaling pathways underlying distinct functional effector states of microglia, and developing strategies to preserve the protective phenotype.

Executive Summary

Strokes occur when the brain does not get enough blood, leading to neuronal death. There are two primary types of strokes, called ischemic and hemorrhagic. A hemorrhagic stroke occurs after a blood vessel bursts, releasing blood contents directly into the brain tissue. Although hemorrhagic strokes are less common than ischemic strokes, they cause greater impairment and more deaths. The scarcity of treatments available for patients who suffer brain bleeding shows the importance of research that focuses on factors underlying brain damage, and what can be done to prevent it. My project uses a cell culture model to mimic hemorrhagic stroke. We take tissue from the brains of rats and experimentally grow neurons, astrocytes and microglia to study the roles of microglia in response to injury that mimics brain bleeding. Microglia can be protective by internalizing the toxic blood products before they have a chance to do damage, but microglia can also be overwhelmed by an excessive amount of blood and become toxic, increasing the amount of damage. Currently, therapies aimed at inhibiting the functions of microglia are being studied to treat strokes and other brain injuries. However, we believe that it is important to recognize the differences between injuries that preserve the protective functions of microglia versus injuries that result in conversion of microglia to states that make injuries worse, called cytotoxic. One would not want to use a microglia inhibitor under injury conditions where microglia are serving a protective role.

To better understand the different states that microglia assume in response to a brain bleed, we used low and high concentrations of the potentially toxic

blood product hemin. We reasoned that the low concentration of hemin would be effectively cleared by the microglia without causing cell damage, whereas the higher concentration of hemin would lead to microglial overload, and generate production of toxic molecules causing death of neurons in the cultures. When hemin is introduced to the environment, microglia and possibly other cells produce the protective enzyme, called heme oxygenase-1 (HO-1), which breaks down the hemin into carbon monoxide, biliverdin, and free iron. The amount of HO-1 mRNA generated was quantified using RT-PCR, which is a technique that measures RNA expression. Our results showed that more HO-1 was induced as the concentration and duration of exposure to hemin was increased. Through staining with hemin-specific antibodies, it was also determined that the HO-1 generation was primarily localized in the microglia. Additionally, we expected there to be iron deposited in the microglia since iron is a product released when hemin is broken down. We performed an iron stain and found that, when the cells are challenged with a low concentration of hemin, the iron is localized within the microglia. However, when they are challenged with a high concentration of hemin, the iron is taken up by all the cell types indicating that the microglia alone, are unable to process the large hemin load. Similarly, there is little or no neuronal death in response to the low concentration of hemin, but there is substantial neuronal death in response to the high concentration of hemin, as assessed using various cell viability assays. Moreover, only the higher concentration of hemin led to the production of several inflammatory molecules, which are markers of cell toxicity. These results suggest that microglia are protective and effectively

break down hemin when exposed to the low concentration. However, increasing the hemin concentration appears to exceed the capacity of the microglia to effectively catabolize the hemin load, resulting in an inflammatory and cytotoxic environment. Using a modification of our culture model, we also observed that addition of a serum protein, albumin, protected the neurons from dying after exposure to hemin. These results suggest that added albumin binds the toxic hemin and serves to protect the neurons. In future studies, our model should prove valuable in developing strategies to protect the brain against toxic blood products as occurs after a hemorrhagic stroke.

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Introduction

Ischemic strokes result from the formation of clots in blood vessels, which prevent delivery of oxygen and nutrients to brain tissue. This type of stroke in the United States accounts for the majority of all stroke types. Hemorrhagic strokes result when vasculature weakens and rupture releasing blood directly into the brain parenchyma in cases of intracerebral hemorrhage. Although hemorrhagic strokes make up only 10-15% of all stroke types, they are much more devastating in terms of mortality and morbidity compared to ischemic stroke. For example, about 35-52% of people who have hemorrhagic strokes die within 30 days (Taylor and Sensing, 2013). Currently, there is a lack of effective treatments for patients who suffer intracerebral hemorrhages, demonstrating the importance of research aimed at understanding the pathogenesis of neuronal damage following hemorrhagic stroke, and possible strategies to prevent or reduce the extent of the injury.

We are using co-cultures of neurons, astrocytes and microglia as a simple and convenient model to assess the functional effector states of microglia in response to different types of injury. The earliest studies on microglia defined two functional states; resting, which coincided with a ramified morphology, and active, which correlated to an amoeboid morphology and expression of a host of gene products that served to mobilize the microglia to the site of injury and promote phagocytosis (Ravish et al. 1999; Streit et al. 2002; Hanisch and Kettenmann 2007; Ransohoff and Perry 2009; Salter and Beggs 2014). Studies done more recently, have shown that microglia assume a range of phenotypes

depending on the duration and extent of injury. Resting microglia exhibit a highly ramified phenotype with numerous processes and a small compact soma. These cells are not stagnant but, instead, are constantly surveying the local environment for minor damage. When exposed to mild or moderate injuries, they transition to a less ramified, phagocytic phenotype in which they are protective and function to remove substances from the environment to limit inflammation and restore homeostasis. However, with more severe injuries, microglia may lose their phagocytic functions and transition to rounded, pro-inflammatory phenotypes that actually enhance tissue damage (Hanisch and Kettenmann 2007; Salter and Beggs 2014). From a clinical prospective, microglia inhibitors are being actively investigated in humans for a variety of brain injuries such as ischemic and hemorrhagic stroke, multiple sclerosis, neurodegenerative diseases, and numerous formed of brain trauma and cerebral edema (Bachstetter et al. 2012; Bachstetter et al. 2013; Sloka et al. 2013; Neher et al. 2013; Xie et al. 2014; Chen et al. 2014; Huang et al. 2014; Kabati et al. 2014). However, we believe it is important to distinguish between the different phenotypes of microglia because treatment to inhibit microglia functions may be useful only when they have converted to the cytotoxic, inflammatory phenotype. If inhibited while assuming the other states, the protective functions of the microglia may be lost, thereby preventing the cells from containing the damage and returning the environment to homeostasis.

In our laboratory, we use different concentrations of the toxic blood product hemin (20 μM and 100 μM) to model a mild to moderate versus a severe hemorrhagic stroke. When a blood vessel ruptures in the brain, the blood cells that

surround brain tissue lyse releasing hemoglobin and other toxic blood products. Hemoglobin then breaks down into heme (or hemin), a product that may be toxic. Microglia take up these blood products and catabolize them. To protect against oxidative injury, there is an induction in the enzyme heme oxygenase-1 (HO-1) within microglia and perhaps other cell types, which catabolize heme into carbon monoxide, biliverdin, and free iron (Robinson et al., 2009; Dang et al., 2011). Our working hypothesis is that microglia transition to distinct functional effector states in response to different hemin loads, corresponding to mild to moderate versus severe injury. When the hemin load is mild or moderate, we predict that microglia are able to effectively internalize the hemin, catabolize it without demonstrating a robust inflammatory response, and return the system to homeostasis, avoiding neuronal damage or death. When the hemin load becomes excessive, we predict the microglia are overwhelmed and are unable to effectively manage the removal and catabolism of hemin from the environment. In such cases, neuronal death and expression of inflammatory markers are expected. To test these hypotheses, after our cultures are exposed to low and high concentrations of hemin, alterations in microglia effector states were examined using immunohistochemistry techniques, real-time RT-PCR assays, an iron deposition assay, a phagocytosis assay, and live-dead assays to assess neuronal survival.

