The University of Toledo
The University of Toledo Digital Repository

Theses and Dissertations

2017

Studies on the role of cofilin signaling in hemin induced microglial activation

Muhammad Shahdaat Bin Sayeed
University of Toledo

Follow this and additional works at: http://utdr.utoledo.edu/theses-dissertations

Recommended Citation
Bin Sayeed, Muhammad Shahdaat, "Studies on the role of cofilin signaling in hemin induced microglial activation" (2017). *Theses and Dissertations*. 2218.
http://utdr.utoledo.edu/theses-dissertations/2218

This Thesis is brought to you for free and open access by The University of Toledo Digital Repository. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of The University of Toledo Digital Repository. For more information, please see the repository's About page.

A Thesis

entitled

Studies on the role of Cofilin signaling in Hemin induced Microglial activation

by

Muhammad Shahdaat Bin Sayeed

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in Pharmaceutical Sciences

____
Dr. Zahoor A. Shah, Committee Chair

____
Dr. Frederick E. Williams, Committee Member

____
Dr. Marcia F. McInerney, Committee Member

____
Dr. Patricia R. Komuniecki, Dean
College of Graduate Studies

The University of Toledo

May 2016

Copyright 2016, Muhammad Shahdaat Bin Sayeed

This document is copyrighted material. Under copyright law, no parts of this document may be reproduced without the expressed permission of the author.

An Abstract of

Studies on the role of Cofilin signaling in Hemin induced Microglial activation

by

Muhammad Shahdaat Bin Sayeed

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the
Master of Science Degree in
Pharmaceutical Sciences

The University of Toledo

May 2016

Intracerebral hemorrhage (ICH) is the most severe form of stroke and further exacerbates brain damage due to secondary injury following the initial burst. This secondary damage is partly due to the toxic effects of hemin, an endogenous breakdown product of hemoglobin. Microglia are CNS resident macrophages and the first to respond to any kind of insult (sterile or nonsterile) in the brain parenchyma. Cofilin is an actin depolymerizing factor and it has been primarily attributed to the control of actin dynamics.

Recently it was shown that cofilin is involved in mediating neuronal cell death in ischemic conditions. Cofilin is also involved in microglial activation induced by bacterial lipopolysaccharide. There are not many studies available that have clearly demonstrated the deleterious effects of extremely high doses of hemin released during ICH and its influence on microglia. Furthermore, the role of cofilin in this respect is still unknown. Therefore, investigations were conducted to study the effects of hemin on microglial activation and subsequent inflammation. The end-points measured were; NO and iNOS expression; cell survival; cytokine production (TNF-α, IL1-β); migration; hemin degrading

protein, HO-1expression; Nrf2 expression; endoplasmic reticulum (ER) stress markers (Wfs-1, XBP-1, spliced XBP-1) and calcium (Ca^{2+}) signaling.

In our studies, we found that hemin induces cofilin expression in microglia and increases NO production dose dependently. There were increased levels of iNOS, TNF- α, HO-1, Nrf2, Wfs-1, XBP-1 and spliced XBP-1 due to hemin treatment and were mediated by cofilin. However, hemin treatment did not affect IL-1 β production. Acute hemin treatment did not evoke Ca^{2+} signaling but long-term treatment of hemin resulted in microglial failure to respond to acetylcholine evoked Ca^{2+} signaling. Knockdown of cofilin by siRNA also reduced acetylcholine evoked Ca^{2+} signaling. These studies demonstrate that cofilin signaling is important in ICH-induced brain injury and cofilin inhibition reduced hemin induced inflammation, oxidative stress, ER stress, microglial migration, and ability to evoke Ca^{2+} signaling and thus its inhibition can become novel drug therapeutics.

Keywords: Intracerebral Hemorrhage, Microglia, Hemin, Cofilin, Cell signaling

For the People of the United States of America

Acknowledgements

I gratefully acknowledge the scientifically stimulating guidance of Dr. Zahoor A. Shah while working under his mentorship. I am highly grateful to him also for giving me freedom of thinking while doing the experiment. His mentorship will always be one of my greatest learning experiences. In the lab, Qasim Alhadidi generously helped me all the time. Kevin Nash helped in discussion of experimental results. Saleh Alaqel helped me to approach experiments in more organized way. I am also grateful to Dr. Jatin, Dr. Aparna, Rami, Emily, Lina, Samaya for their generous help in the lab.

I am thankful to Dr. Sonia M. Najjar, Dr. Shahnawaz Imam, Dr. Lucia Russo, Dr. Michael P. Morran, Dr. Saja S. Khuder, Sujan Chandra Das and Hilda E Ghadieh for their generous help.

For calcium signaling I am indebted to Dr. David R Giovannucci. He explained calcium signaling in a mind-blowing way. I am also grateful to his lab members: Dr. Sumit, Jack Imbery and Saroj.

I am grateful to Dr. Johnnie L. Early, II, Dr. Marcia McInerney, Dr. Ezdihar A. Hassoun, Dr. Frederick E. Williams, Dr. Steinmiller, Dr. Sarver, Dr. Trendel, Dr. Hall, Dr. Wall, Dr. Slama, Dr. Liu for their constant support throughout my degree program.

I am thankful to my parents, siblings, neighbors and friends: Kamal, Saad, Ali, Yeasir, Anish, Fawas, Qasim, Rupa, Shirin, Zach, Junayet, Touhid, Rayan, Pervaiz, Ibrahim including others for making life comfortable during study and research.

Table of Contents

Abstract iii

Acknowledgements vi

Table of Contents vii

List of Tables x

List of Figures xi

List of Abbreviations xiii

1 Introduction............ 1

1.1 Intracerebral hemorrhage 1

1.1.1 Pathophysiology 1

1.1.2 Oxidative stress and intracerebral hemorrhage 3

1.1.3 Inflammation and intracerebral hemorrhage 5

1.2 Hemin............ 6

1.2.1 Chemistry of hemin 6

1.2.2 Catabolism and toxicity of hemin 7

1.2.3 Hemin and intracerebral hemorrhage 10

1.3 Microglia............ 11

1.3.1 Microglia and Intracerebral hemorrhage 13

1.4 Cofilin............ 15

1.4.1 Cofilin and actin dynamics 16

1.4.2 Regulation of cofilin activity 17

1.4.3 Cofilin and intracerebral hemorrhage 17

1.5 Aim and hypothesis..........19

2 Materials and Methods..........22

2.1 Cell Culture..........22

2.2 Microglial transfection by siRNA..........22

2.3 Hemin induced microglial activation and nitrite oxide production 23

2.4 ELISA assay..........23

2.5 MTT-cell viability assay 24

2.6 Scratch migration assay 24

2.7 Subcellular fractionation and Western blotting 24

2.8 RT-qPCR..........25

2.9 Calcium signaling 26

2.10 Statistical analysis 27

3 Results..........28

3.1 Cofilin knockdown changed Microglial cells morphology 28

3.2 Hemin dose adjustment and Nitrite Oxide production 29

3.3 Hemin induces increased Cofilin expression..........30

3.4 Cofilin mediates hemin induced iNOS production..........31

3.5 Cofilin knockdown inhibits Hemin induced microglial TNF-α production but not IL-1β 33

3.6 Cofilin knockdown reduces hemin treatment-induced microglial cell death as well as protects undifferentiated PC-12 cells from neurotoxicity from conditioned medium of hemin activated microglia ………………. 35

3.7 Cofilin knockdown inhibits microglial cell migration...................................36

3.8 Cofilin knockdown increases Hemin-induced HO-1 and Nrf2 expression. 39

3.9 Cofilin knockdown decreases hemin treatment-induced endoplasmic reticulum stress………………...42

3.10 Hemin alone does not evoke Calcium signaling...44

3.11 Long term exposure of hemin and cofilin knockdown decreases acetylcholine evoked calcium signaling in microglia..45

4 Discussion……………………...48

References..55

List of Tables

2.1 Primers sequences 27

List of Figures

1-1 Cascade of neural injury initiated by intracerebral hemorrhage. 3

1-2 Chemical structure of hemin (Fe^{3+}-protoporphyrin). 7

1-3 Microglia activation following CNS injury. 12

1-4 Human Cofilin Structure...15

3-1 SIMA9 Cell morphology and cofilin expression after transfection by scramble and cofilin siRNA. 29

3-2 Dose dependent hemin induced nitrite oxide (NO) production. 30

3-3 Hemin induced increased expression of cofilin... 31

3-4 Cofilin mediates hemin treatment-induced increase in iNOS production. 32

3-5 Hemin induced microglial cytokine production. 34

3-6 Cell viability assay after; hemin treatment in SIM-A9 cells and exposure of undifferentiated PC-12 cells to conditioned media from SIM-A9 cells…………………....36

3-7 Cofilin mediated microglial migration after hemin treatment 40

3-8 Cofilin mediates hemin treatment-induced hemoxygenase- 1 expression............41

3-9 Cofilin mediates hemin treatment-induced nuclear factor (erythroid-derived 2)-like 2 expression 42

3-10 Cofilin mediates hemin treatment-induced endoplasmic reticulum (ER) stress markers..43

3-11 Hemin treatment alone neither evoke calcium signaling nor affect acetyl choline evoked calcium signaling....45

3-12 Acetylcholine evoked calcium signaling due to hemin treatment. 46

List of Abbreviations

μl ....Microliter
μM.............................Micromolar
ABCG2 ....ATP-binding cassette sub-family G member 2
ADF............................Actin depolymerizing factor
Aip1............................Actin-interacting protein-1
ATP ....Adenosine tri phosphate
BBB............................Blood–brain barrier
BMP ....Bone morphogenic protein
BSA............................Bovine Serum Albumin
Ca^{2+} ....Calcium ion
CaMK.........................Ca^{2+}/calmodulin-dependent protein kinase
CAP1..........................Cyclase-associated protein-1
cDNA ....Complementary DNA
CNS............................Central nervous system
CO ....Carbon Monooxide
DAMP........................Danger associated molecular patterns
DNA...........................Deoxyribonucleic Acid
DTT............................Dithiothreitol
ELISA ....Enzyme-linked immunosorbent assay
ER ....Endoplasmic reticulum
ERK............................Extracellular-signal-regulated kinases
E-selectin ....Endothelial leukocyte adhesion molecules
FBS ....Fetal bovine serum
FLVCR1.....................Feline leukemia virus subgroup C receptor
GAPDH......................Glyceraldehyde 3-phosphate dehydrogenase
GPx ....Glutathione peroxidase
GSH...........................Glutathione
GSK3β ....Glycogen synthase kinase 3β
H ....Hour
H_2O_2 ....Hydrogen peroxide
Hb ....Hemoglobin
HEPES ....4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGB1 ....High mobility group box 1
HO.............................Hemoxygenase
Hp..............................Haptoglobin

HRP............................Horseradish peroxidase
HS ....Horse serum
HSP32 ....Heat shock protein 32
Hx..............................Hemopexin
ICAM-1 ....Intracellular adhesion molecules
ICH............................Intracerebral hemorrhage
IL-10 ....Interleukin-10
IL-1β ....Interleukin 1- beta
IL-2 ....Interleukin-2
INFγ ....Interfereon gamma
iNOS ....Induced nitric oxide synthase
KCl.............................Potassium Chloride
LIMK ....LIM kinase
LPS.............................Lipopolysaccharide
LRP1 ....Low density lipoprotein receptor
MAPK........................p38 mitogen-activated protein kinase
MAPKAPK-2/MK2 ...MAPK)-activated protein kinase-2
MgCl2 ....Magnesium chloride
mM.............................Millimolar
MMP ....Matrix metalloprotein mM
MPTP ....Mitochondrial permeability transition pore
MTT ....3-[4,5-dimethyl- thiazol-2-yl]-2,5-diphenyltetrazolium bromide
Na...............................Sodium
NaF.............................Sodium Flouride
nM ....Nanomolar
NMDA ....N-methyl-D-aspartate
NO.............................Nitrite oxide
NOS...........................Nitrite Oxide Synthase
NOX...........................NADPH oxidase
NP 40 ....Nonidet P-40
Nrf2 ....Nuclear factor (erythroid-derived 2)-like 2
PC-12 ....Pheochromocytoma cell with embryonic origin from neural crest
PCFT ....Proton-coupled folate transporter
PGE2 ....Prostaglandin E 2
PI3K ....Phosphoinositide 3-kinase
PKD............................Protein kinase D
PMN...........................Polymorphonuclear cells
PMSF ....Phenylmethylsulfonyl fluoride
PP1/PP2A...................Phosphoprotein phosphatases
PPARγ........................Peroxisome proliferator activated receptor-γ
PtdIns (3,4) P2 ....Phosphatidylinositol (3,4)-bisphosphate
PVDF ....Polyvinylidene fluoride
RNA ....Ribonucleic Acid
RNS............................Reactive nitrase species
ROCK ....Rho-associated kinase
ROS............................Reactive oxygen species

RT-qPCR....................Real time quantitative polymerase chain reaction
SDS ....Sodium dodecyl sulfate
SIM-A9 ....Spontaneously Immortalized Microglia
siRNA ....Small interfering RNA
SOD............................Superoxide dismutase
SSH ....Slingshot
TGF-β.........................Transforming growth factor-β
TLR ....Toll-like receptor
TNF-α.........................Tumor necrosis factor- α
Trem2 ....Triggering receptor expressed on myeloid cells 2
UPR............................Uncoupled Protein Response
VCAM-1 ....Vascular cell adhesion molecule1
VEGF ....Vascular endothelial growth factor
WB ....Western blotting
Wfs-1..........................Wolframin ER transmembrane glycoprotein
XBP1..........................X-box binding protein-1

Chapter 1

Introduction

1.1 Intracerebral Hemorrhage

Intracerebral hemorrhage (ICH), a subtype of stroke, is a devastating form of cerebrovascular disorder, with an annual incidence of 10–30 per 100,000, and about 2 million cases occurring worldwide. The very high morbidity and mortality rate reaches up to 40% within a month following ICH. Those who survive are left with severe neurological deficits (Mozaffarian, Benjamin et al. 2015). Six months after the onset of ICH, only 20% of ICH survivors are able to live independently (Kuramatsu, Huttner et al. 2013). The rate of ICH is expected to increase as a result of the aging population, but there are only few effective medical and surgical treatment strategies available currently. The mortality rates after one month of ICH have not decreased in the past three decades (van Asch, Luitse et al. 2010).

1.1.1 Pathophysiology

ICH causes immediate physical damage to the brain cellular architecture adjacent to the hemorrhage. After the first ictus of bleeding, about 20–40% of patients experience hematoma enlargement during the first day (Keep, Hua et al. 2012). The precise

mechanism for the hematoma growth is not known yet, and there are not much alternatives to stop this initial insult. However, the secondary brain injury after ICH is caused by a complicated cascade of events where therapeutic intervention could help to prevent the further damage (**Figure 1-1**). Immediately after ICH, the activation of hemostatic cascade occurs as an initial reaction to limit bleeding. Thrombin is produced in the brain immediately after hemorrhage affecting endothelial cells, astrocytes, neurons, and microglia (Hua, Keep et al. 2007, Keep, Hua et al. 2012), thereby contributing to early blood–brain barrier (BBB) disruption and edema formation (Keep, Zhou et al. 2014). The release of hemoglobin (Hb) and subsequent formation of hemin and iron from the hematoma is considered another prime contributor to the ICH-induced brain injury (Wagner, Sharp et al. 2003, Hua, Keep et al. 2007). Erythrocyte lysis occurs within 24 hour (h) after ICH, peaking at day 1–7, and hemoglobin- and iron-mediated brain injuries occur along with lysis of extravagated erythrocytes (Qureshi, Mendelow et al. 2009, Keep, Zhou et al. 2014).

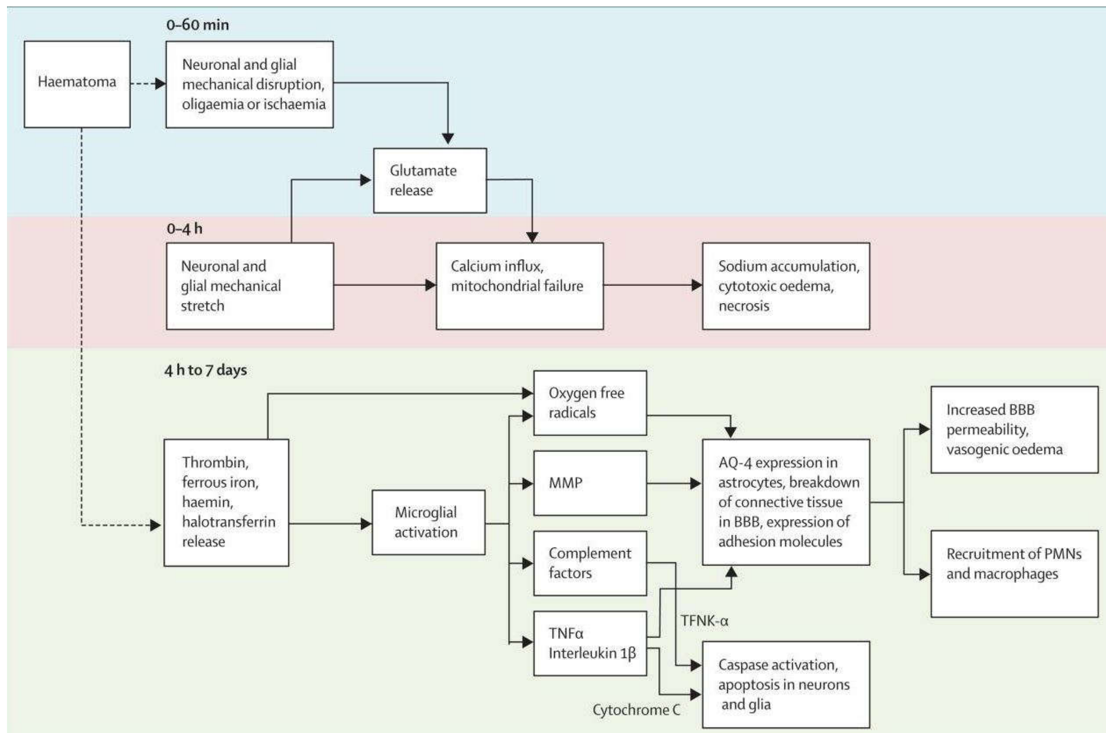


Figure 1-1: Cascade of neural injury initiated by intracerebral hemorrhage. The steps in the first 4 h are related to the direct effect of the hematoma; later steps are due to the products released from the hematoma. BBB=blood–brain barrier. MMP=matrix metallopeptidase. TNF=tumor necrosis factor. PMN=polymorphonuclear cells (Adapted from (Qureshi, Mendelow et al. 2009) with permission from Elsevier) .

