

# Iron Regulation and Heat Shock Proteins in Ischemic Stroke

By

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## Abstract

Ischemic stroke is one of the leading causes of brain injury and death. Many mechanisms underlying ischemia-induced neuronal death have been investigated. Dysregulation of brain iron homeostasis is one of these mechanisms. It is well known that accumulation of intercellular iron in the brain induces free radical production, lipid peroxidation, and DNA damage. The overall aim of this project was to investigate a novel therapeutic mechanism that could be targeted to reduce or prevent iron-induced ischemic brain injury. We hypothesize that heat shock protein 90 (HSP90) plays a role in regulating intracellular iron accumulation upon ischemia and that inhibiting HSP90 rescues the brain from ischemic injury.

First, in order to measure iron levels, the function of a newly developed hydroxylamine compound was tested as a ferrous ion probe. We found that this probe was able to indicate the level of ferrous ion both in cells and in solution with high sensitivity and reliability. Few interfering effects were observed by the presence of other metal ions and reducing agents on the iron measurement. KU32 is a HSP90 inhibitor that was used to investigate whether pharmacological inhibition of HSP90 reduced ischemic injury. We found that KU32 decreased levels of intercellular iron and expression of hepcidin, a central iron regulator. In addition, KU32 reduced neuronal death following ischemia. Taken together, this project has found a new method for measuring ferrous iron levels and a possible link among heat shock proteins in cellular iron regulation. These data suggest that targeting HSP90 may be a potential therapeutic approach to reduce ischemia-induced iron neurotoxicity.

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# Chapter 1 Introduction

## 1.1. Stroke

Stroke is a very serious disease affecting human health, and as the second leading cause of death and disability worldwide. According to epidemiological studies, approximately 12% of deaths in western countries all caused by stroke<sup>1</sup>, and this proportion is up to 9% worldwide<sup>2</sup>. In 2000s, 2-4% of worldwide total health-care cost was spent on stroke, while in developed countries the percentage was higher, up to more than 4%<sup>3</sup>. The problem of disability-adjusted life years (DALYs) caused by stroke-related disability makes stroke rank as sixth among all the common causes of DALYs in 2002<sup>4</sup>. By 2030, this rank is estimated to be fourth in Western countries<sup>5</sup>. With the increase in the aging population, the risk of stroke is projected to increase.

Stroke was defined by the World Health Organization as a "neurological deficit of cerebrovascular cause that persists beyond 24 hours or is interrupted by death within 24 hours"<sup>6</sup>. There are mainly two types of stroke, hemorrhagic and ischemic. The following sections will explain these stroke types in more details.

### 1.1.1. Hemorrhagic stroke

Hemorrhagic stroke is caused by a blood vessel rupturing within or surrounding the skull cavity. With bleeding into the brain tissue or cranial vault, hematomas are formed and compress the brain tissue, which can lead to brain injury. In addition, the elevated pressure in the skull can also limit the blood supply causing infarction. Furthermore, the blood irrigated in the brain has detrimental effects on brain tissue, in which inflammatory mediators, such as matrix

metalloproteinases (MMPs), nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase (HO), and iron toxicity plays a secondary role causing further brain damage<sup>7</sup>.

### **1.1.2. Ischemic stroke**

Ischemic stroke is caused by reduction of blood supply to the brain. There are four major reasons contributing to this pathological condition: (1) an obstruction in peripheral blood vessel forming by a blood clot (thrombosis), (2) an obstruction in peripheral blood vessel formed by an embolus (embolism)<sup>3</sup>, (3) a general decrease of blood supply (systemic hypoperfusion)<sup>8</sup>, (4) cerebral venous sinus thrombosis<sup>9</sup>. Heart failure is associated with about 9%<sup>10</sup> of all strokes and is a major risk factor for ischemic strokes since it causes systemic hypoperfusion<sup>10</sup>.

Due to the loss of blood supply in the brain, an ischemic cascade is initiated<sup>11</sup>. The loss of blood supply directly results in the deprivation of oxygen and nutrients, such as glucose, delivered to the brain. This restriction of oxygen and nutrients impairs the brain tissue functions within one to two minutes following the infarction. A long restriction of blood supply to the brain (> 3 hours) can cause irreversible tissue damage with a massive death of cells in the ischemic area.

Glutamate plays an important role in the tissue injury during oxygen and glucose deprivation in stroke. In the absence of oxygen and glucose, low levels of ATPs are generated, resulting in reduction of the energy source for the ion pump which maintains concentration gradients of ions (mainly sodium) across the cell membrane. Downregulation of transmembrane ions may induce glutamate release into the extracellular area, activating numerous receptors, such as the *N*-methyl-D-aspartate (NMDA) receptor which stimulates cellular influx of calcium<sup>12</sup>. This calcium influx activates the enzymes to digest the components of the cell, causing cell death. At same time, calcium influx can also cause mitochondrial dysfunction, followed by energy depletion and apoptosis<sup>13</sup>.