Methods

Tissue Culture:

Granule cell-enriched cultures were prepared as described by Vallano et al. (1996). However, for this study, the mitotic inhibitor AraC was omitted from the growth medium to promote the growth of astrocytes and microglia (Beaman-Hall et al. 1998). Briefly, the cerebella of 8-day-old Sprague-Dawley rats were minced, trypsinized, and triturated to separate cells. The cells were plated at a density of 1.25×10^6 cells/ml of medium onto 12-well Corning dishes (for live-dead assays, and RT-PCR assays) or 24-well Corning dishes containing glass coverslips precoated with poly-L-lysine (for immunocytochemistry). They were then incubated at 37°C in a humidified atmosphere containing 5% CO_2/95% air (pH 7.4). Cultures with abundant microglia and astrocytes were grown in basal Eagle's medium with Earle's salts, enhanced with heat-inactivated fetal bovine serum (10%), gentamicin sulfate (100 μg/ml), and L-glutamine (2mM). To ensure the lasting survival of cerebellar granule neurons (CGNs), 20mM KCl (25mM f.c.) was added to the growth medium (Thangnipon et al. 1983; Kingsbury et al. 1985; Gallo et al. 1987). At 7-8 days *in vitro* (DIV), the CGNs mature and develop sensitivity to addition of excitotoxic agents (Leahy et al. 1994), and hemin experiments were done at this phase of growth. Although the medium was not replaced during this time, the addition of 5.5 mM glucose at 6 DIV ensured the lasting survival of the cells (Schramm et al. 1990). Phase contrast microscopy was utilized in evaluating the viability of the cells regularly.

Excitotoxicity:

Cell viability was assessed using a lactate dehydrogenase (LDH) assay as described by Decker and Lohmann-Matthes (1988). Fluorescent staining techniques were also used to visualize live and dead cells using a live/dead kit in which calcein was used to stain living cells and PI to stain dead cells (Invitrogen). Hemin (20 μM or 100 μM) was added and, at 24 hours, the relative amounts of living and dead cells were quantified. Up to four fields per coverslip comprising of at least one hundred cells were photographed. The total number of living and dead CGNs, in relation to microglia and astrocytes were also quantified to estimate the relative proportions of glia and neurons in the cultures. In one series of experiments, kainic acid was used to model ischemic injury and, for comparative purposes, I will present some of these data that were done by another laboratory member.

Immunocytochemistry:

Phase-contrast microscopy and immunocytochemical staining with the microglial markers ED1 and Iba1 (estimated as ~11% of cells at 7-8 DIV), and the abundant neuronal protein β-tubulin (not shown) were used to observe the production of abundant microglia and neurons in culture. Astrocytes were labeled using an antibody against GFAP (estimated as ~10% of cells at 7-8 DIV). Cultures were fixed with 4% paraformaldehyde 24 hrs after toxicity (7-8 DIV), permeabilized with 0.3% Triton X-100 and 4% normal donkey serum in phosphate-buffered saline (PBS) was added for 20 min at room temperature. Following the manufacturer's protocol, the following antibodies were added to

the cultures: ED1(1:300) mouse anti-rat monoclonal antibody, Iba1(1:1000) anti-rabbit polyclonal, GFAP (1:1000) anti-rabbit polyclonal antibody, iNOS (1:1000) anti-rabbit polyclonal were added for 24 hrs at 4°C. Cultures were then washed 3x with PBS and incubated in Alexa fluor 488 donkey anti-mouse secondary antibody (1:400) or Alexa fluor 488 donkey anti-rabbit (1-400), and Alexa fluor 594 donkey anti-rabbit (1:400) for 1 hr at room temperature. Before they were photographed under a fluorescent microscope, Vectashield Antifade (Vector Laboratories) or Vectashield Antifade with PI if ED1 was used to mount coverslips. Cells positively labeled with Iba1 in two randomly photographed fields per coverslip were counted in 3 different cell culture preparations.

Phagocytosis assay:

A phagocytosis assay was performed using 5.5 μm polystyrene beads as previously described (Bocchini et al. 1988a, 1988b). Briefly, cultures were prepared as above and plated on 12mm glass coverslips at a density of 1.25 x 10^6 cells/ml and cultured for 7-8 DIV. Then, 1.5 μl/ml of 5.5 μm fluorescent microspheres were added directly to the cultures and incubated for 1 hr at 37^0C. To remove lingering microspheres, cells were washed three times with PBS before being fixed with 4% formalin in PBS. Iba1 or GFAP antibodies were used as detailed above to label cells. Four random fields were photographed.

Iron stain:

To visualize hemin uptake into cells, iron deposition was examined. After hemin exposure for 24 hrs, cultures were washed three times with PBS and then incubated with 5% potassium ferrocyanide for 30 minutes. After the removal of

the 5% potassium ferrocyanide, the cultures were exposed to HCl for 30 minutes. DAB-NI without H_2O_2 from the Vector Kit was added for 15 minutes followed by DAB-NI with H_2O_2 for 15 minutes. The cultures were washed with PBS three times before four random fields were photographed.

RT-PCR:

Real-time quantitative RT-PCR (qRT-PCR) analysis was done to examine the induction of mRNAs reflecting the expression of heat shock protein (HO-1 and inflammatory molecules (TNF-α, COX-2, iNOS) using a Light-cycler 480 System (Roche Applied Sci) using SYBR Green, a fluorescent double-stranded DNA Dye as previously described by our laboratory (Gerber et al. 2010). Samples were analyzed in triplicate PCR reactions on a single 384 well plate and the data was quantified. Amplification in the absence of template was evaluated to ensure lack of signal due to primer dimerization and extension. To verify the presence of a single amplicon in each reaction well, end point melt-curve analysis was done. The ΔΔCT method (with 18S as an internal reference) was done to analyze the data, and a repeated measures ANOVA model using the ΔCT data. Validity of the ΔΔCT method was confirmed by indicating equivalent efficiencies of amplification of the 18S RNA normalization control versus the target mRNAs over a range of serial dilutions. The primer sets used are shown in Table 1.

Statistical analysis:

Values are presented as mean ± standard deviation of the mean. Analysis of Variance (ANOVA) and Fisher's Protected Least Significant Difference

(PLSD) or Tukey/Kramer post hoc test were used to compare the groups statistically. A probability of $p \leq 0.05$ is considered statistically significant.

Results

Co-cultures of microglia, astrocytes and neurons:

Figure 1 is a representative image showing the presence of three primary cell types in our co-cultures, which were fixed at 8 DIV. Numerous rounded microglia are shown in green using the microglial marker Iba1. Many fibrous astrocytes are shown in red using the astrocyte marker GFAP. The nuclear stain Hoeschst was used to stain the nuclei of all cells in the culture. The image shows several clusters of blue nuclei throughout the culture that are not labeled with microglia or astrocytes markers, and corresponding to granule neurons.

Phagocytic activity of microglia:

Past studies have demonstrated that both microglia and astrocytes have phagocytotic activity, but most studies show that microglia are the primary phagocytotic cells in the brain. (Bechmann and Nitsch 1997; Ito et al. 2007; Koizumi et al. 2007; Neher et al. 2013). To determine whether the microglia are the primary phagocytic cells in our co-cultures, 5.5μm fluorescently labeled polystyrene beads were utilized. The cells were grown for 7-8 DIV and the beads were added for 1 hour. The cultures were then washed, fixed and stained with an antibody against Iba1 to label the microglia or an antibody against GFAP to label the astrocytes. As shown in the representative example in Figure 2, many beads (red) were observed within the Iba1 labeled microglia (green), but not the GFAP labeled astrocytes (green). Similar results were observed in four different culture preparations. These data demonstrate that the microglia in our cultures are indeed, the primary phagocytic cells.