1.1.2 Oxidative stress and intracerebral hemorrhage

Oxidative stress is one of the major contributors of ICH, metabolites of Hb-heme-iron axis are key contributors to oxidative brain damage after ICH, although other factors, such as neuroinflammation and prooxidases play the primary role (Hu, Tao et al. 2016). The oxidative products of macromolecules significantly increase, whereas antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase,

correspondingly decrease as a result of ICH (Nakamura, Keep et al. 2005, Nakamura, Keep et al. 2006). Mitochondrial dysfunction has been reported to be responsible for the decreased oxygen metabolites after ICH (Kim-Han, Kopp et al. 2006). The mechanism of excessive ROS formation by mitochondria after ICH is not clear, but this has been attributed to the mitochondrial permeability transition pore (MPTP) since the inhibition of MPTP can attenuate ROS production (Ma, Chen et al. 2014).

Hb is released from erythrocyte protein into the extracellular space via complement-mediated cell lysis in the hours after ICH. This release is considered as the potent mediator of oxidative stress-induced injury (Regan and Panter 1993, Hua, Xi et al. 2000). Hb leads to increased reactive oxygen species (ROS), reactive nitrate species (RNS) (Yang, Chen et al. 2013). Heme from methemoglobin, and its oxidized product, hemin, trigger oxidative damage in brain tissue around the hematoma (Hu, Tao et al. 2016). Iron overload in the brain starts within 24 h and peaks at 7 days after hemorrhage, remains at least for a month, and consequently produces toxicity based on the Fenton reaction (Chaudhary, Gemmete et al. 2013). In experimental ICH models, it was confirmed that oxidative deoxyribonucleic acid (DNA) damage caused by free radicals occurs after ICH, and it leads to neuronal death (Nakamura, Keep et al. 2005). Neuroinflammation has been linked to increased oxidative stress after ICH (Matsuo, Kihara et al. 1995). Inhibition of NADPH oxidase (NOX) and nitric oxide synthase (NOS) has been found to improve ICH condition (Matveev 1966, Zia, Csiszar et al. 2009). The role of antioxidant system such as hemoxygenase (HO) is very complex and found to affect ICH in different ways based on context and site of action (Chen-Roetling, Lu et al. 2015). It has been found that in the free radical scavenging systems, SOD is severely impaired after ICH and therefore contribute to further increased

ROS, RNS induced brain damage (Wu, Hua et al. 2002). ROS can activate the Keap1/Nrf2/ARE pathway to counteract oxidative damage after ICH as an adaptive response mechanism but due to excessive oxidative stress the cells eventually fail to protect themselves (Shang, Yang et al. 2013).

1.1.3 Inflammation and intracerebral hemorrhage

Inflammation is the key contributor of ICH-induced secondary brain injury. Inflammation progresses in response to various stimuli released after ICH (Zhou, Wang et al. 2014). Hematoma components initiate inflammatory signaling, with the production of thrombin playing a prominent role via activation of microglia and subsequently releasing proinflammatory cytokines and chemokines to attract peripheral inflammatory mediators (Keep, Hua et al. 2012). Hb, heme and iron released after red blood cell lysis aggravate ICH-induced inflammatory injury. Bilirubin, a Hb breakdown product, can also induce neutrophil entrance into brain but does not activate microglia (Loftspring, Johnson et al. 2011). Many preclinical studies have shown that targeting such signaling can affect ICH-induced brain injury. Inflammation is involved not only in early ICH-induced brain injury, but also in tissue repair (Egashira, Hua et al. 2015). Danger associated molecular patterns (DAMPs) such as the high mobility group box 1 protein (HMGB1) which is released from damaged or dead cells, trigger inflammation in the late stage of ICH (Lei, Lin et al. 2013). Preclinical studies have identified inflammatory signaling pathways that are involved in microglial activation, leukocyte infiltration, toll-like receptor (TLR) activation, and DAMP regulation in ICH. Recent advances in understanding the pathogenesis of ICH-induced inflammatory injury have facilitated the identification of

several novel therapeutic targets for the treatment of ICH such as thrombin inhibitors, desferrioxamine, minocycline, statins and therapeutic hypothermia; but none of these agents has proven to be effective in clinical settings (Belur, Chang et al. 2013).

1.2 Hemin

Hemin is an erythrocyte derived endogenous chemical substance that is released due to breakdown of erythrocyte during different pathological conditions.

1.2.1 Chemistry of hemin

Hemin is an iron–protoporphyrin complex consisting of four substituted pyrrole rings linked by –CH group (**Figure 1-2**). When the iron atom is in the ferrous state, the complex is called ferroprotoporphyrin or heme and the molecule is electrically neutral. When the iron atom is in the ferric state, the complex is called ferriprotoporphyrin or hemin, and the molecule carries a unit positive charge (and is consequently associated with an anion, chloride). Hemin is hydrophobic in nature and contains methyl groups in positions 1, 3, 5 and 8; vinyl groups in positions 2 and 4; propionic acid groups in positions 6 and 7 (Kumar and Bandyopadhyay 2005).

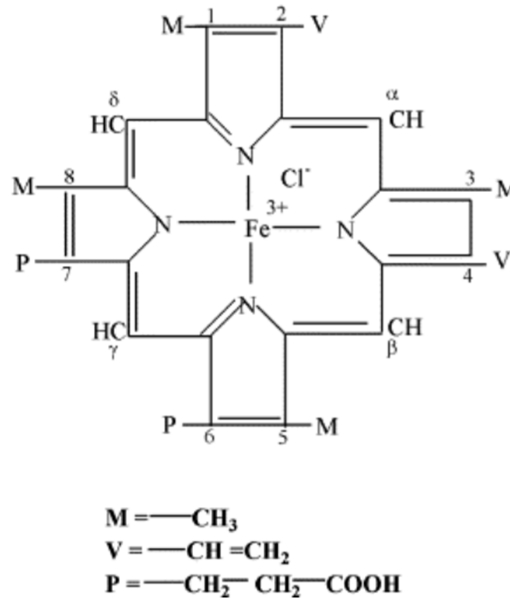


Figure 1-2: Chemical structure of Hemin (Fe^{3+}-protoporphyrin). Adapted from Kumar and Bandyopadhyay, 2005 with permission from Elsevier.

1.2.2 Catabolism and toxicity of hemin

Hemin is catabolized by the microsomal hemeoxygenase (HO) system consisting of HO and NADPH–cytochrome P450 reductase. This system catalyzes the oxidation of heme to biliverdin, carbon monoxide (CO) and ferrous iron, in equimolar amounts. This reaction requires the presence of oxygen and NADPH. Once biliverdin is formed, it is rapidly metabolized to bilirubin by biliverdin reductase (Liu and Ortiz de Montellano 2000, Wagener, Volk et al. 2003, Li and Stocker 2009). There are two isoforms of HO that are found in the CNS: HO-1 and HO-2. The former is also known as heat shock protein 32 (HSP32) and is highly expressed by microglia and astrocytes but is weakly expressed by neurons (Geddes, Pettigrew et al. 1996, Turner, Bergeron et al. 1998, Scapagnini, D'Agata et al. 2002). HO-1 expression can be induced by a wide range of conditions including

hemorrhage (Syapin 2008). On the other hand, HO-2 is primarily expressed by neurons and is not inducible, even by injections of whole or lysed blood into the subarachnoid space (Matz, Turner et al. 1996). It is generally considered that HO activity improves cellular viability only in instances where the amounts of hemin to be metabolized are within the capacity of the cells to sequester the iron that is released. Conversely, HO activity becomes detrimental whenever excessive amounts of free iron are produced (Robinson, Dang et al. 2009) and no longer be stored in ferritin; this unbound iron is redox-active and produces toxic hydroxyl radicals.

Hemin can also be cleared by forming a non-toxic heme-complex (e.g., hemopexin (Hx), albumin, heme-binding protein, etc.) or by its degradation (e.g., glutathione (GSH), xanthine oxidase, NADPH–cytochrome P-450 reductase, etc.) or by scavenging free redox-active iron (ferritin) released after heme catabolism (Kumar and Bandyopadhyay 2005). Hx-mediated transport of hemin is an active process, requiring cellular energy, Ca^{2+} and a minimum temperature for heme uptake and also depends on the ability of the cells to respond adequately (Smith and Morgan 1981). Haptoglobin (Hp), albumin, lipocalin alpha 1-microglobulin, heme binding proteins have been suggested but detailed mechanism is yet to be discovered (Van Vlierberghe, Langlois et al. 2004, Kumar and Bandyopadhyay 2005).

Hemin in the aqueous phase frequently aggregates in the membrane and promotes oxidation, which leads to the enhancement of permeability and membrane disorder. Oxidation of membrane components may promote cell lysis and death (Vincent 1989, Schmitt, Frezzatti et al. 1993). Free hemin is a pro-inflammatory molecule, activates neutrophil responses. Hemin increases the expression of interleukin-2 (IL-2) receptors in

human peripheral blood mononuclear cells and causes induction of mitogenicity, cytotoxicity and cytokine production (Tsuji, Wang et al. 1993). Hemin induces neutrophil chemotaxis, cytoskeleton reorganization as well as increased expression of chemokine interleukin-8 *in vitro* (Graca-Souza, Arruda et al. 2002). Hemin causes substantial hemolysis by affecting red cell membrane, stimulating potassium loss and swelling (Chou and Fitch 1981). This neurotoxic effect of hemin is iron-dependent, oxidative, and predominantly necrotic (Goldstein, Teng et al. 2003). In endothelial cells, hemin stimulates the expression of intracellular adhesion molecules (ICAM-1), vascular cell adhesion molecule1 (VCAM-1) and endothelial leukocyte adhesion molecules (E-selectin), probably through hemin-mediated generation of intracellular ROS and its subsequent signaling pathways (Shono, Ono et al. 1996).

Hemin catalyzes the degradation of proteins to small peptide fragments (Aft and Mueller 1984), oxidizes low- and high-density lipoproteins and forms conjugated dienes, thiobarbituric acid reacting substances (Camejo, Halberg et al. 1998), causes nicking and subsequent degradation of DNA (Kumar and Bandyopadhyay 2005). Even though hemin has been suggested to cause mitochondrial DNA damage (Suliman, Carraway et al. 2002), degradation product of hemin has been proposed to protect mitochondria (Mullebner, Moldzio et al. 2015). CO formed via degradation of hemin has been suggested to offer neuroprotection from hippocampal cell damage (Han, Yi et al. 2015) but bilirubin has been shown to induce endoplasmic reticulum (ER) stress in hepatocytes (Mullebner, Moldzio et al. 2015). Iron released from hemin breakdown is thought to damages lipids, proteins, and DNA through the generation of ROS (Rodriguez, Kemp et al. 2003).

1.2.3 Hemin and intracerebral hemorrhage

There is approximately 2.5 mM hemoglobin in blood (Robinson, Dang et al. 2009) and during ICH deposits of approximately 10 mM suspension of hemin is found in brain parenchyma (Letarte, Lieberman et al. 1993). To put this value into perspective, cell culture experiments have shown that 3–30 μM hemin is sufficient to kill 60–70% of cultured neurons and astrocytes within 4–14 h (Regan, Wang et al. 2001, Regan, Chen et al. 2004). Although initially sequestered within erythrocytes, some is released and accounts for the high micromolar concentration of hemin that is present in one week old intracranial hematomas (Robinson, Dang et al. 2009). The exact mechanisms responsible for this hemin accumulation to cytotoxic levels have not been defined clearly. Obviously brain tissue is a poor microenvironment for erythrocytes survival. Whether their lysis are due to complement activation (Hua, Xi et al. 2000), heme-mediated oxidative stress (Baysal, Monteiro et al. 1990) or other mechanisms is still poorly defined (Wagner and Dwyer 2004, Jaremko, Chen-Roetling et al. 2010).

At the physiological temperatures, extracellular Hb is oxidized spontaneously to ferric methemoglobin, releasing a superoxide ion in the reaction (Chen-Roetling, Lu et al. 2015) and then it transfers them to higher-affinity lipid and protein binding sites (Bunn and Jandl 1968). Due to brain microenvironment's low concentration of the Hx, there is likelihood of increased hemin uptake by vulnerable cell populations such as neurons and astrocytes. Hb in the hematoma is scavenged by microglia and infiltrating macrophages, which phagocytose intact erythrocytes as well as take up Hb and Hb-haptoglobin (Hp) complexes (Kristiansen, Graversen et al. 2001, Schaer, Schaer et al. 2006). After being taken up by these phagocytic cells, the fate of hemoglobin-bound heme remains unclear. It

is likely that some percentage is ejected into the extracellular space by different exporters such as ATP-binding cassette sub-family G member 2 (ABCG2) and Feline leukemia virus subgroup C receptor (FLVCR1) where it is expected to take part in free radical reactions (Yang, Philips et al. 2010, Chen-Roetling, Cai et al. 2014). The accumulation of hemin to toxic concentrations within the hematoma and its remediation provides an obvious therapeutic target for ICH.

1.3 Microglia

Microglia are the central nervous system (CNS) resident innate immune cells constituting approximately 5–10% of the brain cell population depending on the brain region. They are the first responders in a defense network that covers the entire CNS parenchyma. They respond to immunogenic stimulus by migrating towards injured regions and releasing a cascade of cytokines and recent studies have shown their involvement in the synapse formation (Tremblay, Stevens et al. 2011, Sierra, Tremblay et al. 2014). Mice microglia originate from a unique primitive myeloid progenitors in the yolk sac (Ginhoux, Greter et al. 2010). These progenitors migrate and colonize the brain during fetal development before brain vasculature arborization is complete and before the BBB is fully formed. In the adult brain, microglia are confined by the fully developed BBB and become an autonomous, long-lived cell population retaining the ability to divide and self-renew throughout life (Ajami, Bennett et al. 2007). Under resting physiological conditions, microglia have a small soma and numerous long and thin processes extending several times the length of the soma into the microenvironment. This unique cytostructure allows them to cover the entire parenchyma in a non-overlapping, mosaic fashion in the normal adult

brain. *In vivo* imaging has shown that microglial processes are highly motile even under 'resting' physiological conditions (Nimmerjahn, Kirchhoff et al. 2005). Microglia exhibit at least four functional behaviors: surveillance, neuroprotection, phagocytosis, and toxicity (Chen and Trapp 2016). Acute insults and chronic disease states induce hypertrophy of their cell bodies, asymmetrical distribution of their processes and increased expression of activation molecules. Microglial morphology has intricately been related to their differential functions against different types of insults (**Figure 1-3**) but quantitative relation has not been fully elucidated yet (Nayak, Roth et al. 2014).

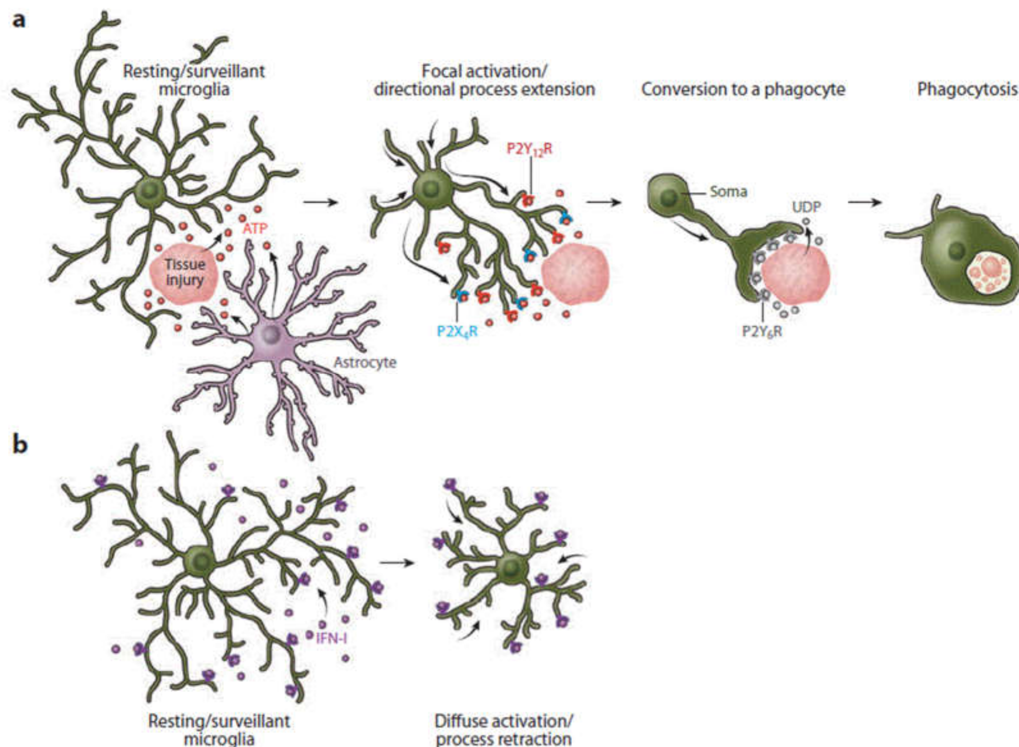


Figure 1-3: Microglia activation following CNS injury. Illustration of (a) focal and (b) diffuse microglia activation resulting from sterile brain injury and viral infection, respectively. (Adapted from Nayak et al., 2014 with permission from Annual Reviews).

1.3.1 Microglia and intracerebral hemorrhage

Microglial activation is a very early event after ICH (Zhao, Grotta et al. 2009, Taylor and Sansing 2013) and 'activated microglia' produce many pro- and anti-inflammatory mediators. The response of microglia is highly dependent on the age of the subject with more prominence in aged than in young (Gong, Hua et al. 2004). Pro-inflammatory M1 phenotype of microglia produce interleukin-1 beta (IL-1β), TNF-α, and ROS (Cherry, Olschowka et al. 2014). Such factors are known to activate other microglia and astrocytes, induce BBB disruption and cause the expression of adhesion molecules on the luminal surface of brain endothelial cells (Obermeier, Daneman et al. 2013), an important step for leukocyte migration into the brain. The M1 phenotype damage neurons by releasing oxidative metabolites and proteases (Kigerl, Gensel et al. 2009), induce a rapid switch towards M1 polarization (Kroner, Greenhalgh et al. 2014) and thus further aggravating the pathological conditions. In contrast, microglial cells with a M2 phenotype are primarily anti-inflammatory and secrete interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and arginase-1 which reduces NO production, phagocytic activity and is also involved in tissue repair (Zhao, Grotta et al. 2009, Cherry, Olschowka et al. 2014). M2 phase has also been suggested to produce pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), and IL-8, and are also thought as a potential contributor for angiogenesis after ICH (Welser, Li et al. 2010). Therefore, there has been considerable interest in targeting microglia activation as a very

early event in the inflammatory cascade or altering microglia phenotypes to M2 like phenotype to reduce ICH-injury.