Iron deposition within microglia & other cell types:

When heme is degraded by HO-1 into carbon monoxide and biliverdin, iron is released as a byproduct (Robinson et al., 2009; Dang et al., 2011). To observe the cell types in which the iron deposition occurs in response to different concentrations of hemin, 20 μM hemin or 100 μM hemin were added for 24 hrs, and then the cultures were stained for the presence of iron. Two representative preparations are shown in Figure 3, and similar results were observed in five different culture preparations. As shown, when the cultures are exposed to the 20 μM hemin, which we propose corresponds to a mild-moderate injury, iron was localized primarily to the microglia. However, the addition of 100 μM hemin corresponding to a more severe injury, appears to overwhelm the microglia resulting in iron deposition in all the cell types throughout the cultures. These data demonstrate that microglia take up hemin from the environment, but there is a limit above which they cannot handle the hemin load. Thus, the hemin is able to gain entry into microglia as well as astrocytes and neurons in the culture.

HO-1 induction within microglia:

HO-1 is a protective enzyme that is induced in response to various injury models including models of hemorrhagic stroke (Robinson et al., 2009; Dang et al., 2011). When hemin enters the microglia, one would expect HO-1 mRNA and protein to be generated. To test this, real-time RT-PCR assays were performd at three different time points after addition of 20 μM hemin or 100 μM hemin to the cultures. As shown, there is a significant induction of HO-1 in response to both concentrations of hemin, and these show the expected concentration and time

dependence (Figure 4A). Specifically, the fold induction of HO-1 in response to 20 μM hemin was significant at the 3 hr (~9-fold increase) and 12 hr (~17-fold increase) time points. An even greater induction of HO-1 was observed in response to addition of 100 μM hemin, and was significant at all time points examined. To verify that HO-1 protein as well as mRNA is induced in a concentration-dependent manner, western immunoblotting was performed 12 hrs after addition of 20 μM hemin or 100 μM hemin. Figure 4E shows that there is little detectable HO-1 in control cultures, and that a concentration-dependent increase in HO-1 protein is observed after hemin challenge (actin was used as a loading control). To determine if microglia are the primary source of HO-1 protein induction, double-staining immunocytochemistry with antibodies against the microglia marker ED1 and antibodies against HO-1 was performed 12 hrs after addition of hemin (Figure 4C). Immunocytochemistry images show the induction of HO-1 (green) is primarily localized in the microglia (red).

Neurotoxicity in response to hemin:

To measure the neurotoxicity in response to 20 μM hemin and 100 μM hemin, a LDH assay was done 24 hrs after the addition of the hemin. Insignificant death was observed in response to the 20 μM hemin challenge, compared to control cultures, but significant cell death was observed in response to the 100 μM hemin (Figure 5A). Using a caspase-3 death marker, it was determined that the cell death occurs in the granule neurons (green) and not in the microglia (Figure 5B-D). Note that the caspase 3 experiments were performed by another member of the laboratory.

Expression of inflammatory molecules:

When exposed to injury, such as an intracerebral hemorrhage, microglia can convert to a cytotoxic phenotype, which is associated with the expression of inflammatory molecules. To determine if there is a difference in the expression of inflammatory molecules in response to different concentrations of hemin, we assayed TNF-α, COX-2 and iNOS mRNA expression using real-time RT-PCR. TNF-α is an early cytokine in the injury cascade, and we observed a strong and significant induction of TNF-α in response to 100 μM at both the 1 and 3 hr time points, approaching 30-fold induction. In contrast, a modest 7-fold induction of TNF-α was observed in response to the 20 μM hemin only at the 3 hr time point (Figure 6). Significant induction of COX-2 mRNA (approaching 65-fold), which is downstream of TNF-α in the injury cascade, is only observed in response to 100 μM hemin and only at the 12 hr time point (Figure 7). Similarly, significant induction of iNOS mRNA (approaching 450-fold) is only observed in response to 100 μM hemin, again only at the 12 hr time point (Figure 8). Also shown is localization of iNOS to microglia in response to 100 μM hemin (Figure 8). Altogether, the cell death and RT-PCR data indicate that the low concentration of hemin does not trigger significant neuronal death and only induces a mild inflammatory response. Alternatively, the high concentration of hemin leads to significant neuronal death, and a robust inflammatory response.

Phagocytosis of dead neurons by microglia:

To measure the ability of microglia to phagocytose dead CGNs, a live-dead assay was performed in which cells were exposed to 20 μM hemin and 100

μM hemin. As shown in Figures 9A-B, there were limited dead CGNs (red) in the control culture as well as the culture challenged with 20 μM hemin after 24 hrs. However, there was significant death in response to the 100 μM hemin (Figure 9C). Despite the presence of microglia, the dead CGNs were not phagocytosed. For comparative purposes, results of a live-dead assay performed in our lab using 250 μM kainate to mimic an ischemic injury are presented. In this injury model, there were very few dead CGNs after 24 hrs indicating that phagocytosis had been completed by this time (Figure 9E). We can be sure there was CGN death after 24 hrs by looking at the histogram presented in Figure 9F in which the addition of AraC (to decrease glial growth) and kainate is shown to increase cell death. To observe the phagocytic activity, cultures were fixed 3 hrs after kainate exposure, and numerous dead neurons (red) were visible within the ED1 labeled phagocytic microglia (Figure 9G). This discrepancy in the activity of the microglia in response to hemin verses the kainate indicates that the microglia are able to engulf dead neurons but the addition of high concentrations of hemin compromises their phagocytic function.

Defined medium and protection by albumin

The presence of serum proteins, such as albumin and hemopexin, can bind blood products such as hemin, and slowly release them to be processed by microglia after a brain bleed (Belayev et al., 2005). We examined the protective effect of serum albumin against hemin-induced neuronal death in our co-cultures. To test this, we used co-cultures grown in a chemically defined medium that does not include fetal bovine serum in conjunction with hemin addition plus or minus co-

addition of albumin, followed 24 hrs later by a live-dead assay. As exemplified in Figure 10 (and observed in 3 separate culture preparations), significant cell death was observed in response to both 20 μM hemin and 100 μM hemin 24 hours after its addition, with essentially total neuronal death in response to the higher concentration. The significant neuronal death observed after addition of 20 μM hemin is not unexpected since the growth medium does not contain serum proteins, unlike the data shown in Figure 9 (top panels). Co-addition of an equal concentration of albumin (20 μM and 100 μM) to the cultures proved to be protective, and significantly decreased the amount of cell death in response to both concentrations of hemin (Figure 10).

Discussion

Although hemorrhagic strokes are less common than ischemic strokes, they have a significantly higher morbidity and mortality (Taylor and Sensing, 2013). Currently, we lack effective treatments for individuals who have intracerebral hemorrhages. Thus, there is a critical need to understand more about the genesis of the injury process, and also to develop strategies to ameliorate the extent of tissue damage. Scientific evidence shows that microglia impact, and are impacted by, astrocytes and neurons (Sudo et al. 1998; Rosenstiel et al. 2001; Kettenmann et al. 2011; Ousman and Kubes 2012). Microglia in different brain regions also demonstrate functional and morphological heterogeneity (Lawson et al. 1990; Elkabes et al. 1996; Ren et al. 1999; de Haas et al. 2008). To accurately explore microglia functional effector states *in vitro*, we co-culture microglia, astrocytes and neurons using cerebellar cortex as our tissue source. Thus, all cells in the co-cultures come from the same brain region. To promote the growth of the glial cells in our co-cultures, we omit the mitotic inhibitor, AraC, which is typically included in the growth medium to enrich CGNs (Thangnipon et al. 1983; Kingsbury et al. 1985). As exemplified in Figure 1, we estimate that at 7-8 DIV microglia and astrocytes represent ~11% and ~10%, respectively, of the cell population, which is similar to what would be found *in vivo*. In summary, we observe that microglia and astrocytes proliferate when AraC is not added to the medium so that neuron-glia co-cultures from the same brain region can be examined in our injury model.

When placed in primary culture, microglia assume an amoeboid shape and they express ED1 indicating that are not true "resting" microglia, which predominate in brain tissue and exhibit a highly ramified morphology, compact cell soma, and no expression of ED1 (Damoiseaux et al. 1994; Ito et al. 2001). The basal state of these microglia in our co-cultures is not characterized by expression of inflammatory molecules (Figures 6-8), and they are effective phagocytes when challenged with a cargo such as latex beads (Figure 2). Similarly, when challenged with the lower concentration of hemin, the microglia effectively contain the hemin load, as shown by iron deposition localized to microglia only (Figure 3), only a modest induction of one of the inflammatory markers at a single time point (TNF-α at 3 hours, Figure 6), and no significant neuronal death (Figure 5). Importantly, there was significant induction of HO-1 protein and mRNA in response to 20 μM hemin, demonstrating that the amount of hemin used to challenge the cultures was sufficient to induce a microglia response. When challenged with the higher concentration of hemin, the microglia appear to convert to an inflammatory (Figure 6-8), possibly cytotoxic phenotype that is unable to phagocytose dead neurons (Figure 5, Figure 9/upper panels). Consistent with this, iron deposition is observed throughout the culture (Figure 3). Not unexpectedly, there was even greater induction of HO-1 protein and mRNA in response to 100 μM hemin (Figure 4). Challenge of co-cultures with a different toxic agent, kainate, was used to demonstrate that microglia in the co-cultures are able to effectively phagocytose essentially all of the dead neurons within a 24 hour time period (Figure 9, lower panels). Possibly, the phagocytic

function of microglia is compromised when they are actively catabolizing hemin. For example, Mairuae and associates (2011) reported that iron accumulation interfered with the ability of microglia to phagocytose zymosan.

For effective treatment of humans with hemorrhagic stroke, it is crucial to distinguish between microglial functional effector states. In cases of mild-to-moderate injury, for example our 20 μM hemin condition, microglia appear able to effectively remove and catabolize the hemin without mounting a robust inflammatory response, and there is no observable cell death. Thus, it would be counter-productive to inhibit these important protective functions of microglia if such a situation was observed in the patient. In contrast, cases of severe or sustained injury, for example our 100 μM hemin condition, microglia appear unable to effectively remove and catabolize the hemin. Consistent with this, iron is deposited in all cell types in the culture (Figure 3). Moreover, they mount a robust inflammatory response, and there is profound cell death. These data are in good agreement with studies using other model systems, where significant induction of TNF-α, COX-2 and iNOS in microglia exacerbate neuronal death (Bal-Price and Brown, 2001; von Bernhardi et al. 2007; Cho et al. 2008; Blaylock 2013; Xing et al. 2015). Thus, it maybe useful to inhibit this potentially cytotoxic state that microglia have assumed, but only if one can preserve the protective functions while so doing. It might prove difficult to establish the appropriate conditions to achieve this goal. Another possible strategy would be to promote microglial proliferation, so as to avoid their being overwhelmed by the hemin load. Finally, our studies using cultures grown in chemically defined medium

indicate that administration of agents that bind toxic blood products, and slowly release them for catabolism by microglia, may attenuate neuronal damage. Future studies using our model will explore the potential value of such strategies.

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Appendix 1

Table 1: Primers used for PCR

Name	Sense	Antisense
TNFα	5'-GTAGCCCACGTCGTAGCAAA-3'	5'-CCCTTCTCCAGCTGGGAGAC-3'
COX2	5'-CCATGTCAAAACCCGTGGTGAATG-3'	5'-ATGGGAGTTGGGCAGTCATCAG-3'
iNOS	5'-GCAGAATGTGACCATCATGG-3'	5'-ACAACCTTGGTGTTGAAGGC-3'
18S	5'-CGCCGCTAGAGGTGAAATTC-3'	5'-TTGGCAAATGCTTTCGCTC-3'

Appendix 2

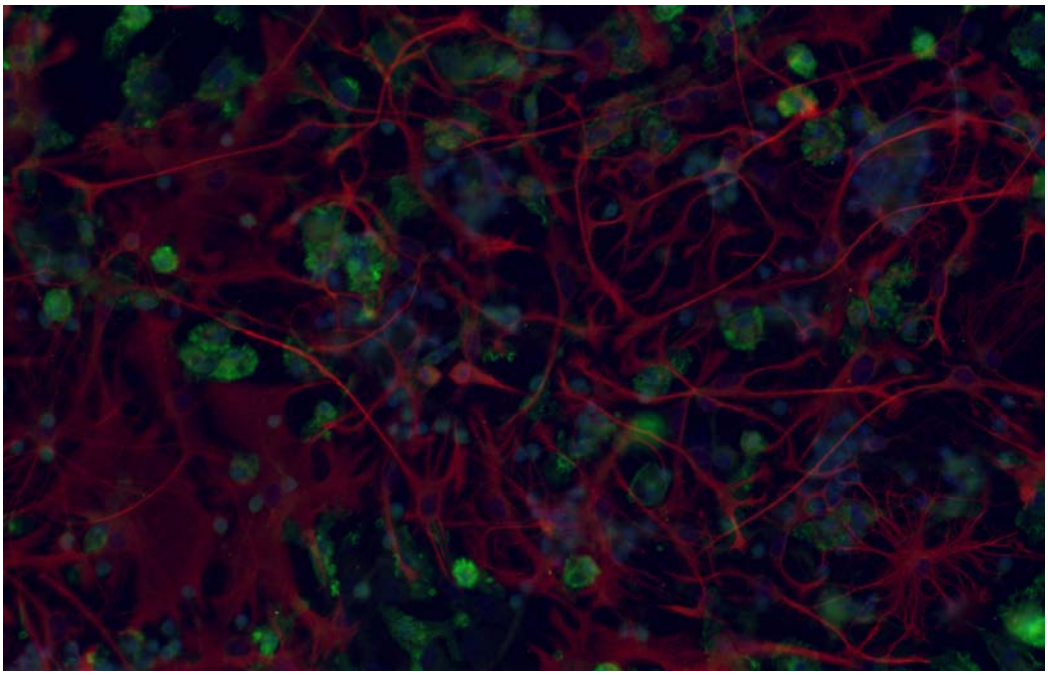


Figure 1: The three primary cell types in our co-cultures. Microglia labeled green with an Iba1 marker, astrocytes labeled red with a GFAP marker, and clusters of neuronal nuclei labeled blue with Hoechst.