After ICH, microglia strongly express HO-1, the inducible isoform of HO (Wang and Dore 2007), that converts hemin to iron, CO and biliverdin (Maines 1988). In order to prevent excessive ROS production, iron is temporarily stored within the phagocytes by iron-binding proteins, such as ferritin (Aronowski and Zhao 2011). This clean-up process persists 3–4 weeks after ICH onset (Zhao, Sun et al. 2007). Limiting extracellular hemoglobin degradation by early inhibition of HO-1 effectively ameliorated hemoglobin-induced brain edema in rats (Huang, Xi et al. 2002). However, in the later phase, HO-1 inhibition may interfere with phagocyte activity and the clean-up process after ICH (Aronowski and Zhao 2011). ICH induced extravasated erythrocytes are also cleared from brain parenchyma by microglia and thus microglia prevent further aggravation of secondary injury following ICH (Xi, Keep et al. 2006). Recent studies have shown that modulating phagocytic signal on erythrocytes could be a potential therapeutic target (Burger, de Korte et al. 2012, Zhou, Xie et al. 2014). It is generally accepted that microglia can have beneficial as well as detrimental effects during and after ICH and the effects of microglia inhibition could be planned time-dependently (Taylor and Sansing 2013). An alternate therapeutic approach might be to modify microglial activation to promote potential beneficial effects such as phagocytosis and tissue repair. In order to promote M2 phagocytic phenotype, peroxisome proliferator activated receptor-γ (PPARγ) agonists have shown benefit in animal ICH models and a PPARγ agonist is now in clinical trial for ICH (Gonzales, Shah et al. 2013). Further investigation into the

interaction of microglia and the mediators of ICH induced pathological cascade might lead to a better therapeutic treatment.

1.4 Cofilin

Cofilin was purified and identified as a protein with molecular weight of 20-kDa that induces depolymerization of actin filaments in an extract from avian or porcine brain (Nishida, Maekawa et al. 1984, Bamburg, McGough et al. 1999). The cofilin family in mammals is composed of non-muscle type, cofilin-1 (also known as n-cofilin), muscle type, cofilin-2 (also known as m-cofilin) and actin depolymerizing factor (ADF) (also known as destrin) (Ono 2007, Poukkula, Kremneva et al. 2011).

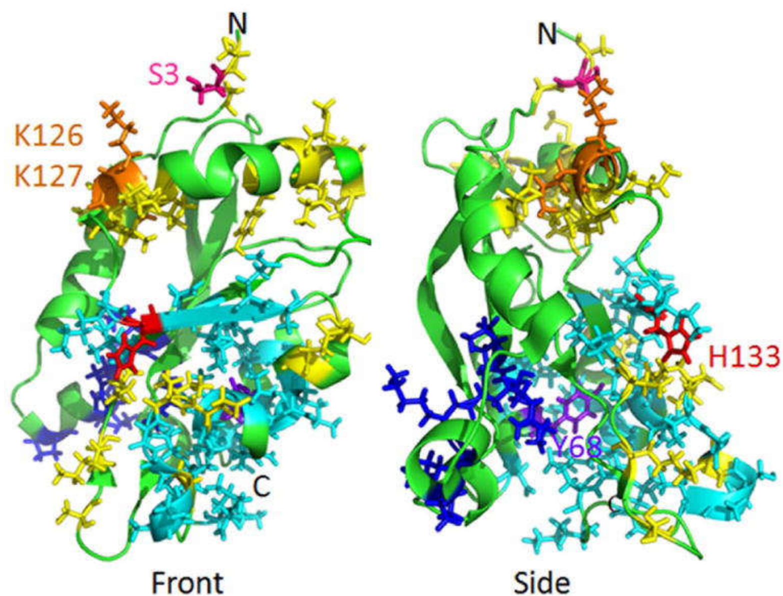


Figure 1-4: Human Cofilin Structure. The residues shown in yellow are the ones those are in the actin-contact regions (right side of the molecule in the side view), those in cyan are in the Phosphatidylinositol (3,4)-bisphosphate (PtdIns (3,4) P2) binding region, and

those in dark blue are in the nuclear localization sequence. Also shown here is the Ser3 regulatory phosphorylation site, the two Lys residues (K126 and K127) to which Ser3 binds when it is phosphorylated, His133 that confers pH-sensitivity to the PtdIns (3,4)P2-binding, and Tyr68 that serves as a Src substrate for phosphorylation prior to ubiquitination and degradation of cofilin (Adapted from (Bernstein and Bamburg 2010) with permission from Elsevier).

The following section will use cofilin to mean cofilin-1. Aside from this canonical cofilin role in actin cytoskeleton modification and thus in cell motility, migration, and cytokinesis, cofilin has also been linked to modulate the intracellular $Ca2+$ signaling, inflammation and different neurodegenerative diseases (Nusco, Chun et al. 2006, Schonhofen, de Medeiros et al. 2014, Ohashi 2015)

1.4.1 Cofilin and actin dynamics

Cofilin is distributed throughout the cytoplasm of the cell. It translocates to the cortical regions of the active cells in which the actin cytoskeleton is highly dynamic. It binds to ADP-bound F-actin and destabilizes and severs the actin filaments, resulting in depolymerization of the short actin filaments and such modulation depends on the ratio of cofilin to actin. At low cofilin/actin ratios, cofilin acts to sever actin filaments, whereas at high cofilin to actin ratio, cofilin nucleates actin assembly and stabilizes F-actin (Pollard and Borisy 2003). It enhances both actin filament disassembly and actin polymerization by severing and by supplying actin monomers, which results in higher dynamics of the actin cytoskeleton. Another suggested proposition of cofilin mediated actin severing is through

generation of short filaments and new barbed ends for actin assembly (Oser and Condeelis 2009). It promotes the generation of actin monomers, nuclei, and newly barbed ends for the assembly of actin structures such as lamellipodia, filopodia, and stress fibers, as well as the turnover of these structures during the cellular morphology changes. On the other hand, the inactivation of cofilin in response to extracellular signals decelerates the actin turnover and contributes to the assembly of actin structures (Ohashi, Fujiwara et al. 2011, Ohashi 2015).

1.4.2 Regulation of cofilin activity

Different regulatory kinases and phosphatases modulate cofilin activity through phosphorylation/dephosphorylation processes. Cofilin is inactivated by phosphorylation at Ser 3 by LIM kinase and testicular (TES) kinase and reactivated through dephosphorylation by the phosphatases like slingshot (SSH), chronophin, and phosphoprotein phosphatases (PP1/PP2A). Oxidative stress, proteins that modulate cofilin-actin interactions, phosphatidylinositol (4, 5) bisphosphate, and pH are also found to regulate cofilin activities. Kinases and phosphatases are activated in response to different intracellular and extracellular signals like ROS, elevation in intracellular Ca^{2+}, and drop in intracellular ATP, resulting in cofilin induced actin dynamic modulation (dos Remedios, Chhabra et al. 2003, Bamburg and Bernstein 2010, Bernstein and Bamburg 2010).

1.4.3 Cofilin and intracerebral hemorrhage

There is not any direct experimental evidence showing the relation between cofilin and ICH but increasing evidence have suggested that the regulators of cofilin are affected

by ICH pathophysiology (Alhadidi, Bin Sayeed et al. 2016). Studies have shown that both the excess cofilin activity as well as defect in cofilin expression inhibits its proper functions in the cells. The dynamic imbalance of cofilin activity is one of the major pathophysiological features of ICH (Rust 2015). ICH leads to phosphatase activation in response to ROS mediated elevation in intracellular Ca^{2+} and drop in intracellular ATP, resulting in cofilin over-activation and actin dynamic disruption (Alhadidi, Bin Sayeed et al. 2016). ICH induced oxidative stress has been proposed to be the mechanism of both cofilin over-activation and excitotoxicity. Increased ROS affect cysteine residue in cofilin molecules resulting in intramolecular disulfide bond formation. Such structural modification disrupts cofilin-actin interaction and frees cofilin to translocate into the mitochondria, where it opens the permeability transition pore and initiates cytochrome C release, eventually leading to apoptosis. Inhibition of both cofilin expression with cofilin siRNA and cofilin oxidation prevented oxidant-induced apoptosis, suggesting the central role of oxidative stress in triggering cofilin-induced apoptosis (Zhang, Wang et al. 2004, Klamt, Zdanov et al. 2009). Studies have shown that oxidation of cofilin leads to oxidant-induced apoptosis (Klamt, Zdanov et al. 2009) as well as caspase-independent necrotic-like programmed cell death (Wabnitz, Goursot et al. 2010).

ICH induced ionic imbalance that in turn inhibits the uptake of glutamate by glutamate transporters and even causes the reversal of the transporters, induces over-accumulation of extracellular glutamate (Rossi, Oshima et al. 2000). Studies have shown that cofilin activation mediate Bax translocation to the mitochondria during excitotoxic neuronal death (Posadas, Perez-Martinez et al. 2012) and both N-methyl-D-aspartate (NMDA) and non-NMDA receptors mediate glutamate stimulation-induced cofilin

dephosphorylation and cofilin-actin rod formation (Chen, Jiang et al. 2012). Following ICH, glial cells release a wide array of pro-inflammatory cytokines, chemokines, and free radicals. Therefore, inhibiting the inflammatory response can reduce injury and improve neurological outcomes. But inhibiting cofilin might not reduce inflamamation fully resulting in low grade inflammatory response during the chronic phase which in turn can lead to repair, regeneration, and functional recovery (Zhou, Wang et al. 2014). Cofilin has also been suggested to play a major role in ICH glial cell migration and proliferation (Li, Brieher et al. 2007, Jonsson, Gurniak et al. 2012). Cofilin knockdown in microglia inhibited NADPH oxidase activity and ROS formation (Rasmussen, Pedersen et al. 2010). Therefore, inhibition of the cofilin might prevent progression of over-activation induced inflammation and might also reduce the levels of the injury.

1.5 Aim and hypothesis

The primary goal of this investigation is to study the role of cofilin in different microglial functions as well as underlying molecular mechanism induced by high doses of hemin exposure to mimic the condition of ICH.

Aim 1: Evaluate the role of cofilin in hemin induced microglia inflammation and survival. This can be done by exposing microglia to high doses of hemin followed by studying the levels of NO, nitric oxide synthase (iNOS), cytokines, and cell viability. To study the role of cofilin, microglia were transfected with cofilin siRNA followed by similar experimental studies. Microglia are the main source of NO in ICH (Zhou, Wang et al.

2014); increased iNOS and higher cytokines levels are the markers of pathogenesis of ICH (Kim, Im et al. 2009, Lin, Yin et al. 2012, Ndisang and Jadhav 2013) and extremely high doses of hemin presumably affect the viability of all cell types of brain parenchyma in ICH (Egashira, Hua et al. 2015). Therefore, these investigations are highly relevant to the context of ICH.

Aim 2: Investigate the role of cofilin in hemin induced microglial migration. This can be accomplished by exposing microglia to hemin followed by streaking a scratch in the culture media and then counting the number of cells migrated to the area of scratch. Similar experimental paradigm would be followed after cofilin siRNA transfection in microglia for studying the role of cofilin. Migration of microglia towards the site of injury is one of the primary functions of microglia (Kettenmann, Hanisch et al. 2011) and it has been suggested to be mediated by cofilin (Bernstein and Bamburg 2010). Hemin at a very high concentration as in the case of ICH is hypothesized to affect migration of microglia.

Aim 3: Illustrate the role of cofilin in microglial antioxidant defense system. By incubating microglia with hemin followed by the protein extraction and expression by Western blotting of hemin degrading enzyme, HO-1 (Chen-Roetling, Lu et al. 2015) and one of the upstream regulators of HO-1, nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Gonzalez-Reyes, Guzman-Beltran et al. 2013) a broad overview of antioxidant defense system of microglia due to hemin exposure could be studied. Microglia transfection by cofilin siRNA followed by the same experimental paradigm would show the role of cofilin in microglial antioxidant defense system relevant to hemin exposure. Studies have shown the cytoprotective role of HO-1 and Nrf2 in different cellular insults (Gozzelino, Jeney et al. 2010, Paine, Eiz-Vesper et al. 2010, Li, Wang et al. 2014) and therefore, the

investigation on the role of cofilin in hemin induced microglial cytoprotective antioxidant system is highly relevant to those in case of ICH.

Aim 4: Elucidate the role of cofilin in hemin induced ER stress and Ca^{2+} signaling in microglia. Incubation of microglia with hemin followed by ribonucleic acid (RNA) extraction, complementary DNA (cDNA) synthesis and then quantifying ER stress makers such as Wolframin ER transmembrane glycoprotein (Wfs-1), X-box binding protein-1 (XBP-1), sliced XBP-1with reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) would provide the extent of ER stress due to hemin exposure. Cofilin siRNA transfection of microglia followed by similar experimental paradigm would give the role of cofilin in hemin induced ER stress markers. Studies have shown that hemin affect cytostructure of the cell and increase ER stress in endothelial cell (Mullebner, Moldzio et al. 2015). Therefore, investigation on the role of cofilin in hemin induced ER stress in microglia is highly relevant to ICH. ER is one the primary sources of intracellular Ca^{2+} that plays a critical role in the process of microglial transformation and migration (Sharma and Ping 2014, Tsai, Seki et al. 2014). Ca^{2+} signaling measurement after acute exposure of hemin as well as after long term exposure of hemin would provide answer to the questions whether hemin evokes Ca^{2+} signaling and whether hemin exposure affect the efficacy of microglial ability to respond to acetylcholine evoked Ca^{2+} signaling respectively. Experimentation with cofilin siRNA transfected microglia would offer the role of cofilin in evoking Ca^{2+} signaling.

Chapter 2

Materials and Methods

2.1 Cell culture

Spontaneously Immortalized Microglial Cells (SIM-A9 Cells) were generous gift from Dr. Kumi Nakamoto-Combs at the University of North Dakota and were grown in Dulbecco's Modified Eagle Medium (DMEM): Nutrient Mixture F-12 (HyClone, Thermo scientific, West Palm Beach, FL, USA) supplemented with 5% horse serum (HS), 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Pheochromocytoma cell with embryonic origin from the neural crest (PC-12) cells (ATCC, Manassas, VA, USA) were grown in DMEM supplemented with 10% horse serum (HS), 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin. To study the impact of cofilin knockdown in hemin activated microglia on the survival of PC-12 cells, a conditioned medium from microglial cells was added to the PC-12 cells and cells were incubated for 24 h.

2.2 Microglial transfection by siRNA

SiGENOME SMARTpool mouse cofilin1 siRNA with the following sequences AGACAAGGACUGCCGCUAU, GGUGGCAGCGCCGUCAUUU, GUUCGCAAGUCUUCAACAC, and GAGAAUGCACCCCUCAAGA was ordered from

GE Healthcare Dharmacon, Inc. In addition, scrambled siRNA was used as a control. SIM-A9 cells were plated at the density of $1.25X10^5$ cells/ml. The cells were transfected with scrambled/cofilin1 siRNA at 50 nanomolar (nM) concentrations according to previously optimized conditions in our lab. Xfect transfection reagent (Clontech Laboratories, Inc) was used to perform the transfection experiment according to the manufacturer's instructions. Briefly, in two separate sets of microtubes, siRNA and Xfect transfection reagent were diluted using Xfect buffer. Then, the transfection reagent was added to the siRNA and was kept at room temperature for 20 min to ensure complex formation. After that, transfection mix was added to the cell culture and then incubated back for 48 h before starting hemin treatment.

2.3 Hemin induced microglial activation and nitrite oxide production

Hemin at 75, 100, 200, 500 micromolar (μM) concentration were used initially to activate microglia and then 100 μM were selected for subsequent experiments due to consistent result in NO production. SIM-A9 cells were plated in 24-well plate and then transfected with scram/cofilin siRNA for 48 h prior to hemin activation. Cell culture medium from the respective wells was mixed with equal volume of Griess reagent (Sigma-Aldrich) in 96-well plate at room temperature. The amount of NO released into cell culture medium was quantified calorimetrically at 540 nm according to the manufacturer instructions.

2.4 ELISA assay

SIM-A9 cells were plated in 24-well plate, transfected with scrambled/cofilin siRNA and then 100 μM hemin was added to the cells. After that, the levels of TNF-α and IL-1β released into cell culture medium were quantified using commercially available

enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA, USA) according to the manufacturer protocol.

2.5 MTT-cell survival assay

Cell viability assays were determined using the 3-[4,5-dimethyl- thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. In this assay, the viability of siRNA transfected SIM-A9 cells treated with hemin as well as PC-12 cells, treated with microglia conditioned medium, were measured. MTT assay protocol involves incubation of the cells (SIM-A9 or PC-12 cells) with MTT reagent (Promega Corporation, Madison, WI, USA) for 3 h in 5% CO_2 at 37°C. After that, the whole medium was discarded and DMSO was added to dissolve formazan crystals. Viable cells were quantified by measuring the absorbance at 570 nm.

2.6 Scratch migration assay

SIM-A9 cells were cultured in 6-cm plate and then transfected with scrambled/cofilin siRNA for 48 h. Hemin at a dose of 100 μM were added to the cells for 20 h. By using a sterile 200 μl pipette tip, a scratch was made in the middle of each well followed by washing with PBS to remove damaged cell remnants. Then similar media as used earlier were added to the cells. At different time intervals (3 h, 12 h, 24 h of scratch and 23 h, 32 h and 44 h of hemin treatment), cells migrated to the scratched area were imaged and then the number of cells were counted by using ImageJ software (NIH, USA).

2.7 Subcellular fractionation and western blotting

To study protein expression levels by Western blotting (WB), SIM-A9 cells were plated in 6-cm plate and then stimulated with 100 μM hemin for 24 h before cell lysis. To

obtain subcellular fractions, SIM-A9 cells were harvested using ice- cold lysis buffer [250 mM sucrose, 1.5 magnesium chloride ($MgCl_2$), 10 mM potassium chloride (KCl), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM sodium fluoride (NaF), 10 mM Na vanadate, 20 mM Na pyrophosphate and protease inhibitor cocktail (Thermo Scientific)]. Cell homogenates were kept on ice for 15 min and then Nonidet P-40 (NP-40) was added at 0.5% for 2 min extra. The resultant supernatant was used for studying protein expression. Bradford reagent (Bio-Rad Laboratories, CA, USA) were used for measuring protein concentrations, and samples were analyzed by loading equivalent amounts of protein (20-30 µg) onto 10–15% SDS-polyacrylamide gels. Proteins separated on the gels were transferred onto a pretreated polyvinylidene fluoride (PVDF) membrane and were blocked with 3% bovine serum albumin (BSA) for 1 h to prevent nonspecific binding. The membrane was then incubated overnight at 4°C with the following primary antibodies: rabbit anti-cofilin, rabbit anti-α-tubulin, rabbit anti-GAPDH, rabbit anti-HO-1, rabbit anti- Nrf2, and rabbit anti- iNOS (1:2000; Cell Signaling Technology, Danvers, MA, USA). Following the incubation, the blots were washed and incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody (1:2000; Jackson ImmunoResearch) for 1 h at room temperature. Anti-rabbit α-Tubulin, rabbit anti-GAPDH were used as a loading control for cytosolic proteins. The images were analyzed using Bio-Rad ChemiDoc™ XRS Image Lab Software.