Appendix 3

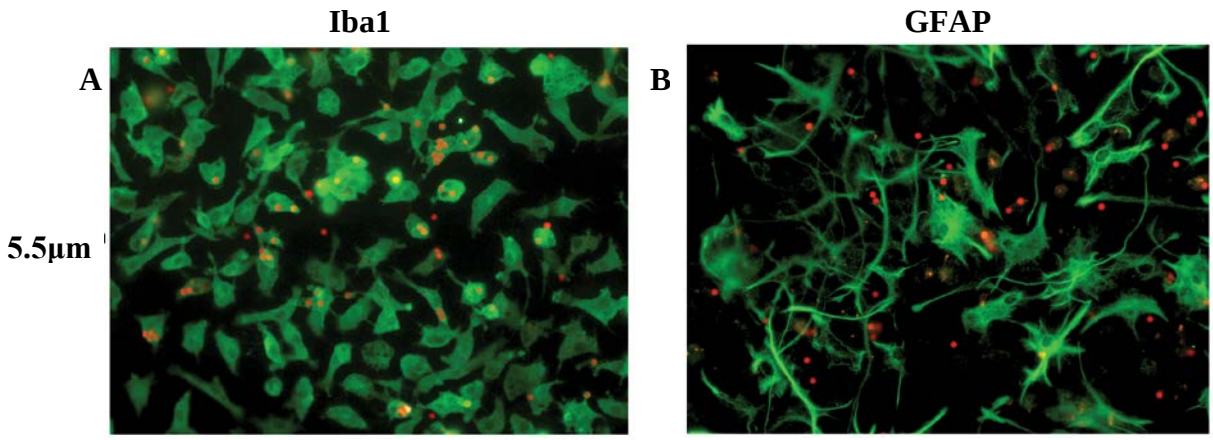


Figure 2: Phagocytic activity of microglia demonstrated by the presence of 5.5 µm beads within the (A) Iba1 labeled microglia and the absence of beads within the (B) GFAP labeled astrocytes.

Appendix 4

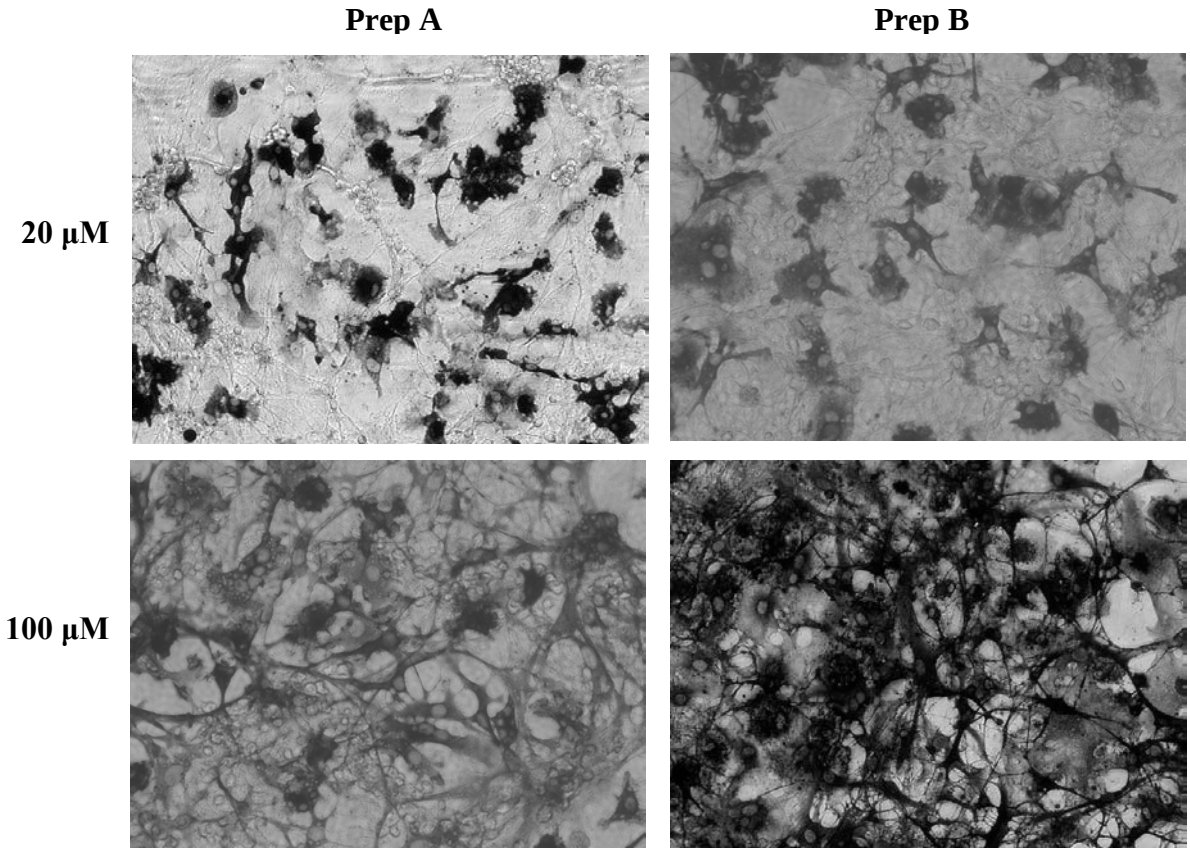


Figure 3: Deposition of iron within microglia and other cell types observed in cell Prep A and cell Prep B after the addition of 20 µM hemin and 100 µM hemin.