2.8 RT-qPCR

SIM-A9 cells were transfected with scramble/cofilin-1 siRNA for 48 h followed by hemin treatment for 24 h. Total RNA was extracted from SIM-A9 cells with the treatments

as mentioned earlier by using TRIzol method (Life technologies) according to the manufacturer's protocol. Then 1 µg total RNA was transcribed into cDNA templates in a 20 µl a reaction mix of 5X iScript reaction mix, iScript reverse transcriptase, and nuclease-free water (Bio Rad, Hercules, CA). PCR was performed in a 10 µl reaction, containing 5 µl cDNA (1/5 diluted), 1X SYBR Green PCR Master Mix (Applied Biosystems) and 100 nM of each primer. Ct values (cycle threshold) were used to calculate the amount of amplified PCR product relative to 18S (the endogenous control). The relative amount of mRNA was calculated using the ΔΔCT method. Minus-reverse transcriptase samples were used as negative controls to test for DNA contamination. The experiments were repeated thrice with duplication. For ER stress, Wfs-1, XBP1 and spliced XBP1 were used whose primers are presented in **Table 2-1**.

Table 2-1: Primers sequences

Genes	Primer Forward Sequence (5'-3')	Primer Reverse Sequence (5'-3')
Wfs-1	CGCCTCGTCAGCAGTGAAT	GGAACAGGTTGGTGGGAATG
XBP-1	TGGCCGGGTCTGCTGAGTCCG	GTCCATGGGAAGATGTTCTGG
Spliced XBP-1	CTGAGTCCGAATCAGGTGCAG	GTCCATGGGAAGATGTTCTGG
18S	TTCGAACGTCTGCCCTATCAA	ATGGTAGGCACGGCGACTA

2.9 Calcium signaling

SIM-A9 cells were transfected with scramble/cofilin siRNA for 48 h followed by hemin treatment for 24 h. Then the cells were incubated with fura-2 AM for 1 h at 37°C. Fluorescence images were obtained using a polychrome IV monochromator-based high-speed digital imaging system (TILL Photonics, Gräfelfing, Germany) ported to a fiber optic guide and epifluorescence condenser and coupled to a Nikon TE2000 microscope equipped with DIC optics. Cytosolic Ca^{2+} concentration in fura-2 AM-loaded SIM-A9 cells was

measured by alternatively illuminating the SIM-A9 cells with dual wavelength light (340 and 380 nm) focused onto the image plane via a DM400 dichroic mirror and Nikon SuperFluor ×40 oil-immersion objective, and fluorescence obtained through a 525 ± 25 nm band-pass filter (Chroma Technologies, Brattleboro, VT, USA). Alternating transmitted light and fluorescent images were acquired using a high-speed Uniblitz VS35 optical shutter (Vincent Associates, Rochester, NY, USA) placed in the tungsten lamp illumination path in experiments. Pairs of transmitted light and fluorescence images (30 and 50 ms exposure, respectively) were collected at 2 Hz. Images were generated by subtracting each transmitted light frame from its preceding frame as described previously (Chen, Warner et al. 2005).

2.10 Statistical Analysis

The experimental results were expressed as the mean ± standard error of mean (SEM) and are accompanied by the number of observations (independent preparations of cultured cells). Data were analyzed by ANOVA followed by Bonferroni post Hoc using Graph Pad Prism (GraphPad Software, San Diego, CA) and IBM SPSS Statistics 22. Furthermore, student's unpaired t-test was used to determine significant differences between two groups. A value of $p<0.05$ is considered to be statistically significant.

Chapter 3

Results

3.1 Cofilin knockdown changed microglial cells morphology

SIM-A9 cells assume ramified morphology in cell culture DMEM/F12 media and most of the cells transfected with scramble siRNA assumed spindle shape. Some assume globular shape indicating microglial functional state to stress conditions (**Figure 3-1A**). On the other hand, SIM-A9 cells transfected with cofilin siRNA assumed globular morphology (**Figure 3-1B**). There was no signs of pointed region in any corner of the cell whereas in scramble siRNA transfected SIM-A9 cells, pointed region in one or more than one regions was observed. Based on our optimized transfection experiment there was more than 80% reduction of cofilin protein expression as observed by the WB protein analysis (**Figure 3-1C**).

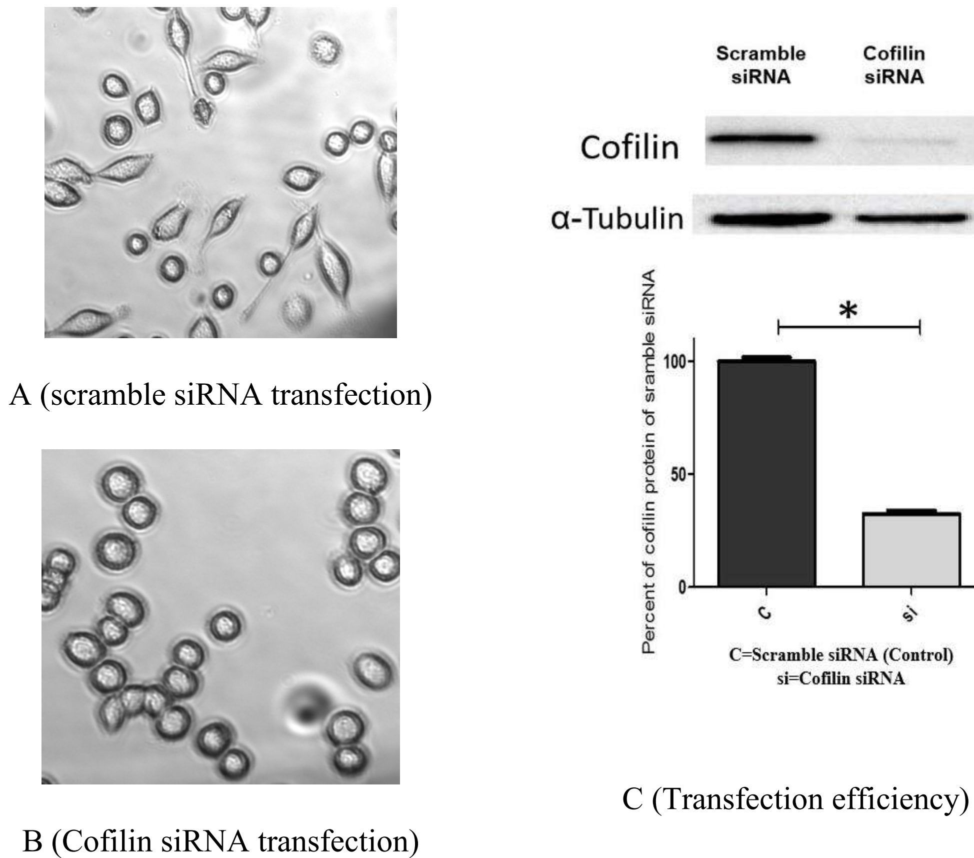


Figure 3-1: SIMA9 cell morphology and cofilin expression after transfection by scramble (A) and cofilin siRNA (B). Images were taken at 20X bright field and protein expression was performed by Western blot analysis (C). The Western blot results were expressed as mean ± SEM of the ratio of cofilin and α-tubulin. One-way ANOVA followed by Bonferroni post hoc was performed and the asterisk (*) indicates statistically significant difference and the value of $p<0.05$ was considered significant (n=3).

3.2 Hemin dose adjustment and nitrite Oxide production

Previously, different doses of hemin were used to induce microglia activation (Huang, Xi et al. 2002, Lin, Yin et al. 2012, Hanafy 2013). In order to mimic ICH conditions, higher dose of hemin such as 75 µM, 100 µM, 200 µM and 500 µM were

initially selected. It was found that there was a dose dependent increase of NO production (**Figure 3-2**) but due to interference with NO detection at higher hemin dose exposure levels (200 and 500 μM), 100 μM hemin was selected for subsequent hemin treatments.

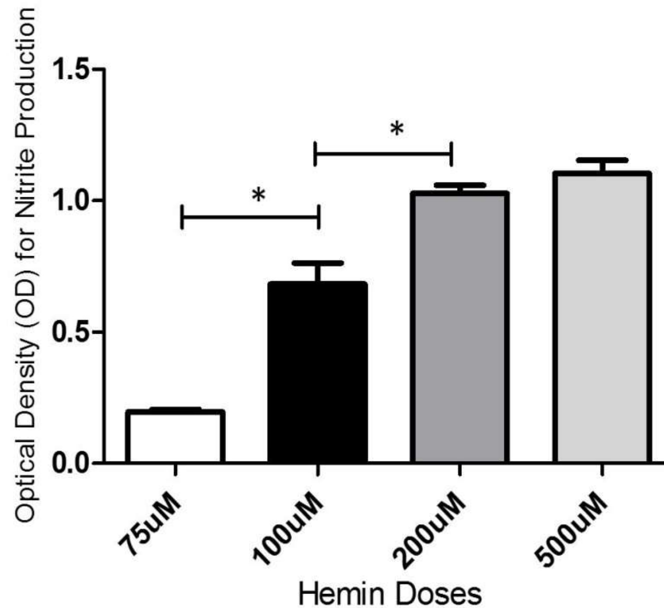


Figure 3-2: Dose dependent hemin treatment-induced nitrite oxide (NO) production. There was gradual increase in the production of NO production due to increase in hemin dose of 75 μM, 100 μM, 200 μM. But further increase of hemin dose after 200 μM lead to interference with optical detection of NO production and therefore giving rise to inconsistence results. The results were expressed as the mean ± SEM of OD; 3 independent experiment in triplicate. One-way ANOVA followed by Bonferroni post hoc was performed and the asterisk (*) indicates statistically significant difference and the value of $p<0.05$ was considered significant.

3.3 Hemin induces increased cofilin expression

Our lab has previously shown that lipopolysaccharide (LPS) increases the expression of cofilin (unpublished results). Similar conditions were applied in case of hemin treatment in microglia in order to investigate the effect of hemin treatment on cofilin expression and it was found that there was significant increase in cofilin expression ($p<0.05$) after hemin treatment (**Figure 3-3**).

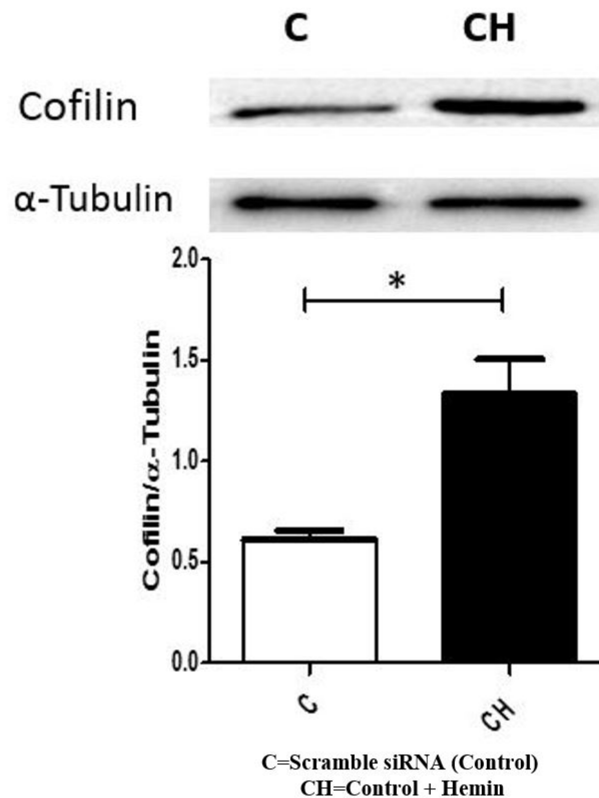


Figure 3-3. **Hemin treatment-induced increased expression of cofilin.** The Western blot results were expressed as mean ± SEM of the ratio of cofilin and α-tubulin. Independent t test was performed and the asterisk (*) indicates statistically significant difference and the value of $p<0.05$ was considered significant (n=3).

3.4 Cofilin mediates hemin treatment-induced iNOS production

There was a significant difference in microglial iNOS levels among different treatment groups with F (3, 8) = 680.4, p<0.0001. Bonferroni post Hoc test indicated that there was no significant difference in iNOS expression levels between scramble siRNA and cofilin siRNA transfected microglia (p>0.05). However, scramble siRNA transfected microglia treated with hemin showed significantly higher levels of iNOS (p=0.000) as compared to scramble siRNA transfected microglia without hemin treatment group (control). On the other hand, significantly reduced (p=0.000) iNOS levels were observed in cofilin siRNA transfected microglia treated with hemin group as compared to scramble siRNA transfected microglia treated with hemin. These results indicate that microglia iNOS expression increase due to long term exposure to hemin treatment is mediated by cofilin **(Figure 3-4)**.

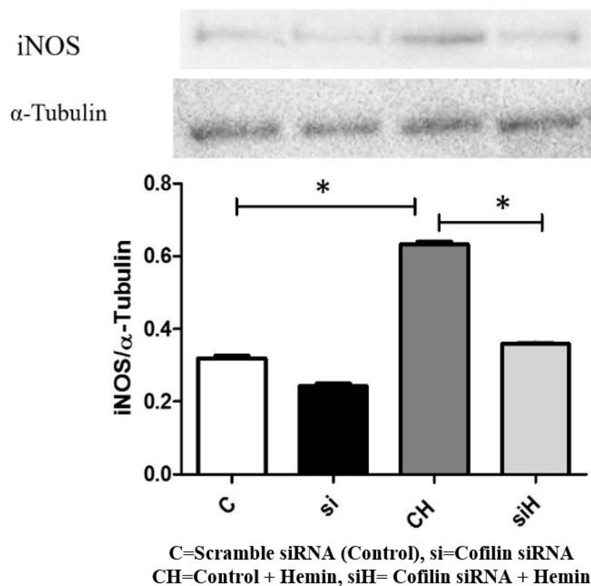


Figure 3-4: Cofilin mediates hemin treatment-induced increase in iNOS production. The Western blot results were expressed as mean ± SEM of the ratio of

iNOS and α-tubulin. One-way ANOVA followed by Bonferroni post hoc was performed and the asterisk (*) indicates statistically significant difference and the value of $p<0.05$ was considered significant (n=3).

3.5 Cofilin knockdown inhibits hemin treatment-induced microglial TNF-α production but not IL-1β

In this investigation, there was a significant difference in microglia derived TNF-α levels among different treatment groups with $F_{(3, 32)} = 28.14$ $p=0.000$. Bonferroni post Hoc test indicated that there was no significant difference in TNF-α levels between scramble siRNA and cofilin siRNA transfected microglia ($p>0.05$). However, scramble siRNA transfected microglia treated with hemin showed significantly higher levels of TNF-α ($p=0.000$) as compared to scramble siRNA transfected microglia without hemin treatment group (control). On the other hand, significantly reduced ($p=0.000$) TNF-α levels were observed in cofilin siRNA transfected microglia treated with hemin and as compared to scramble siRNA transfected microglia treated with hemin. These results indicate that increased TNF-α released from microglia is due to long term exposure to hemin treatment which is mediated by cofilin (**Figure 3-5A**).

On the other hand, there was no significant difference in microglia derived IL-1β levels among different treatment groups with $F_{(3, 32)} = 0.6468$, $p= 0.5907$. Bonferroni post Hoc test indicated that there was no significant difference in IL-1β levels between scramble siRNA and cofilin siRNA transfected microglia ($p>0.05$). Difference was also not observed in scramble siRNA transfected microglia treated with hemin derived IL-1β ($p=1.000$) as compared to scramble siRNA transfected microglia without hemin treatment

group (control). Similarly, there was no significant difference observed in IL-1β level (p=0.067) between cofilin siRNA transfected microglia treated with hemin and scramble siRNA transfected microglia treated with hemin. These results indicate that increased IL-1β released from microglia is not affected due to long term exposure to hemin treatment (**Figure 3-5B**).

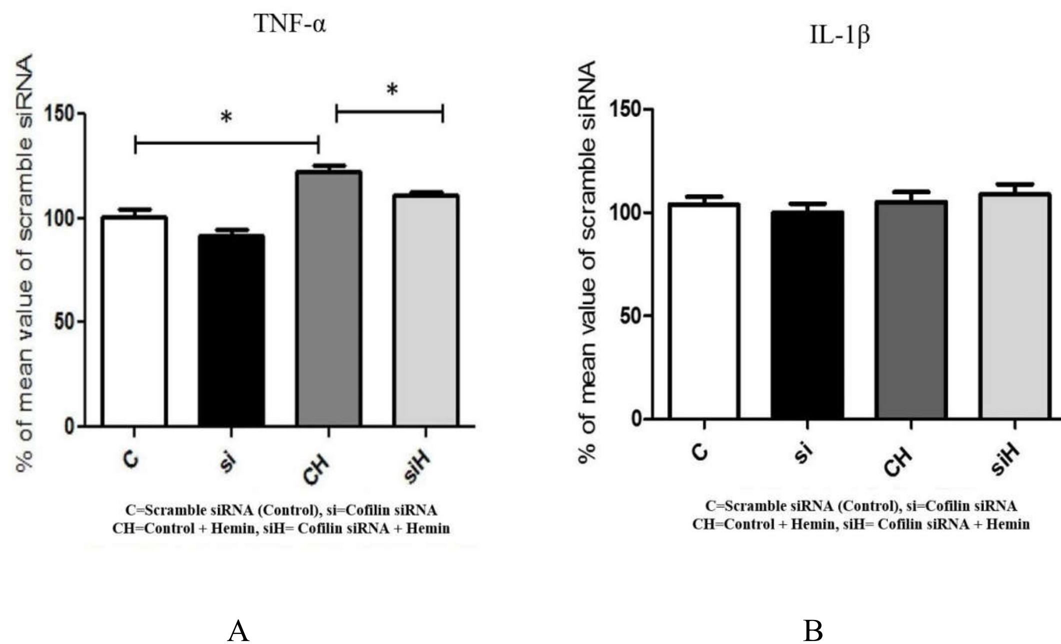


Figure 3-5: Hemin induced cytokine production. The ELISA results for TNF-α (A) and IL-1β (B) were expressed as mean ± SEM of the percent of the mean optical density (OD) of the control group. One-way ANOVA followed by Bonferroni post hoc was performed and the asterisk (*) indicates statistically significant difference and the value of $p<0.05$ was considered significant (n=3 in triplicate).

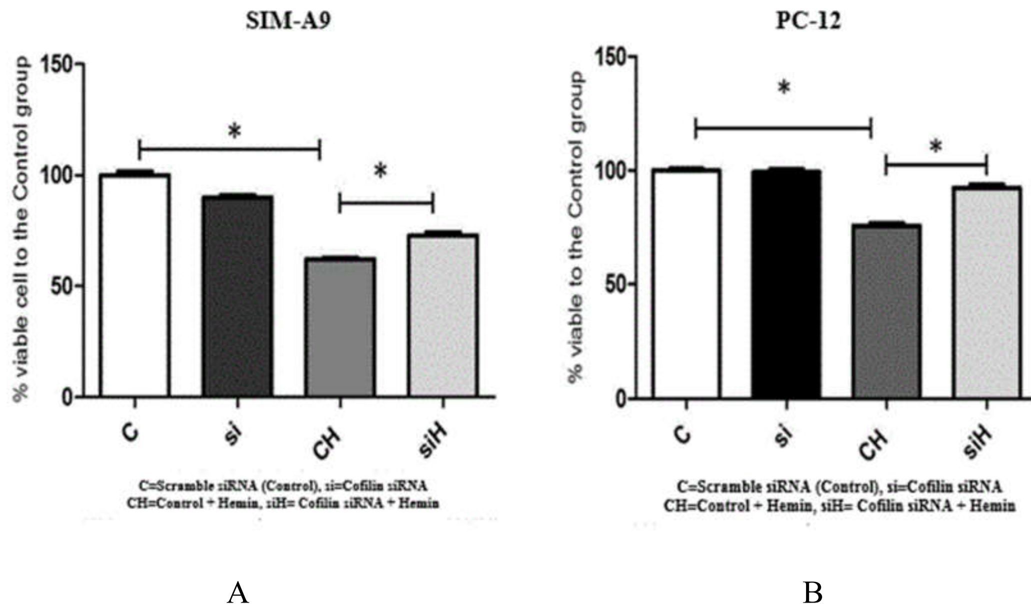
3.6 Cofilin knockdown reduces hemin treatment-induced microglial cell death as well as protects undifferentiated PC-12 cells from neurotoxicity from conditioned medium of hemin activated microglia

There was a significant difference in microglial viability between different treatment groups with F (3, 32) = 81.05, p=0.000. Bonferroni post Hoc test indicated that there was no significant difference in cell viability between scramble siRNA and cofilin siRNA transfected microglia (p>0.05). However, scramble siRNA transfected microglia treated with hemin showed significantly lower cell viability (p=0.000) as compared to scramble siRNA transfected microglia without hemin treatment group (control). On the other hand, significantly higher cell viability (p=0.000) were observed in cofilin siRNA transfected microglia treated with hemin and as compared to scramble siRNA transfected microglia treated with hemin. These results indicate that decreased microglial viability due to long term exposure to hemin treatment is mediated by cofilin **(Figure 3-6A)**.

To test the neurotoxic potential of the activated microglia, conditioned medium of transfected and hemin treated microglia was added to undifferentiated PC-12 cells and incubated for 24 h. The viability of treated PC-12 cells was measured using MTT assay.

There was a significant difference in PC-12 viability between different treatment groups with F (3, 32) = 86.11, p=0.000. Bonferroni post Hoc test indicated that there was no significant difference in cell viability between scramble siRNA and cofilin siRNA transfected group (p>0.05). However, scramble siRNA transfected group treated with hemin showed significantly lower cell viability (p=0.000) as compared to scramble siRNA transfected group without hemin treatment group (control). On the other hand, significantly higher cell viability (p=0.000) was observed in cofilin siRNA transfected group treated

with hemin and as compared to scramble siRNA transfected group treated with hemin. These results indicate that decreased PC-12 viability due to long term exposure to hemin treatment is mediated by cofilin (**Figure 3-6B**).



A B

Figure 3-6: Cell viability assay after; hemin treatment in SIM-A9 cells (A) and exposure of undifferentiated PC-12 cells to conditioned media from SIM-A9 cells (B). The MTT assay results were expressed as mean ± SEM of the percent of the viable cells to the control group. One-way ANOVA followed by Bonferroni post hoc was performed and the asterisk (*) indicates statistically significant difference and the value of $p<0.05$ was considered significant (n=3 in triplicate).

3.7 Cofilin knockdown inhibits microglial cell migration

Scratch assay was conducted to test the effect of hemin as well as cofilin knockdown on microglial cells migration **(Figure 3-7).** Only at 3 h of scratch and 23 h of

hemin treatment there was significant reduction of cell migration ($p<0.05$) but in all time points the number of microglia migrated to the scratched area was significantly less in cofilin siRNA transfected cells (**Figure 3-7**). Different time periods were considered to reduce the impact of cell proliferation in the scratched area.

At 3 h of scratch and 23 h of hemin treatment, there was significant difference among different treatment groups with $F(3, 44) = 6.944$, $p=0.001$. Bonferroni post Hoc test indicated that there was a significant difference in migration between scramble siRNA and cofilin siRNA transfected microglia ($p=0.031$). Similar results were obtained ($p=0.025$) in case of scramble siRNA transfected microglia treated with hemin as compared to scramble siRNA transfected microglia without hemin treatment group (control). There was significantly reduced number of microglia ($p=0.000$) in cofilin siRNA transfected microglia treated with hemin group as compared to scramble siRNA transfected microglia treated with hemin. This indicated that microglial migration is reduced due to both hemin treatment as well as cofilin knockdown at 3 h after scratch and 23 h after hemin treatment.

At 12 h of scratch and 32 h of hemin treatment, there was significant difference among different treatment groups with $F(3, 44) = 10.152$, $p=0.000$. Bonferroni post Hoc test indicates that there was significant difference in migration between scramble siRNA and cofilin siRNA transfected microglia ($p=0.010$). However, such difference was not observed ($p=1.00$) in case of scramble siRNA transfected microglia treated with hemin and as compared to scramble siRNA transfected microglia without hemin treatment group (control). There was significantly reduced number of microglia ($p=0.000$) in cofilin siRNA transfected microglia treated with hemin group as compared to scramble siRNA transfected microglia treated with hemin. This indicated that microglial migration at 12 h of scratch

and 32 h of hemin treatments is reduced due to cofilin knockdown but hemin was not found to affect migration of microglia neither in scramble siRNA group nor in cofilin siRNA group.

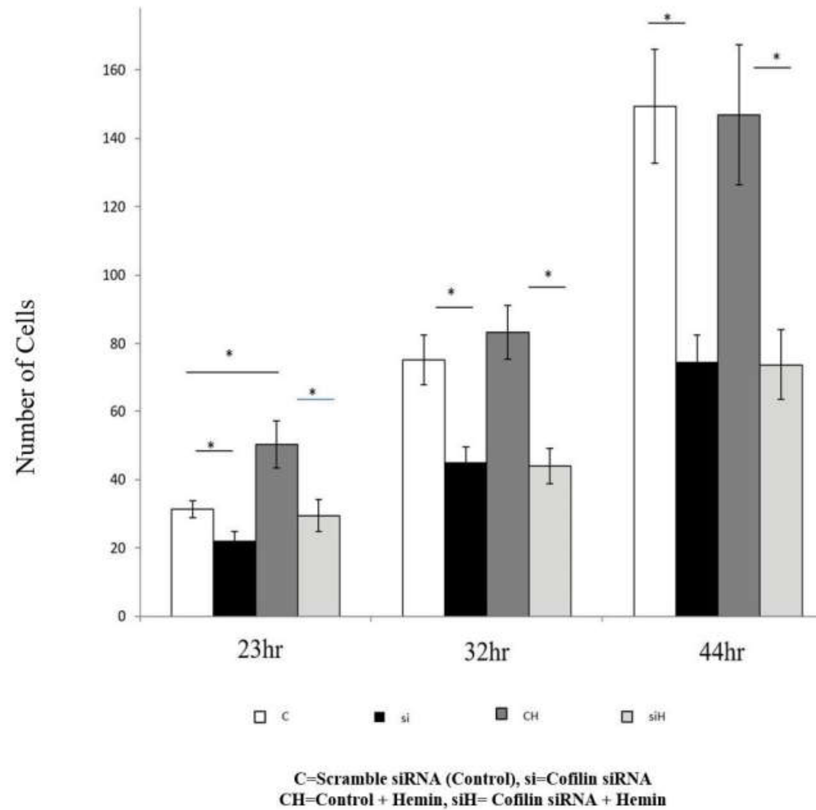


Figure 3-7: **Cofilin mediated microglial migration after hemin treatment**. The number of microglia migrated to the area of scratch in the arbitrary dimension of 330X800 or 800X330 depending of the orientation of the scratch. The cells were counted manually with the help of Image J software (NIH, US). The results were expressed in mean ± SEM of the number of the cells migrated to the randomly chosen area with fixed dimension within scratch area. One-way ANOVA followed by Bonferroni post hoc was

performed and the asterisk (*) indicates statistically significant differences and the value $p<0.05$ was considered significant (n=3).

At 24 h of scratch and 44 h of hemin treatment of scratch, there was significant difference among different treatment groups with $F (3, 44) = 8.428$, $p=0.000$. Bonferroni post Hoc test indicates that there was significant difference in migration between scramble siRNA and cofilin siRNA transfected microglia ($p=0.005$). However, such difference was not observed ($p=1.00$) in case of scramble siRNA transfected microglia treated with hemin as compared to scramble siRNA transfected microglia without hemin treatment group (control). There was significantly reduced number of microglia ($p=0.000$) in cofilin siRNA transfected microglia treated with hemin and as compared to scramble siRNA transfected microglia treated with hemin. This indicated that microglial migration at 24 h of scratch and 44 h of hemin treatments is reduced due to cofilin knockdown but hemin was not found to affect migration of microglia neither in scramble siRNA group nor in cofilin siRNA group.

3.8 Cofilin knockdown increases hemin treatment-induced HO-1 and Nrf2 expression

There was a significant difference in microglial HO-1 expression among different treatment groups with $F (3, 8) = 28.35$, $p<0.0001$. Bonferroni post Hoc test indicated that there was no significant difference in HO-1 expression levels between scramble siRNA and cofilin siRNA transfected microglia ($p>0.05$). However, scramble siRNA transfected microglia treated with hemin showed significantly higher levels of HO-1 expression ($p=0.000$) as compared to scramble siRNA transfected microglia without hemin treatment

group (control). On the other hand, significantly higher (p=0.000) HO-1 expression levels were observed in cofilin siRNA transfected microglia treated with hemin and as compared to scramble siRNA transfected microglia treated with hemin. These results indicate that microglial HO-1 expression is increased due to long term exposure to hemin treatment and cofilin knockdown further increased HO-1 expression induced by hemin treatment **(Figure 3-8)**.

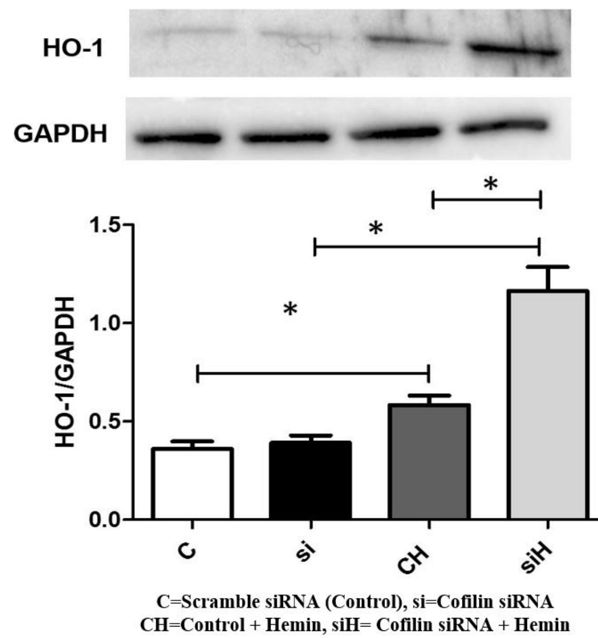


Figure 3-8: Cofilin mediates hemin treatment-induced hemoxygenase- 1 (HO-1) expression. The Western blot results were expressed as mean ± SEM of the ratio of HO-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). One-way ANOVA followed by Bonferroni post hoc was performed and the asterisk (*) indicates statistically significant difference and the value $p<0.05$ was considered significant (n=3).

There was a significant difference in microglial Nrf2 expression among different treatment groups with $F(3, 8) = 51.15$, $p<0.0001$. Bonferroni post Hoc test indicated that there was no significant difference in Nrf2 expression levels between scramble siRNA and cofilin siRNA transfected microglia ($p>0.05$). However, scramble siRNA transfected microglia treated with hemin showed significantly higher levels of Nrf2 expression ($p=0.000$) as compared to scramble siRNA transfected microglia without hemin treatment group (control). On the other hand, significantly higher ($p=0.000$) Nrf2 expression levels were observed in cofilin siRNA transfected microglia treated with hemin group as compared to scramble siRNA transfected microglia treated with hemin. These results indicate that microglial Nrf2 expression is increased due to long term exposure to hemin treatment and cofilin knockdown further increased Nrf2 expression induced by hemin treatment **(Figure 3-9)**.

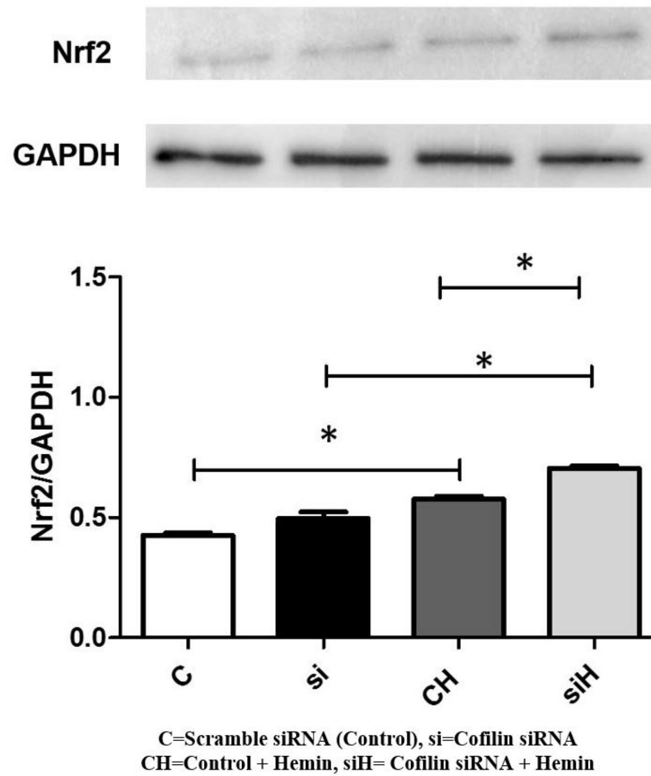


Figure 3-9: Cofilin mediates hemin treatment-induced nuclear factor (erythroid-derived 2)-like 2 (Nrf2) expression. The Western blot results were expressed as mean ± SEM of the ratio of Nrf2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). One-way ANOVA followed by Bonferroni post hoc was performed and the asterisk (*) indicates statistically significant difference and the value of $p<0.05$ was considered significant (n=3).

3.9 Cofilin knockdown decreases hemin treatment-induced endoplasmic reticulum stress

There was significant difference in microglial Wfs-1, XBP-1, spliced XBP-1 expression with $F (3, 20) = 852.94$, $p=0.000$; $F (3, 20) = 38.50$, $p=0.000$; $F (3, 20) = 48.61$, $p=0.000$ respectively (**Figure 3-10**).

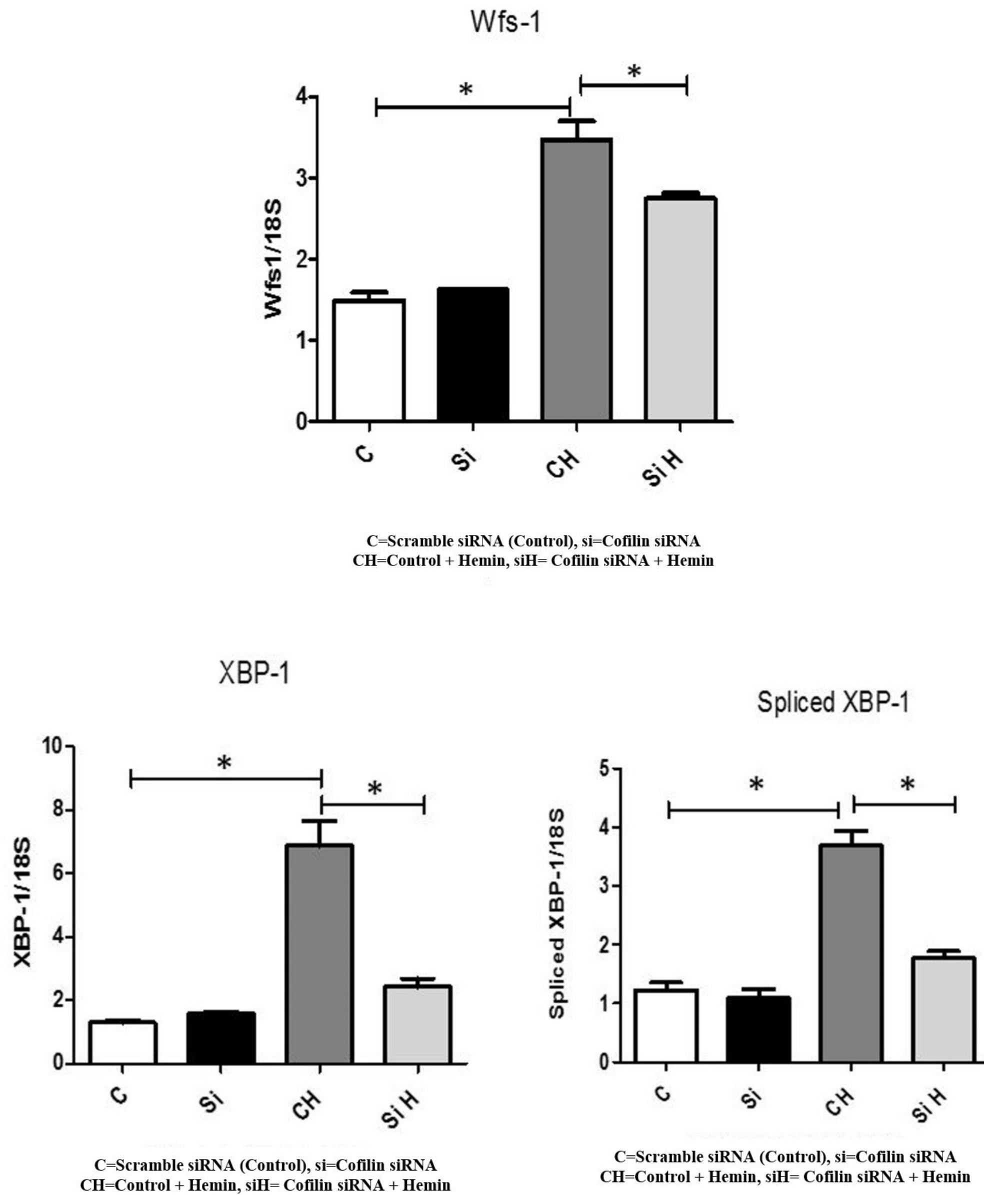


Figure 3-10: Cofilin mediates hemin treatment-induced endoplasmic reticulum (ER) stress markers (Wfs-1, XBP-1, spliced XBP-1). RT-qPCR results were expressed

in fold change as mean ± SEM (n=3 with duplicate). One-way ANOVA followed by Bonferroni post hoc was performed and the asterisk (*) indicates statistically significant difference and value of $p<0.05$ was considered significant.