Appendix 5

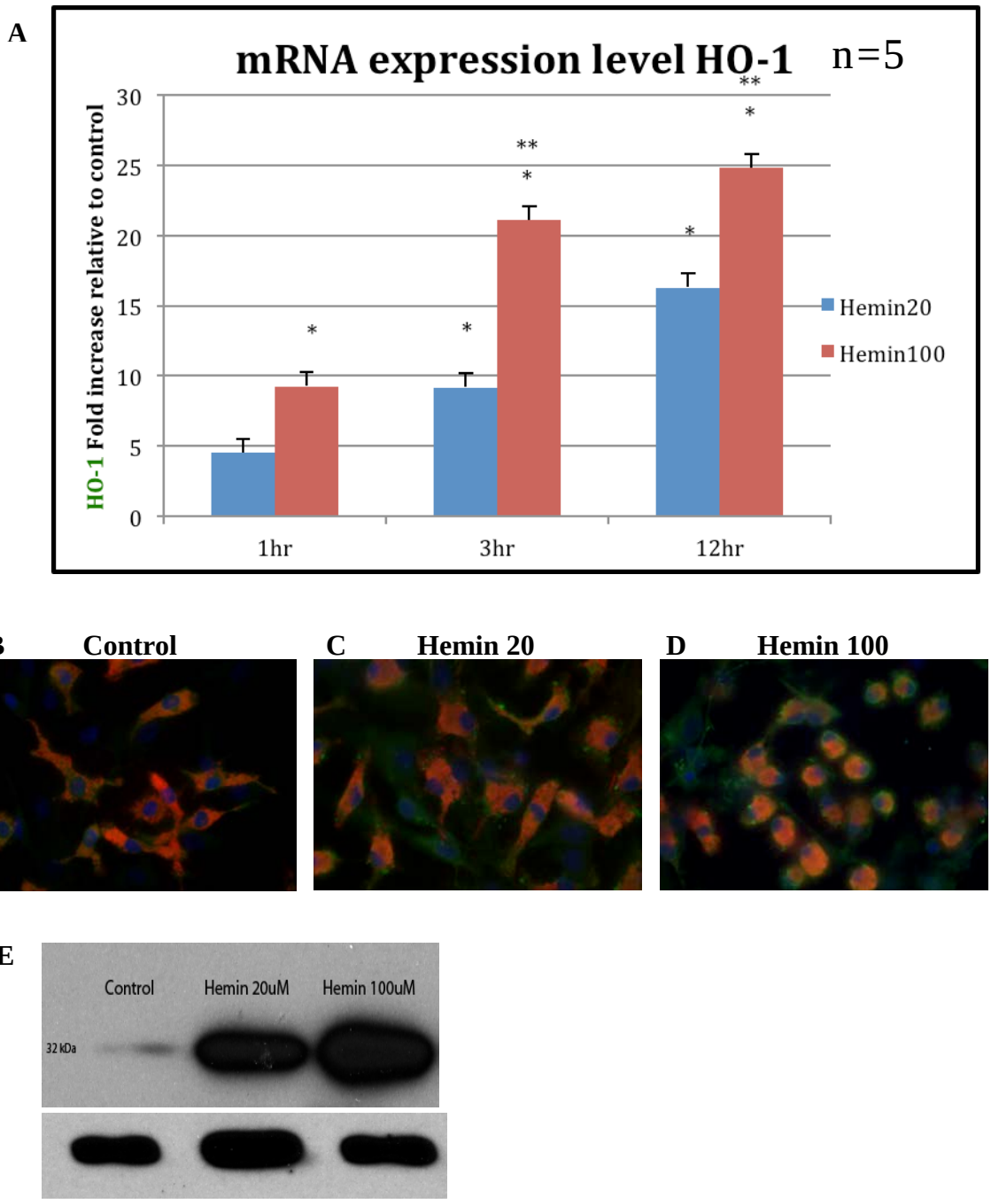
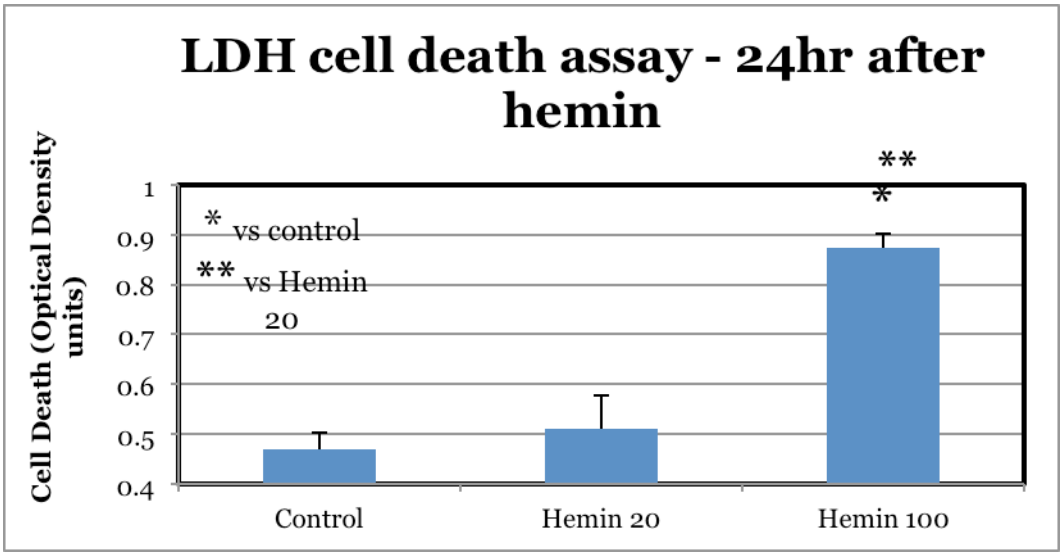


Figure 4: (A) Induction of HO-1 mRNA in response to 20 μM hemin and 100 μM hemin, as assessed by real time RT-PCR assays. (B-D) Immunocytochemistry images taken 12 hours after exposure to 20 μM hemin and 100 μM hemin showing the localization of green-labeled HO-1 protein within the red-labeled ED1 protein that is a microglia marker. E. Western immunoblot of hemin protein expression 12 hrs after addition of control, 20 μM hemin, or 100 μM hemin. Actin loading controls are shown in the lower panels.

Appendix 6

A



B **Control** **C** **Hemin 20** **D** **Hemin 100**

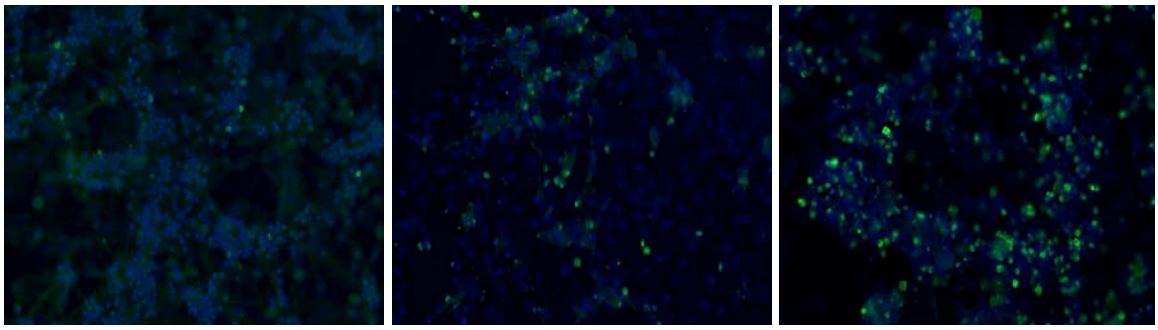


Figure 5: (A) Neurotoxicity measured in response to 20 μM hemin and 100 μM hemin using a LDH assay, performed 24 hours following hemin addition. (B-D) Cell death observed in granule neurons labeled green using a caspase-3 death marker (green label).

Appendix 7

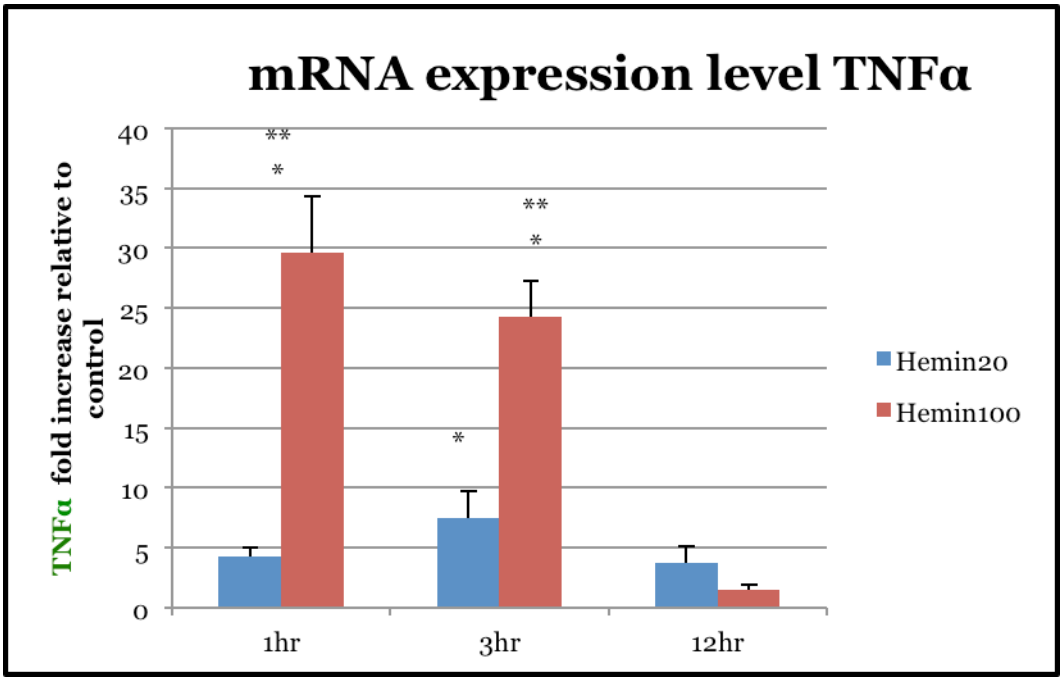


Figure 6: TNFα mRNA induction in response to 20 μM hemin and 100 μM hemin, as assessed by real-time RT-PCR assays.