Bonferroni post Hoc test indicated that there was no significant difference in Wfs-1, XBP-1, spliced XBP-1 expression levels between scramble siRNA and cofilin siRNA transfected microglia ($p>0.05$ for all). However, scramble siRNA transfected microglia treated with hemin showed significantly higher levels of Wfs-1, XBP-1, spliced XBP-1 expression ($p=0.000$ for all) as compared to scramble siRNA transfected microglia without hemin treatment group (control). On the other hand, significantly higher ($p=0.000$) Wfs-1, XBP-1, spliced XBP-1 expression levels were observed in scrambled siRNA transfected microglia treated with hemin group as compared to cofilin siRNA transfected microglia treated with hemin. These results indicate that microglial Wfs-1, XBP-1, spliced XBP-1 expressions are increased due to long term exposure to hemin treatment and cofilin mediate the process.

3.10 Hemin treatment alone does not evoke calcium signaling

In order to study whether hemin evokes calcium signaling, different concentration of hemin was exposed to microglia and it was found that hemin alone cannot evoke Ca^{2+} signaling. However, due to short-term treatment of hemin, microglia do not lose their ability to respond to acetylcholine evoked calcium signaling induced by acetylcholine agonist carbachol **(Figure 3-11)**.

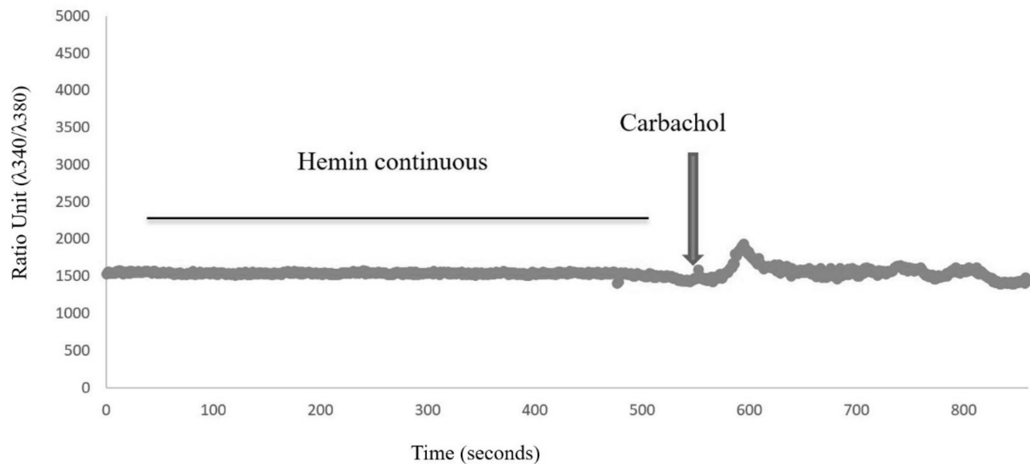
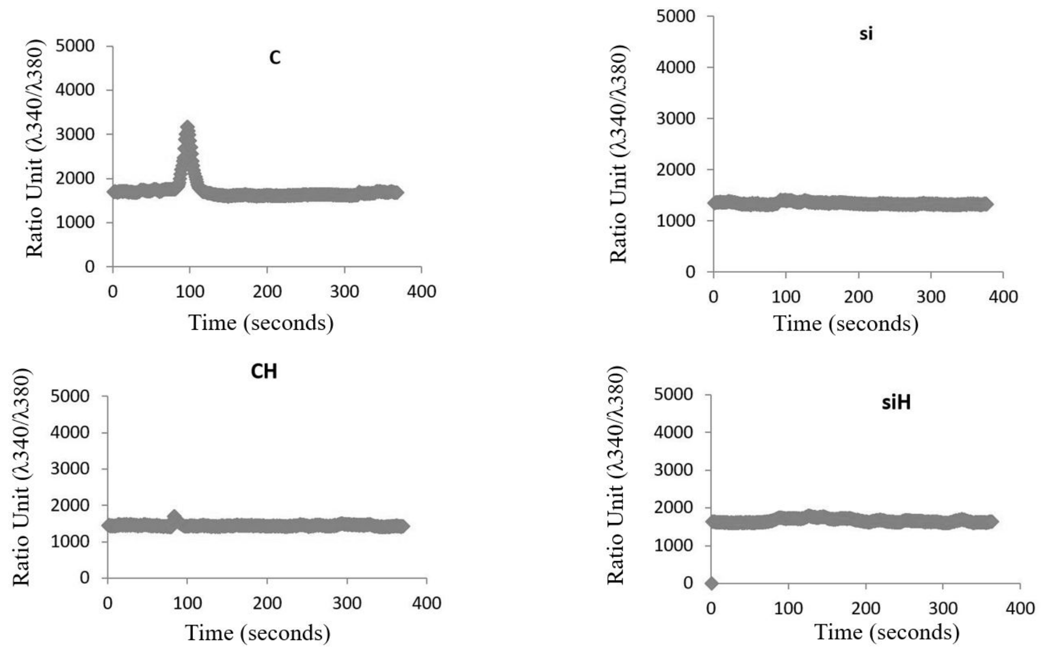


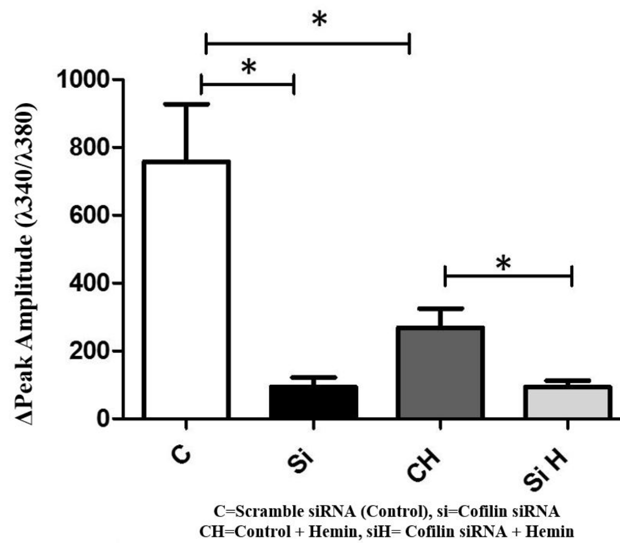
Figure 3-11: **Hemin treatment alone neither evoke calcium signaling nor affect acetyl choline evoked calcium signaling.** 100 µM hemin was continually passed over microglia culture media for 550 seconds followed by 100 µM carbachol (acetylcholine agonist) running over the media for next 250 seconds. The experiments were repeated trice with 10-15 cells stimulated in each independent experiment.

3.11 Long term exposure of hemin and cofilin knockdown decreases acetylcholine evoked calcium signaling in microglia

For evoking Ca^{2+} signaling 100 µM carbachol was used which was based on the previous investigations on microglia (Whittemore, Korotzer et al. 1993) and SIMA9 cells responded to acetylcholine evoked Ca^{2+} signaling (**Figure 3-11 and Figure 3-12A)**



A



B

Figure 3-12: Acetylcholine evoked calcium signaling due to hemin treatment. (A) Cofilin knockdown as well as hemin treatment decreased microglial ability to respond

to acetylcholine evoked calcium signaling. Locke solution was passed for 60 seconds over the microglia media followed by 100 µM carbachol (acetylcholine agonist) passing for up to 300 seconds. Experiments were conducted in triplicate with 8-14 microglia in each experiments. (B) The results were expressed in fold change as mean ± SEM. One-way ANOVA followed by Bonferroni post hoc was performed and the asterisk (*) indicates statistically significant difference and the value of $p<0.05$ was considered significant.

There was a significant difference in acetyl choline evoked Ca^{2+} signaling among different treatment groups in microglia with $F(3, 47) = 16.61$, $p=0.000$. Bonferroni post Hoc test indicated that there was statistically significant difference in Ca^{2+} signaling between scramble siRNA and cofilin siRNA transfected microglia ($p=0.000$). Scramble siRNA transfected microglia treated with hemin showed significantly lower levels of Ca^{2+} ($p=0.000$) as compared to scramble siRNA transfected microglia without hemin treatment group (control). Significantly lower ($p=0.000$) Ca^{2+} signaling were observed in cofilin siRNA transfected microglia treated with hemin group as compared to scramble siRNA transfected microglia treated with hemin. These results indicate that that microglial ability to respond to acetylcholine evoked Ca^{2+} signaling is mediated by cofilin as well as hemin treatment for 24 h (**Figure 3-12B**).

Chapter 4

Discussion

In the present investigation, we found that hemin induces microglial inflammation which was observed to be mediated by cofilin. Two previously reported results are relevant in this context. One is on the report that hemin induced inflammation via MyD88/TRIF signaling pathway (Lin, Yin et al. 2012), and other in hemorrhage, that reported inflammation is not due to hemin but due to methemoglobin (Gram, Sveinsdottir et al. 2013). Our investigations are aligned with the former but contradict with the later. This apparent contradiction could be explained by the fact that microglial inflammatory responses *in vitro* is slightly different from those of *in vivo* and the studies on microglia *in vitro* tend to provide increased inflammatory response (Kettenmann, Hanisch et al. 2011). Extremely high dose of hemin induced increased oxidative stress might also lead to microglial inflammation (Hu, Tao et al. 2016). Similar explanation could be provided for hemin induced increased production of TNF-α production but further investigation is warranted in this regard. The observed decrease in TNF-α production in cofilin siRNA transfected microglia might be due to the conditions that lead to decreased cofilin-actin rod formation which is in compliance with previously reported role of cofilin in mediating cellular stress in several disease conditions (Cichon, Sun et al. 2012).

Hemin treatment reduced the viability of the cells which is opposite to the previous report (Mullebner, Moldzio et al. 2015). The explanation lies in the dose use. Previous study used low dose that increase cellular protective mechanism but extremely high dose as in ICH or in our study conditions give rise to cell death. Cofilin knockdown increased cell viability which could be due to decreased hemin take up due to globular microglial cell surface or decreased mitochondrial transition pore permeability induced cell death (Klamt, Zdanov et al. 2009, Posadas, Perez-Martinez et al. 2012). This is also in compliance with previous report with cellular stress induced by chemicals or oxygen glucose deprivation (Madineni, Alhadidi et al. 2016). Hemin treated conditioned media from microglia induced increased cell death of undifferentiated PC-12 cell could be attributed to the increased production of TNF-α or other toxic cytokines from microglia.

Time dependent hemin exposure effects on microglial migration could be due to differential microglial response. Acute hemin treatment affects microglial migration negatively but over time microglia recover by themselves to assume their normal migratory function. However, cofilin knockdown decreased microglial migration is due to dysregulation of cytoskeletal dynamics (Sidani, Wessels et al. 2007).

Since hemin is a known inducer of HO-1 and therefore it is likely that microglia expressed higher HO-1 levels due to hemin treatment. Hemin induced higher expression of Nrf2 by microglia implies that HO-1 might also be upregulated due to Nrf2 translocation to the nucleus and upregulation of antioxidant system (Andreadi, Howells et al. 2006, Li, Dong et al. 2014). Cofilin knockdown alone cannot induce HO-1 and Nrf2 expression but cofilin knockdown microglia treated with hemin have shown increased HO-1 and Nrf2 expression indicating that cofilin knockdown did not reduce microglial ability to degrade

hemin. We found that there was more HO-1 and Nrf2 expression in cofilin knockdown microglia in comparison to scramble siRNA transfected hemin treated microglia. One possible explainable could be that due to cofilin knockdown microglia lose the ability to change the morphology against stress which is not enough to upregulate HO-1 and Nrf2 but when stressor like hemin is used to challenge microglia, the higher expression of HO-1 and Nrf2 might be the work of preconditioning like effects of microglia preparing cells to protect via antioxidant defense mechanism (Cai, Cho et al. 2011).

Wfs-1 is a cellular ER stress marker that attenuates unfolded protein response (UPR) and protects cells from apoptosis (Fonseca, Fukuma et al. 2005, Ueda, Kawano et al. 2005, Zatyka, Da Silva Xavier et al. 2015). XBP1 and spliced XBP-1 are markers of ER stress and indicate adaptive response of the cells (Xu, Bailly-Maitre et al. 2005, van Schadewijk, van't Wout et al. 2012). The increased ER stress due to hemin treatment as observed in our study could be due to increased oxidative stress caused by hemin exposure. Increasing evidence suggest that protein folding and generation of ROS in the ER are closely linked events. Activation of the UPR on exposure to oxidative stress is an adaptive mechanism to preserve cell function and survival (Malhotra and Kaufman 2007). Even though increased ER stress have increased protective HO-1 expression (Liu, Peyton et al. 2005) but such protective mechanisms fail due to extremely high amounts of hemin. In such context, there is sudden increase of oxidative stress both in the cytoplasm as well as in the ER. Even though it was reported earlier that hemin acts as a generic and potent protein misfolding inhibitor (Liu, Carver et al. 2014), in our experiment condition microglia might have lost their ability to tolerate persistent oxidative stress and protein misfolding. Another possibility is that the antioxidant system like SOD, GPx and catalase

inside microglia might have lost their ability to rescue increased oxidative stress caused by hemin exposure. This failure to scavenge free radicle might increase in ER stress. Based on previous studies on different cell types, it can be said that hemin induced ER stress in microglia is due to iron and bilirubin formed from hemin degradation (Barateiro, Vaz et al. 2012, Mullebner, Moldzio et al. 2015). Since cofilin knockdown did not increase Wfs-1, XBP-1 and spliced XBP-1 expression indicating that actin dynamics dysregulation alone is not sufficient to induce ER stress in microglia. However, the cofilin knockdown microglia treated with hemin had reduced expression of Wfs-1, XBP-1 and spliced XBP-1 indicating that cofilin mediate hemin induced ER stress. Such effect could be due to microglial decreased ability to sense external environment or increased protective Nrf2 expression but further study is warranted to substantiate this finding (Glover-Cutter, Lin et al. 2013).

Hemin neither evokes Ca^{2+} signaling nor affect acetylcholine evoked Ca^{2+} due to short term exposure to microglia indicating that acute hemin exposure cannot induce Ca^{2+} dependent downstream effector functions of microglia or other machinery that evoke Ca^{2+} signaling. On the other hand, hemin exposure of 24 h reduced acetylcholine evoked Ca^{2+} signaling in microglia. This apparently opposite results implies that microglia respond differently based on the duration of hemin treatment (Moller 2002).

Since the mechanism as well as the rate of hemin uptake in microglia has not been determined yet, it cannot be said whether this differential function is due to the interaction of hemin with the surface receptors of microglia or due to interaction with intracellular component of microglia by hemin or degradation products of hemin such as iron, biliverdin bilirubin, and CO. Iron stimulates ryanodine receptor-mediated Ca^{2+} release through ROS

produced via the Fenton reaction leading to stimulation of the extracellular-signal-regulated kinases (ERK) signaling pathway (Hidalgo and Nunez 2007). In case of acute hemin exposure, the non-stimulation of Ca^{2+} signaling might be due to the fact that, within that time frame, hemin could not enter inside the cell in order to stimulate the cell. Or it could be the fact that hemin enters inside cell but the degradation of hemin does not occur for iron production in order to stimulate the Ca^{2+} signaling. Another possibility is that after degradation of hemin, the released iron is taken up by iron-storage protein, ferritin (Ponka, Beaumont et al. 1998) and therefore there is not sufficient iron to stimulate Ca^{2+} signaling. On the other hand, long term exposure to hemin causes microglia to take up more hemin, increased production of iron but over production of iron might lead to increased oxidative stress to the cells that is beyond the ability of microglia to cope up with. It might be possible that ferritin is no longer able to store iron and thus causes the cells to turn on their defense system resulting in reducing microglial ability to respond to stimuli. CO produced from hemin breakdown has shown to increase Ca^{2+} signaling (Wilkinson and Kemp 2011) and this could be due to increased generation of mitochondrial oxidative stress. In acute hemin treatment on microglia, no effect on Ca^{2+} signaling might also be due to the inability of microglia to generate CO adequately within the short period of time. On the other hand, long-term exposure of hemin might cause overproduction of CO resulting in increasing the microglial protective mechanism. Thus, affecting reduced physiological response of microglia to respond to external stimuli. Another possibility is that microglia might lose their ability to process sufficient oxygen which is a requirement for hemin breakdown (Tenhunen, Marver et al. 1968) resulting in their inability to evoke Ca^{2+} signaling adequately. Biliverdin and subsequent production of bilirubin from hemin breakdown

might also affect Ca^{2+} signaling but the mechanism is poorly understood. This response might be similar to those of iron and CO.

Acetylcholine evoked Ca^{2+} signaling in microglia has been suggested to be due to release of Ca^{2+} from internal stores such as ER (Whittemore, Korotzer et al. 1993) but it is not clear how hemin interact in this process. Since other signaling pathways have not been investigated, the possibility that hemin or its degradation products affect other internal Ca^{2+} signaling pathways cannot be ruled out. Further investigation is suggested to substantiate this finding.

Cofilin knockdown reduced the microglial ability to respond to acetylcholine evoked Ca^{2+} signaling could be explained from the fact that the role of cofilin in actin cytoskeleton dynamic is the basis of several effector function (Uhlemann, Gertz et al. 2015). One of the important morphological modification due to cofilin knockdown is that microglia assume globular shape. This shape is different from 'activated microglia' or 'phagocytic microglia'. It is assumed that the actin cytoskeleton dynamic is affected due to the loss of depolymerization of actin by cofilin. Cofilin knockdown results in reduced microglial ability to respond to external stimuli and thus might lose their ability to respond to acetylcholine evoked Ca^{2+} signaling but how this interaction translated into near 'non-respond cell to acetylcholine evoked microglia' signaling is still not clear (Han, Stope et al. 2007). Therefore, further study is needed to unravel the detailed mechanism.

In this investigation, our hypothesis was that inhibition of cofilin might be a potential therapeutic target for the treatment of ICH. Previous studies in our lab have shown the beneficial effect of cofilin inhibition in ischemic stroke as well as preventing LPS induced microglial inflammation (Madineni, Alhadidi et al. 2016). As an extension of the

project, in this current study we have demonstrated that cofilin inhibition might be a potential therapy to prevent hemin induced microglial excitotoxicity, inflammation, oxidative stress and ER stress. However, the ability of microglia to migrate towards the injurious site is negatively affected therefore affecting negatively on the microglial phagocytosis of cellular debris. Investigation using animal models of hemorrhage will provide further insight into the possibility of cofilin inhibition as a therapeutic target for ICH.

References

Aft, R. L. and G. C. Mueller (1984). "Hemin-mediated oxidative degradation of proteins." J Biol Chem 259(1): 301-305.

Ajami, B., J. L. Bennett, C. Krieger, W. Tetzlaff and F. M. Rossi (2007). "Local self-renewal can sustain CNS microglia maintenance and function throughout adult life." Nat Neurosci 10(12): 1538-1543.

Alhadidi, Q., M. S. Bin Sayeed and Z. A. Shah (2016). "Cofilin as a Promising Therapeutic Target for Ischemic and Hemorrhagic Stroke." Transl Stroke Res 7(1): 33-41.

Andreadi, C. K., L. M. Howells, P. A. Atherfold and M. M. Manson (2006). "Involvement of Nrf2, p38, B-Raf, and nuclear factor-kappaB, but not phosphatidylinositol 3-kinase, in induction of hemeoxygenase-1 by dietary polyphenols." Mol Pharmacol 69(3): 1033-1040.

Aronowski, J. and X. Zhao (2011). "Molecular pathophysiology of cerebral hemorrhage: secondary brain injury." Stroke 42(6): 1781-1786.

Bamburg, J. R. and B. W. Bernstein (2010). "Roles of ADF/cofilin in actin polymerization and beyond." F1000 Biol Rep 2: 62.

Bamburg, J. R., A. McGough and S. Ono (1999). "Putting a new twist on actin: ADF/cofilins modulate actin dynamics." Trends Cell Biol 9(9): 364-370.

Barateiro, A., A. R. Vaz, S. L. Silva, A. Fernandes and D. Brites (2012). "ER stress, mitochondrial dysfunction and calpain/JNK activation are involved in oligodendrocyte precursor cell death by unconjugated bilirubin." Neuromolecular Med 14(4): 285-302.

Baysal, E., H. P. Monteiro, S. G. Sullivan and A. Stern (1990). "Desferrioxamine protects human red blood cells from hemin-induced hemolysis." Free Radic Biol Med 9(1): 5-10.

Belur, P. K., J. J. Chang, S. He, B. A. Emanuel and W. J. Mack (2013). "Emerging experimental therapies for intracerebral hemorrhage: targeting mechanisms of secondary brain injury." Neurosurg Focus 34(5): E9.

Bernstein, B. W. and J. R. Bamburg (2010). "ADF/cofilin: a functional node in cell biology." Trends Cell Biol 20(4): 187-195.

Bunn, H. F. and J. H. Jandl (1968). "Exchange of heme among hemoglobins and between hemoglobin and albumin." J Biol Chem 243(3): 465-475.

Burger, P., D. de Korte, T. K. van den Berg and R. van Bruggen (2012). "CD47 in Erythrocyte Ageing and Clearance - the Dutch Point of View." Transfus Med Hemother 39(5): 348-352.

Cai, Y., G. S. Cho, C. Ju, S. L. Wang, J. H. Ryu, C. Y. Shin, H. S. Kim, K. W. Nam, A. M. Jalin, W. Sun, I. Y. Choi and W. K. Kim (2011). "Activated microglia are less vulnerable to hemin toxicity due to nitric oxide-dependent inhibition of JNK and p38 MAPK activation." J Immunol 187(3): 1314-1321.

Camejo, G., C. Halberg, A. Manschik-Lundin, E. Hurt-Camejo, B. Rosengren, H. Olsson, G. I. Hansson, G. B. Forsberg and B. Ylhen (1998). "Hemin binding and oxidation of lipoproteins in serum: mechanisms and effect on the interaction of LDL with human macrophages." J Lipid Res 39(4): 755-766.

Chaudhary, N., J. J. Gemmete, B. G. Thompson, G. Xi and A. S. Pandey (2013). "Iron--potential therapeutic target in hemorrhagic stroke." World Neurosurg 79(1): 7-9.

Chen-Roetling, J., Y. Cai, X. Lu and R. F. Regan (2014). "Hemin uptake and release by neurons and glia." Free Radic Res 48(2): 200-205.

Chen-Roetling, J., X. Lu and R. F. Regan (2015). "Targeting heme oxygenase after intracerebral hemorrhage." Ther Targets Neurol Dis 2(1).

Chen, B., M. Jiang, M. Zhou, L. Chen, X. Liu, X. Wang and Y. Wang (2012). "Both NMDA and non-NMDA receptors mediate glutamate stimulation induced cofilin rod formation in cultured hippocampal neurons." Brain Res 1486: 1-13.

Chen, Y., J. D. Warner, D. I. Yule and D. R. Giovannucci (2005). "Spatiotemporal analysis of exocytosis in mouse parotid acinar cells." Am J Physiol Cell Physiol 289(5): C1209-1219.

Chen, Z. and B. D. Trapp (2016). "Microglia and neuroprotection." J Neurochem 136 Suppl 1: 10-17.

Cherry, J. D., J. A. Olschowka and M. K. O'Banion (2014). "Neuroinflammation and M2 microglia: the good, the bad, and the inflamed." J Neuroinflammation 11: 98.

Chou, A. C. and C. D. Fitch (1981). "Mechanism of hemolysis induced by ferriprotoporphyrin IX." J Clin Invest 68(3): 672-677.

Cichon, J., C. Sun, B. Chen, M. Jiang, X. A. Chen, Y. Sun, Y. Wang and G. Chen (2012). "Cofilin aggregation blocks intracellular trafficking and induces synaptic loss in hippocampal neurons." J Biol Chem 287(6): 3919-3929.

dos Remedios, C. G., D. Chhabra, M. Kekic, I. V. Dedova, M. Tsubakihara, D. A. Berry and N. J. Nosworthy (2003). "Actin binding proteins: regulation of cytoskeletal microfilaments." Physiol Rev 83(2): 433-473.

Egashira, Y., Y. Hua, R. F. Keep and G. Xi (2015). "Intercellular cross-talk in intracerebral hemorrhage." Brain Res 1623: 97-109.

Fonseca, S. G., M. Fukuma, K. L. Lipson, L. X. Nguyen, J. R. Allen, Y. Oka and F. Urano (2005). "WFS1 is a novel component of the unfolded protein response and maintains homeostasis of the endoplasmic reticulum in pancreatic beta-cells." J Biol Chem 280(47): 39609-39615.

Geddes, J. W., L. C. Pettigrew, M. L. Holtz, S. D. Craddock and M. D. Maines (1996). "Permanent focal and transient global cerebral ischemia increase glial and neuronal expression of heme oxygenase-1, but not heme oxygenase-2, protein in rat brain." Neurosci Lett 210(3): 205-208.

Ginhoux, F., M. Greter, M. Leboeuf, S. Nandi, P. See, S. Gokhan, M. F. Mehler, S. J. Conway, L. G. Ng, E. R. Stanley, I. M. Samokhvalov and M. Merad (2010). "Fate mapping analysis reveals that adult microglia derive from primitive macrophages." Science 330(6005): 841-845.

Glover-Cutter, K. M., S. Lin and T. K. Blackwell (2013). "Integration of the unfolded protein and oxidative stress responses through SKN-1/Nrf." PLoS Genet 9(9): e1003701.

Goldstein, L., Z. P. Teng, E. Zeserson, M. Patel and R. F. Regan (2003). "Hemin induces an iron-dependent, oxidative injury to human neuron-like cells." J Neurosci Res 73(1): 113-121.

Gong, Y., Y. Hua, R. F. Keep, J. T. Hoff and G. Xi (2004). "Intracerebral hemorrhage: effects of aging on brain edema and neurological deficits." Stroke 35(11): 2571-2575.

Gonzales, N. R., J. Shah, N. Sangha, L. Sosa, R. Martinez, L. Shen, M. Kasam, M. M. Morales, M. M. Hossain, A. D. Barreto, S. I. Savitz, G. Lopez, V. Misra, T. C. Wu, R. El

Khoury, A. Sarraj, P. Sahota, W. Hicks, I. Acosta, M. R. Sline, M. H. Rahbar, X. Zhao, J. Aronowski and J. C. Grotta (2013). "Design of a prospective, dose-escalation study evaluating the Safety of Pioglitazone for Hematoma Resolution in Intracerebral Hemorrhage (SHRINC)." Int J Stroke 8(5): 388-396.

Gonzalez-Reyes, S., S. Guzman-Beltran, O. N. Medina-Campos and J. Pedraza-Chaverri (2013). "Curcumin pretreatment induces Nrf2 and an antioxidant response and prevents hemin-induced toxicity in primary cultures of cerebellar granule neurons of rats." Oxid Med Cell Longev 2013: 801418.

Gozzelino, R., V. Jeney and M. P. Soares (2010). "Mechanisms of cell protection by heme oxygenase-1." Annu Rev Pharmacol Toxicol 50: 323-354.

Graca-Souza, A. V., M. A. Arruda, M. S. de Freitas, C. Barja-Fidalgo and P. L. Oliveira (2002). "Neutrophil activation by heme: implications for inflammatory processes." Blood 99(11): 4160-4165.

Gram, M., S. Sveinsdottir, K. Ruscher, S. R. Hansson, M. Cinthio, B. Akerstrom and D. Ley (2013). "Hemoglobin induces inflammation after preterm intraventricular hemorrhage by methemoglobin formation." J Neuroinflammation 10: 100.

Han, L., M. B. Stope, M. L. de Jesus, P. A. Oude Weernink, M. Urban, T. Wieland, D. Rosskopf, K. Mizuno, K. H. Jakobs and M. Schmidt (2007). "Direct stimulation of receptor-controlled phospholipase D1 by phospho-cofilin." EMBO J 26(19): 4189-4202.

Han, Y., W. Yi, J. Qin, Y. Zhao, J. Zhang and X. Chang (2015). "Carbon monoxide offers neuroprotection from hippocampal cell damage induced by recurrent febrile seizures through the PERK-activated ER stress pathway." Neurosci Lett 585: 126-131.

Hanafy, K. A. (2013). "The role of microglia and the TLR4 pathway in neuronal apoptosis and vasospasm after subarachnoid hemorrhage." J Neuroinflammation 10: 83.

Hidalgo, C. and M. T. Nunez (2007). "Calcium, iron and neuronal function." IUBMB Life 59(4-5): 280-285.

Hu, X., C. Tao, Q. Gan, J. Zheng, H. Li and C. You (2016). "Oxidative Stress in Intracerebral Hemorrhage: Sources, Mechanisms, and Therapeutic Targets." Oxid Med Cell Longev 2016: 3215391.

Hua, Y., R. F. Keep, J. T. Hoff and G. Xi (2007). "Brain injury after intracerebral hemorrhage: the role of thrombin and iron." Stroke 38(2 Suppl): 759-762.

Hua, Y., G. Xi, R. F. Keep and J. T. Hoff (2000). "Complement activation in the brain after experimental intracerebral hemorrhage." J Neurosurg 92(6): 1016-1022.

Huang, F. P., G. Xi, R. F. Keep, Y. Hua, A. Nemoianu and J. T. Hoff (2002). "Brain edema after experimental intracerebral hemorrhage: role of hemoglobin degradation products." J Neurosurg 96(2): 287-293.

Jaremko, K. M., J. Chen-Roetling, L. Chen and R. F. Regan (2010). "Accelerated hemolysis and neurotoxicity in neuron-glia-blood clot co-cultures." J Neurochem 114(4): 1063-1073.

Jonsson, F., C. B. Gurniak, B. Fleischer, G. Kirfel and W. Witke (2012). "Immunological responses and actin dynamics in macrophages are controlled by N-cofilin but are independent from ADF." PLoS One 7(4): e36034.

Keep, R. F., Y. Hua and G. Xi (2012). "Intracerebral haemorrhage: mechanisms of injury and therapeutic targets." Lancet Neurol 11(8): 720-731.

Keep, R. F., N. Zhou, J. Xiang, A. V. Andjelkovic, Y. Hua and G. Xi (2014). "Vascular disruption and blood-brain barrier dysfunction in intracerebral hemorrhage." Fluids Barriers CNS 11: 18.

Kettenmann, H., U. K. Hanisch, M. Noda and A. Verkhratsky (2011). "Physiology of microglia." Physiol Rev 91(2): 461-553.

Kigerl, K. A., J. C. Gensel, D. P. Ankeny, J. K. Alexander, D. J. Donnelly and P. G. Popovich (2009). "Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord." J Neurosci 29(43): 13435-13444.

Kim-Han, J. S., S. J. Kopp, L. L. Dugan and M. N. Diringer (2006). "Perihematomal mitochondrial dysfunction after intracerebral hemorrhage." Stroke 37(10): 2457-2462.

Kim, D. W., S. H. Im, J. Y. Kim, D. E. Kim, G. T. Oh and S. W. Jeong (2009). "Decreased brain edema after collagenase-induced intracerebral hemorrhage in mice lacking the inducible nitric oxide synthase gene. Laboratory investigation." J Neurosurg 111(5): 995-1000.

Klamt, F., S. Zdanov, R. L. Levine, A. Pariser, Y. Zhang, B. Zhang, L. R. Yu, T. D. Veenstra and E. Shacter (2009). "Oxidant-induced apoptosis is mediated by oxidation of the actin-regulatory protein cofilin." Nat Cell Biol 11(10): 1241-1246.

Kristiansen, M., J. H. Graversen, C. Jacobsen, O. Sonne, H. J. Hoffman, S. K. Law and S. K. Moestrup (2001). "Identification of the haemoglobin scavenger receptor." Nature 409(6817): 198-201.

Kroner, A., A. D. Greenhalgh, J. G. Zarruk, R. Passos Dos Santos, M. Gaestel and S. David (2014). "TNF and increased intracellular iron alter macrophage polarization to a detrimental M1 phenotype in the injured spinal cord." Neuron 83(5): 1098-1116.

Kumar, S. and U. Bandyopadhyay (2005). "Free heme toxicity and its detoxification systems in human." Toxicol Lett 157(3): 175-188.

Kuramatsu, J. B., H. B. Huttner and S. Schwab (2013). "Advances in the management of intracerebral hemorrhage." J Neural Transm (Vienna) 120 Suppl 1: S35-41.

Lei, C., S. Lin, C. Zhang, W. Tao, W. Dong, Z. Hao, M. Liu and B. Wu (2013). "High-mobility group box1 protein promotes neuroinflammation after intracerebral hemorrhage in rats." Neuroscience 228: 190-199.

Letarte, P. B., K. Lieberman, K. Nagatani, R. A. Haworth, G. B. Odell and T. A. Duff (1993). "Hemin: levels in experimental subarachnoid hematoma and effects on dissociated vascular smooth-muscle cells." J Neurosurg 79(2): 252-255.

Li, C. and R. Stocker (2009). "Heme oxygenase and iron: from bacteria to humans." Redox Rep 14(3): 95-101.

Li, J., W. M. Brieher, M. L. Scimone, S. J. Kang, H. Zhu, H. Yin, U. H. von Andrian, T. Mitchison and J. Yuan (2007). "Caspase-11 regulates cell migration by promoting Aip1-Cofilin-mediated actin depolymerization." Nat Cell Biol 9(3): 276-286.

Li, L., H. Dong, E. Song, X. Xu, L. Liu and Y. Song (2014). "Nrf2/ARE pathway activation, HO-1 and NQO1 induction by polychlorinated biphenyl quinone is associated with reactive oxygen species and PI3K/AKT signaling." Chem Biol Interact 209: 56-67.

Li, T., H. Wang, Y. Ding, M. Zhou, X. Zhou, X. Zhang, K. Ding, J. He, X. Lu, J. Xu and W. Wei (2014). "Genetic elimination of Nrf2 aggravates secondary complications except

for vasospasm after experimental subarachnoid hemorrhage in mice." Brain Res 1558: 90-99.

Lin, S., Q. Yin, Q. Zhong, F. L. Lv, Y. Zhou, J. Q. Li, J. Z. Wang, B. Y. Su and Q. W. Yang (2012). "Heme activates TLR4-mediated inflammatory injury via MyD88/TRIF signaling pathway in intracerebral hemorrhage." J Neuroinflammation 9: 46.

Liu, X. M., K. J. Peyton, D. Ensenat, H. Wang, A. I. Schafer, J. Alam and W. Durante (2005). "Endoplasmic reticulum stress stimulates heme oxygenase-1 gene expression in vascular smooth muscle. Role in cell survival." J Biol Chem 280(2): 872-877.

Liu, Y., J. A. Carver, L. H. Ho, A. K. Elias, I. F. Musgrave and T. L. Pukala (2014). "Hemin as a generic and potent protein misfolding inhibitor." Biochem Biophys Res Commun 454(2): 295-300.

Liu, Y. and P. R. Ortiz de Montellano (2000). "Reaction intermediates and single turnover rate constants for the oxidation of heme by human heme oxygenase-1." J Biol Chem 275(8): 5297-5307.

Loftspring, M. C., H. L. Johnson, R. Feng, A. J. Johnson and J. F. Clark (2011). "Unconjugated bilirubin contributes to early inflammation and edema after intracerebral hemorrhage." J Cereb Blood Flow Metab 31(4): 1133-1142.

Ma, Q., S. Chen, Q. Hu, H. Feng, J. H. Zhang and J. Tang (2014). "NLRP3 inflammasome contributes to inflammation after intracerebral hemorrhage." Ann Neurol 75(2): 209-219.