Appendix 8

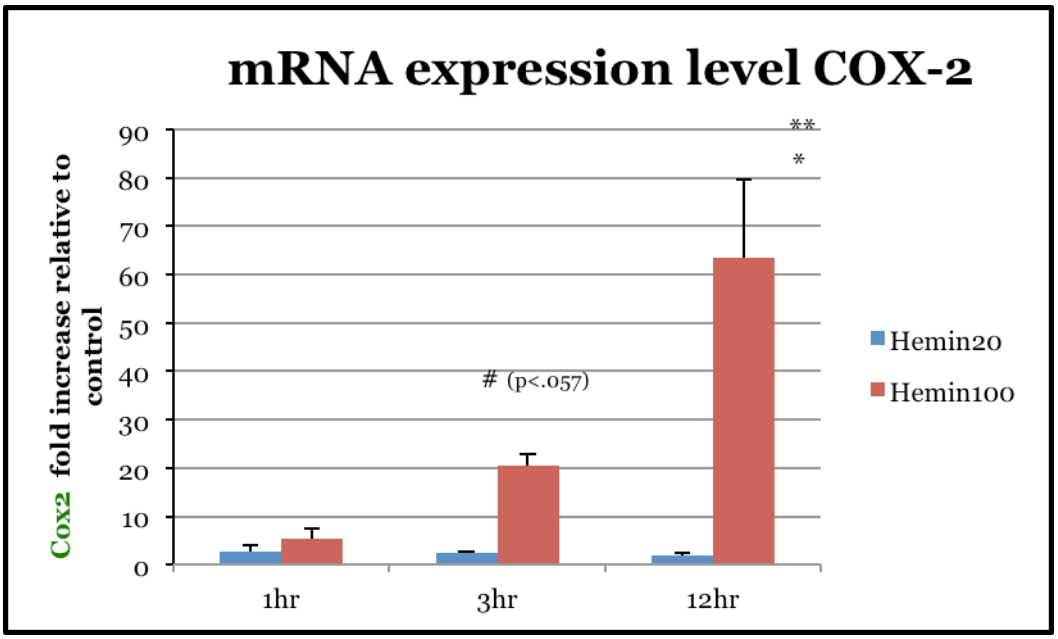
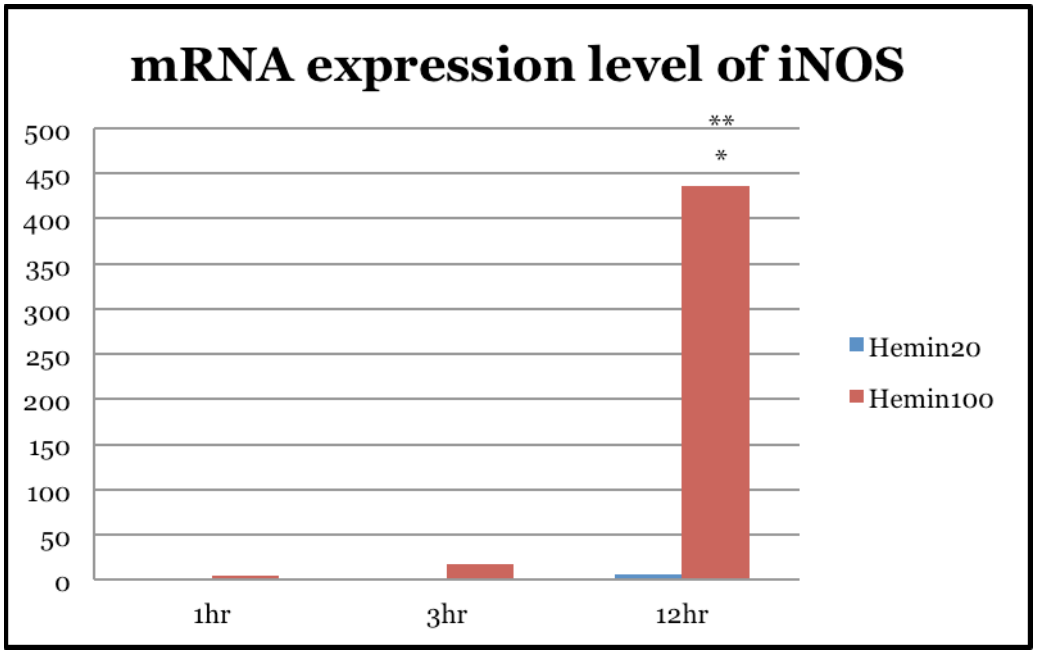


Figure 7: COX-2 mRNA induction in response to 20 μM hemin and 100 μM hemin, as assessed by real-time RT-PCR assays.

Appendix 9

A



B

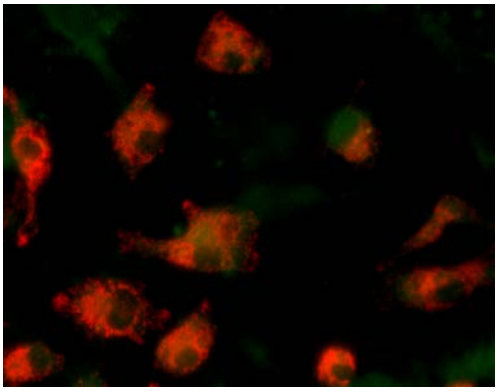


Figure 8: A. iNOS mRNA induction in response to 20 μM hemin and 100 μM hemin, as assessed by real-time RT-PCR assay. B. Immunocytochemical staining with ED1 antibodies to label microglia (red) co-localize with iNOS expression (green) after addition of 100 μM hemin.