Madineni, A., Q. Alhadidi and Z. A. Shah (2016). "Cofilin Inhibition Restores Neuronal Cell Death in Oxygen-Glucose Deprivation Model of Ischemia." Mol Neurobiol 53(2): 867-878.

Maines, M. D. (1988). "Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications." FASEB J 2(10): 2557-2568.

Malhotra, J. D. and R. J. Kaufman (2007). "Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword?" Antioxid Redox Signal 9(12): 2277-2293.

Matsuo, Y., T. Kihara, M. Ikeda, M. Ninomiya, H. Onodera and K. Kogure (1995). "Role of neutrophils in radical production during ischemia and reperfusion of the rat brain: effect of neutrophil depletion on extracellular ascorbyl radical formation." J Cereb Blood Flow Metab 15(6): 941-947.

Matveev, L. V. (1966). "[Rupture of the 3d peroneus muscle in bulls]." Veterinariia 43(3): 74-75.

Matz, P., C. Turner, P. R. Weinstein, S. M. Massa, S. S. Panter and F. R. Sharp (1996). "Heme-oxygenase-1 induction in glia throughout rat brain following experimental subarachnoid hemorrhage." Brain Res 713(1-2): 211-222.

Moller, T. (2002). "Calcium signaling in microglial cells." Glia 40(2): 184-194.

Mozaffarian, D., E. J. Benjamin, A. S. Go, D. K. Arnett, M. J. Blaha, M. Cushman, S. de Ferranti, J. P. Despres, H. J. Fullerton, V. J. Howard, M. D. Huffman, S. E. Judd, B. M. Kissela, D. T. Lackland, J. H. Lichtman, L. D. Lisabeth, S. Liu, R. H. Mackey, D. B. Matchar, D. K. McGuire, E. R. Mohler, 3rd, C. S. Moy, P. Muntner, M. E. Mussolino, K. Nasir, R. W. Neumar, G. Nichol, L. Palaniappan, D. K. Pandey, M. J. Reeves, C. J. Rodriguez, P. D. Sorlie, J. Stein, A. Towfighi, T. N. Turan, S. S. Virani, J. Z. Willey, D. Woo, R. W. Yeh, M. B. Turner, C. American Heart Association Statistics and S. Stroke Statistics (2015). "Heart disease and stroke statistics--2015 update: a report from the American Heart Association." Circulation 131(4): e29-322.

Mullebner, A., R. Moldzio, H. Redl, A. V. Kozlov and J. C. Duvigneau (2015). "Heme Degradation by Heme Oxygenase Protects Mitochondria but Induces ER Stress via Formed Bilirubin." Biomolecules 5(2): 679-701.

Nakamura, T., R. F. Keep, Y. Hua, J. T. Hoff and G. Xi (2005). "Oxidative DNA injury after experimental intracerebral hemorrhage." Brain Res 1039(1-2): 30-36.

Nakamura, T., R. F. Keep, Y. Hua, S. Nagao, J. T. Hoff and G. Xi (2006). "Iron-induced oxidative brain injury after experimental intracerebral hemorrhage." Acta Neurochir Suppl 96: 194-198.

Nayak, D., T. L. Roth and D. B. McGavern (2014). "Microglia development and function." Annu Rev Immunol 32: 367-402.

Ndisang, J. F. and A. Jadhav (2013). "Hemin therapy suppresses inflammation and retroperitoneal adipocyte hypertrophy to improve glucose metabolism in obese rats co-morbid with insulin-resistant type-2 diabetes." Diabetes Obes Metab 15(11): 1029-1039.

Nimmerjahn, A., F. Kirchhoff and F. Helmchen (2005). "Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo." Science 308(5726): 1314-1318.

Nishida, E., S. Maekawa and H. Sakai (1984). "Cofilin, a protein in porcine brain that binds to actin filaments and inhibits their interactions with myosin and tropomyosin." Biochemistry 23(22): 5307-5313.

Nusco, G. A., J. T. Chun, E. Ercolano, D. Lim, G. Gragnaniello, K. Kyozuka and L. Santella (2006). "Modulation of calcium signalling by the actin-binding protein cofilin." Biochem Biophys Res Commun 348(1): 109-114.

Obermeier, B., R. Daneman and R. M. Ransohoff (2013). "Development, maintenance and disruption of the blood-brain barrier." Nat Med 19(12): 1584-1596.

Ohashi, K. (2015). "Roles of cofilin in development and its mechanisms of regulation." Dev Growth Differ 57(4): 275-290.

Ohashi, K., S. Fujiwara, T. Watanabe, H. Kondo, T. Kiuchi, M. Sato and K. Mizuno (2011). "LIM kinase has a dual role in regulating lamellipodium extension by decelerating the rate of actin retrograde flow and the rate of actin polymerization." J Biol Chem 286(42): 36340-36351.

Ono, S. (2007). "Mechanism of depolymerization and severing of actin filaments and its significance in cytoskeletal dynamics." Int Rev Cytol 258: 1-82.

Oser, M. and J. Condeelis (2009). "The cofilin activity cycle in lamellipodia and invadopodia." J Cell Biochem 108(6): 1252-1262.

Paine, A., B. Eiz-Vesper, R. Blasczyk and S. Immenschuh (2010). "Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential." Biochem Pharmacol 80(12): 1895-1903.

Pollard, T. D. and G. G. Borisy (2003). "Cellular motility driven by assembly and disassembly of actin filaments." Cell 112(4): 453-465.

Ponka, P., C. Beaumont and D. R. Richardson (1998). "Function and regulation of transferrin and ferritin." Semin Hematol 35(1): 35-54.

Posadas, I., F. C. Perez-Martinez, J. Guerra, P. Sanchez-Verdu and V. Cena (2012). "Cofilin activation mediates Bax translocation to mitochondria during excitotoxic neuronal death." J Neurochem 120(4): 515-527.

Poukkula, M., E. Kremneva, M. Serlachius and P. Lappalainen (2011). "Actin-depolymerizing factor homology domain: a conserved fold performing diverse roles in cytoskeletal dynamics." Cytoskeleton (Hoboken) 68(9): 471-490.

Qureshi, A. I., A. D. Mendelow and D. F. Hanley (2009). "Intracerebral haemorrhage." Lancet 373(9675): 1632-1644.

Rasmussen, I., L. H. Pedersen, L. Byg, K. Suzuki, H. Sumimoto and F. Vilhardt (2010). "Effects of F/G-actin ratio and actin turn-over rate on NADPH oxidase activity in microglia." BMC Immunol 11: 44.

Regan, R. F., J. Chen and L. Benvenisti-Zarom (2004). "Heme oxygenase-2 gene deletion attenuates oxidative stress in neurons exposed to extracellular hemin." BMC Neurosci 5: 34.

Regan, R. F. and S. S. Panter (1993). "Neurotoxicity of hemoglobin in cortical cell culture." Neurosci Lett 153(2): 219-222.

Regan, R. F., Y. Wang, X. Ma, A. Chong and Y. Guo (2001). "Activation of extracellular signal-regulated kinases potentiates hemin toxicity in astrocyte cultures." J Neurochem 79(3): 545-555.

Robinson, S. R., T. N. Dang, R. Dringen and G. M. Bishop (2009). "Hemin toxicity: a preventable source of brain damage following hemorrhagic stroke." Redox Rep 14(6): 228-235.

Rodriguez, F., R. Kemp, M. Balazy and A. Nasjletti (2003). "Effects of exogenous heme on renal function: role of heme oxygenase and cyclooxygenase." Hypertension 42(4): 680-684.

Rossi, D. J., T. Oshima and D. Attwell (2000). "Glutamate release in severe brain ischaemia is mainly by reversed uptake." Nature 403(6767): 316-321.

Rust, M. B. (2015). "ADF/cofilin: a crucial regulator of synapse physiology and behavior." Cell Mol Life Sci 72(18): 3521-3529.

Scapagnini, G., V. D'Agata, V. Calabrese, A. Pascale, C. Colombrita, D. Alkon and S. Cavallaro (2002). "Gene expression profiles of heme oxygenase isoforms in the rat brain." Brain Res 954(1): 51-59.

Schaer, D. J., C. A. Schaer, P. W. Buehler, R. A. Boykins, G. Schoedon, A. I. Alayash and A. Schaffner (2006). "CD163 is the macrophage scavenger receptor for native and chemically modified hemoglobins in the absence of haptoglobin." Blood 107(1): 373-380.

Schmitt, T. H., W. A. Frezzatti, Jr. and S. Schreier (1993). "Hemin-induced lipid membrane disorder and increased permeability: a molecular model for the mechanism of cell lysis." Arch Biochem Biophys 307(1): 96-103.

Schonhofen, P., L. M. de Medeiros, C. P. Chatain, I. J. Bristot and F. Klamt (2014). "Cofilin/actin rod formation by dysregulation of cofilin-1 activity as a central initial step in neurodegeneration." Mini Rev Med Chem 14(5): 393-400.

Shang, H., D. Yang, W. Zhang, T. Li, X. Ren, X. Wang and W. Zhao (2013). "Time course of Keap1-Nrf2 pathway expression after experimental intracerebral haemorrhage: correlation with brain oedema and neurological deficit." Free Radic Res 47(5): 368-375.

Sharma, P. and L. Ping (2014). "Calcium ion influx in microglial cells: physiological and therapeutic significance." J Neurosci Res 92(4): 409-423.

Shono, T., M. Ono, H. Izumi, S. I. Jimi, K. Matsushima, T. Okamoto, K. Kohno and M. Kuwano (1996). "Involvement of the transcription factor NF-kappaB in tubular morphogenesis of human microvascular endothelial cells by oxidative stress." Mol Cell Biol 16(8): 4231-4239.

Sidani, M., D. Wessels, G. Mouneimne, M. Ghosh, S. Goswami, C. Sarmiento, W. Wang, S. Kuhl, M. El-Sibai, J. M. Backer, R. Eddy, D. Soll and J. Condeelis (2007). "Cofilin

determines the migration behavior and turning frequency of metastatic cancer cells." J Cell Biol 179(4): 777-791.

Sierra, A., M. E. Tremblay and H. Wake (2014). "Never-resting microglia: physiological roles in the healthy brain and pathological implications." Front Cell Neurosci 8: 240.

Smith, A. and W. T. Morgan (1981). "Hemopexin-mediated transport of heme into isolated rat hepatocytes." J Biol Chem 256(21): 10902-10909.

Suliman, H. B., M. S. Carraway, L. W. Velsor, B. J. Day, A. J. Ghio and C. A. Piantadosi (2002). "Rapid mtDNA deletion by oxidants in rat liver mitochondria after hemin exposure." Free Radic Biol Med 32(3): 246-256.

Syapin, P. J. (2008). "Regulation of haeme oxygenase-1 for treatment of neuroinflammation and brain disorders." Br J Pharmacol 155(5): 623-640.

Taylor, R. A. and L. H. Sansing (2013). "Microglial responses after ischemic stroke and intracerebral hemorrhage." Clin Dev Immunol 2013: 746068.

Tenhunen, R., H. S. Marver and R. Schmid (1968). "The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase." Proc Natl Acad Sci U S A 61(2): 748-755.

Tremblay, M. E., B. Stevens, A. Sierra, H. Wake, A. Bessis and A. Nimmerjahn (2011). "The role of microglia in the healthy brain." J Neurosci 31(45): 16064-16069.

Tsai, F. C., A. Seki, H. W. Yang, A. Hayer, S. Carrasco, S. Malmersjo and T. Meyer (2014). "A polarized Ca2+, diacylglycerol and STIM1 signalling system regulates directed cell migration." Nat Cell Biol 16(2): 133-144.

Tsuji, A., J. Wang, K. H. Stenzel and A. Novogrodsky (1993). "Immune stimulatory and anti-tumour properties of haemin." Clin Exp Immunol 93(3): 308-312.

Turner, C. P., M. Bergeron, P. Matz, A. Zegna, L. J. Noble, S. S. Panter and F. R. Sharp (1998). "Heme oxygenase-1 is induced in glia throughout brain by subarachnoid hemoglobin." J Cereb Blood Flow Metab 18(3): 257-273.

Ueda, K., J. Kawano, K. Takeda, T. Yujiri, K. Tanabe, T. Anno, M. Akiyama, J. Nozaki, T. Yoshinaga, A. Koizumi, K. Shinoda, Y. Oka and Y. Tanizawa (2005). "Endoplasmic reticulum stress induces Wfs1 gene expression in pancreatic beta-cells via transcriptional activation." Eur J Endocrinol 153(1): 167-176.

Uhlemann, R., K. Gertz, W. Boehmerle, T. Schwarz, C. Nolte, D. Freyer, H. Kettenmann, M. Endres and G. Kronenberg (2015). "Actin dynamics shape microglia effector functions." Brain Struct Funct.

van Asch, C. J., M. J. Luitse, G. J. Rinkel, I. van der Tweel, A. Algra and C. J. Klijn (2010). "Incidence, case fatality, and functional outcome of intracerebral haemorrhage over time, according to age, sex, and ethnic origin: a systematic review and meta-analysis." Lancet Neurol 9(2): 167-176.

van Schadewijk, A., E. F. van't Wout, J. Stolk and P. S. Hiemstra (2012). "A quantitative method for detection of spliced X-box binding protein-1 (XBP1) mRNA as a measure of endoplasmic reticulum (ER) stress." Cell Stress Chaperones 17(2): 275-279.

Van Vlierberghe, H., M. Langlois and J. Delanghe (2004). "Haptoglobin polymorphisms and iron homeostasis in health and in disease." Clin Chim Acta 345(1-2): 35-42.

Vincent, S. H. (1989). "Oxidative effects of heme and porphyrins on proteins and lipids." Semin Hematol 26(2): 105-113.

Wabnitz, G. H., C. Goursot, B. Jahraus, H. Kirchgessner, A. Hellwig, M. Klemke, M. H. Konstandin and Y. Samstag (2010). "Mitochondrial translocation of oxidized cofilin

induces caspase-independent necrotic-like programmed cell death of T cells." Cell Death Dis 1: e58.

Wagener, F. A., H. D. Volk, D. Willis, N. G. Abraham, M. P. Soares, G. J. Adema and C. G. Figdor (2003). "Different faces of the heme-heme oxygenase system in inflammation." Pharmacol Rev 55(3): 551-571.

Wagner, K. R. and B. E. Dwyer (2004). "Hematoma removal, heme, and heme oxygenase following hemorrhagic stroke." Ann N Y Acad Sci 1012: 237-251.

Wagner, K. R., F. R. Sharp, T. D. Ardizzone, A. Lu and J. F. Clark (2003). "Heme and iron metabolism: role in cerebral hemorrhage." J Cereb Blood Flow Metab 23(6): 629-652.

Wang, J. and S. Dore (2007). "Heme oxygenase-1 exacerbates early brain injury after intracerebral haemorrhage." Brain 130(Pt 6): 1643-1652.

Welser, J. V., L. Li and R. Milner (2010). "Microglial activation state exerts a biphasic influence on brain endothelial cell proliferation by regulating the balance of TNF and TGF-beta1." J Neuroinflammation 7: 89.

Whittemore, E. R., A. R. Korotzer, A. Etebari and C. W. Cotman (1993). "Carbachol increases intracellular free calcium in cultured rat microglia." Brain Res 621(1): 59-64.

Wilkinson, W. J. and P. J. Kemp (2011). "Carbon monoxide: an emerging regulator of ion channels." J Physiol 589(Pt 13): 3055-3062.

Wu, J., Y. Hua, R. F. Keep, T. Schallert, J. T. Hoff and G. Xi (2002). "Oxidative brain injury from extravasated erythrocytes after intracerebral hemorrhage." Brain Res 953(1-2): 45-52.

Xi, G., R. F. Keep and J. T. Hoff (2006). "Mechanisms of brain injury after intracerebral haemorrhage." Lancet Neurol 5(1): 53-63.

Xu, C., B. Bailly-Maitre and J. C. Reed (2005). "Endoplasmic reticulum stress: cell life and death decisions." J Clin Invest 115(10): 2656-2664.

Yang, S., Y. Chen, X. Deng, W. Jiang, B. Li, Z. Fu, M. Du and R. Ding (2013). "Hemoglobin-induced nitric oxide synthase overexpression and nitric oxide production contribute to blood-brain barrier disruption in the rat." J Mol Neurosci 51(2): 352-363.

Yang, Z., J. D. Philips, R. T. Doty, P. Giraudi, J. D. Ostrow, C. Tiribelli, A. Smith and J. L. Abkowitz (2010). "Kinetics and specificity of feline leukemia virus subgroup C receptor (FLVCR) export function and its dependence on hemopexin." J Biol Chem 285(37): 28874-28882.

Zatyka, M., G. Da Silva Xavier, E. A. Bellomo, W. Leadbeater, D. Astuti, J. Smith, F. Michelangeli, G. A. Rutter and T. G. Barrett (2015). "Sarco(endo)plasmic reticulum ATPase is a molecular partner of Wolfram syndrome 1 protein, which negatively regulates its expression." Hum Mol Genet 24(3): 814-827.

Zhang, Y., H. Wang, J. Li, D. A. Jimenez, E. S. Levitan, E. Aizenman and P. A. Rosenberg (2004). "Peroxynitrite-induced neuronal apoptosis is mediated by intracellular zinc release and 12-lipoxygenase activation." J Neurosci 24(47): 10616-10627.

Zhao, X., J. Grotta, N. Gonzales and J. Aronowski (2009). "Hematoma resolution as a therapeutic target: the role of microglia/macrophages." Stroke 40(3 Suppl): S92-94.

Zhao, X., G. Sun, J. Zhang, R. Strong, W. Song, N. Gonzales, J. C. Grotta and J. Aronowski (2007). "Hematoma resolution as a target for intracerebral hemorrhage treatment: role for peroxisome proliferator-activated receptor gamma in microglia/macrophages." Ann Neurol 61(4): 352-362.

Zhou, X., Q. Xie, G. Xi, R. F. Keep and Y. Hua (2014). "Brain CD47 expression in a swine model of intracerebral hemorrhage." Brain Res 1574: 70-76.

Zhou, Y., Y. Wang, J. Wang, R. Anne Stetler and Q. W. Yang (2014). "Inflammation in intracerebral hemorrhage: from mechanisms to clinical translation." Prog Neurobiol 115: 25-44.

Zia, M. T., A. Csiszar, N. Labinskyy, F. Hu, G. Vinukonda, E. F. LaGamma, Z. Ungvari and P. Ballabh (2009). "Oxidative-nitrosative stress in a rabbit pup model of germinal matrix hemorrhage: role of NAD(P)H oxidase." Stroke 40(6): 2191-2198.