Appendix 10

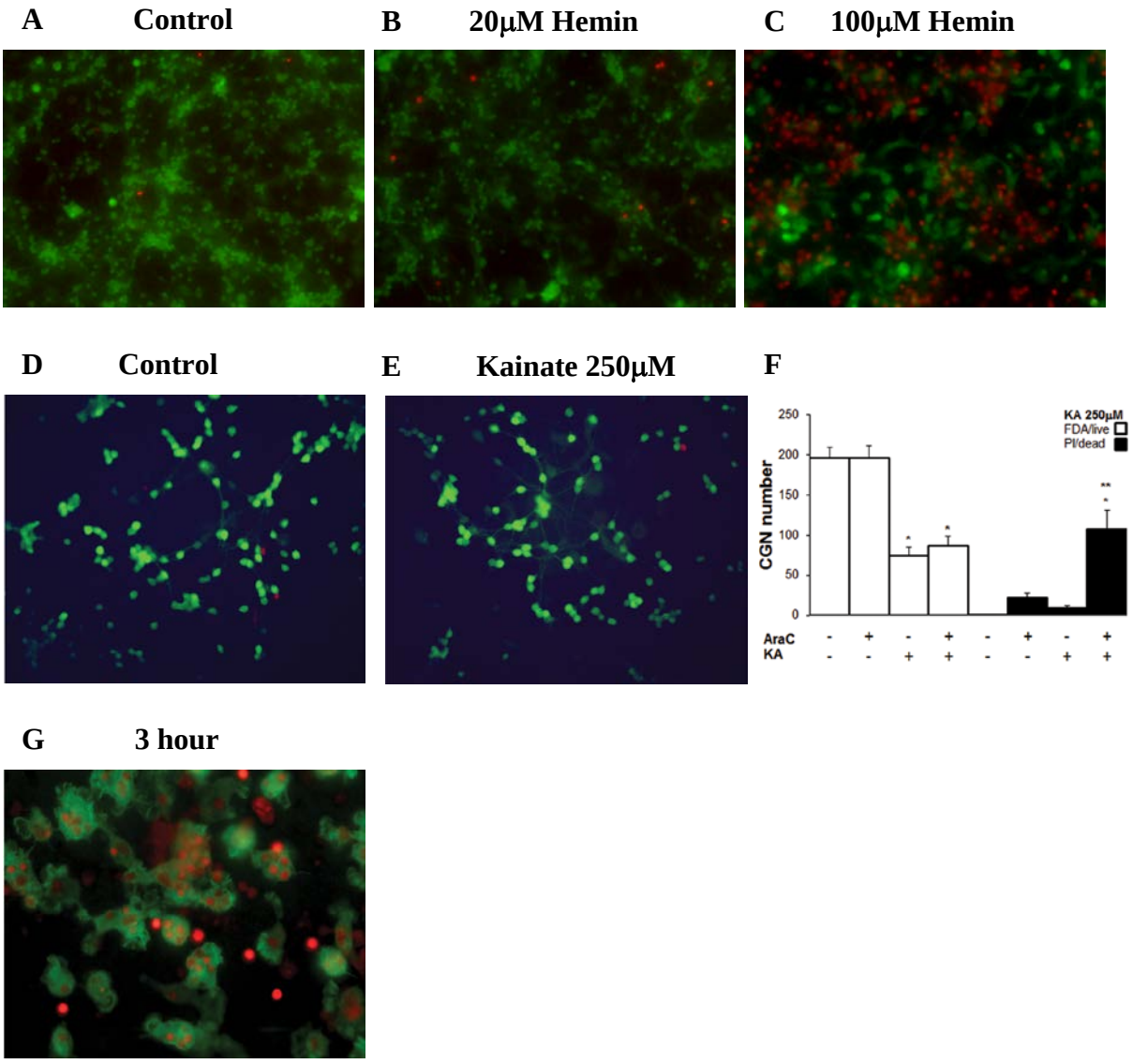


Figure 9: (A-C) Challenge with 20 μM hemin results in little death while exposure to 100 μM hemin results in extensive CGN death. Despite the presence of microglia, the dead CGNs (red) were not engulfed after 24 hrs. (D-E) When challenged with 250 μM kainate, to mimic an ischemic injury, there is an absence of dead (red) CGNs after 24 hrs indicating that phagocytosis is complete by this time. (F) The histogram in panel F shows that there was significant CGN death after 24 hrs (Re: -AraC conditions showing ~66% cell death after kainite exposure). (G) To observe the phagocytic activity directly, the cultures were fixed 3 hrs after exposure to kainate. This image reveals the presence of numerous dead

neurons (red) within Iba1 labeled phagocytic microglia (green). Note that the magnification in this image is ~ 2x greater than those in images A-E.

Appendix 11

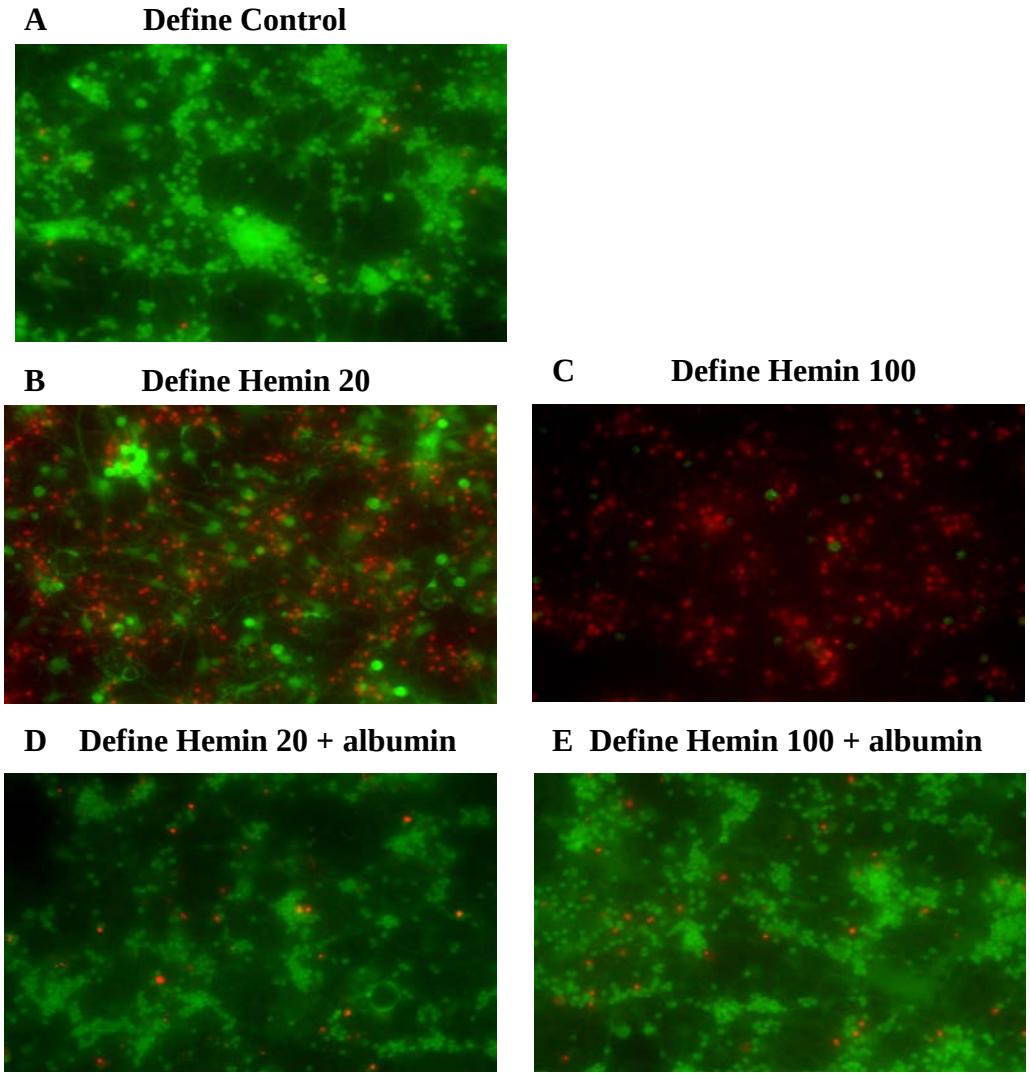


Figure 10: Co-cultures were grown in a chemically defined medium that does not include fetal bovine serum. Vehicle control (A) or hemin was added in the absence (B, C) or presence (D, E) of an equal amount of albumin (20 μM or 100 μM), and live-dead assays were done 24 hours later. Living cells are shown in green; dead cells are shown in red.