Reactive oxygen species (ROS), such as oxygen free radicals are generated during ischemia. Prooxidant enzymes are involved in this production of oxidative stress, which could be divided into three major classes based on their products: (1) nitric oxide synthases (NOS), producing NO<sup>•</sup>; (2) cyclooxygenases (COX), xanthine dehydrogenase (XDH), xanthine oxidase (XO) and NADPH oxidase (NADPHO), producing O<sub>2</sub><sup>•-</sup>; (3) myeloperoxidase (MPO) and monoamine oxidase (MAO), producing HOCl and H<sub>2</sub>O<sub>2</sub>, respectively<sup>14</sup>. It is well known that ROS oxidize and damage the macromolecular cell components, lipids, proteins and DNA, leading to cell death and brain injury<sup>15-17</sup>. Mitochondria, apurinic/aprimidinic endonuclease/redox factor-1 (APE/Ref-1), a DNA repair enzyme, and transcription factors are reported to be the major targets of ROS that contribute to the cell death<sup>18,19</sup>.

### **1.1.3. Reperfusion injury**

The reperfusion following ischemia could exacerbate tissue damage and cause further necrosis, though the mechanism of ischemia/reperfusion injury is still far from being understood<sup>20</sup>.

Although reperfusion gets the blood flow back to normal levels after ischemia, the injury unexpectedly turns out to be more serious. With some aspects still in debate, the mechanisms underlying reperfusion injury may include: (1) the increase of oxidative/nitrosative stress generated by refueling of oxygen and glucose along with the replenishment of blood; (2) calcium overload; (3) the opening of the mitochondrial permeability transition (MPT) pore; (4) dysfunction of endothelial cells; (5) appearance of a prothrombogenic phenotype; (6) and pronounced inflammatory responses<sup>21</sup>.

The brain is the most susceptible organ to the ischemia reperfusion injury. This is because of the high demand and low store of energy resources, such as glucose<sup>22,23</sup>. In addition, the relatively

lower level of antioxidant, such as superoxide dismutase (SOD), catalase, glutathione peroxidase<sup>24</sup> and heme oxygenase-1<sup>25</sup> possessed by the brain is another contributes to the susceptibility of the brain to ischemia reperfusion. Furthermore, polyunsaturated fatty acids, one of the major energy resources in brain, are also vulnerable to oxidative stress<sup>24</sup>. Additionally, some neuro transmitters could induce calcium overload<sup>22</sup>, which is involved in inducing ischemia reperfusion injury in brain as well.

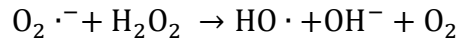
## **1.2. Iron regulation in central nervous system (CNS)**

Iron is an indispensable element for human body. It is widely known as a key component of hemoglobin, delivering oxygen throughout the whole body. In the central nervous system (CNS), iron also plays a key role in various metabolic regulations. Numerous studies have suggested that iron dysregulation is involved in most neurodegeneration diseases, such as Parkinson's disease, amyotrophic lateral sclerosis and cognitive deficits<sup>26-28</sup>. Moreover, iron chelation is also being used clinically as a therapy for some neurodegeneration diseases including Alzheimer's disease<sup>29</sup>, Parkinson's disease<sup>30</sup> and Friedreich's ataxia<sup>31</sup>. Although mechanisms still remain unclear, iron accumulation is widely assumed to mediate free radical generation and enhancing oxidative stress, which contributes to cell death in the CNS<sup>30,32</sup>.

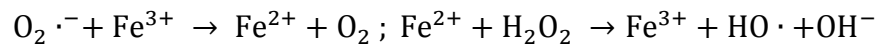
### **1.2.1. Iron and ischemic stroke**

Several studies have reported that iron overload induces free radical production and cell death in stroke<sup>34-36</sup>. During ischemic stroke, the hypoxic condition induces iron to unbind from the iron-storage proteins (i.e. ferritin). Ferric iron is released from ferritin and reduced into ferrous in the presence of ROS. In addition, the increased acidic level during hypoxia catalyzes the ferric iron releasing from transferrin. These series of reactions elevates the iron level in ischemic brain.

Disruption of iron homeostasis induces ischemic brain injuries<sup>37,38</sup>. The Haber–Weiss reaction catalyzes the conversion of superoxide ( $O_2 \cdot^-$ ) to the highly reactive toxic hydroxyl free radicals ( $HO \cdot$ )<sup>39,40</sup>, which is involved in tissue injury.



Ferrous iron works as a catalyzer in this reaction through the Fenton reaction<sup>39</sup>:



These hydroxyl free radicals lead to apoptosis and cause tissue damage. In addition, iron can also cause lipid peroxidation<sup>41</sup> and is associated with some conditions that predispose to ischemic stroke<sup>42-44</sup>.

### 1.2.2. Iron transport in CNS

Transferrin plays a major role in mediating iron entrance into endothelial cells from the blood<sup>45,46</sup>. The divalent metal transporter 1 (DMT1) is involved in iron uptake by endothelial cells<sup>47</sup> while ferroportin contributes to the iron export to the abluminal side of endothelium<sup>47</sup>. Astrocytes are involved in the release of iron from endothelium at the abluminal surface<sup>48,49</sup>. In the brain interstitium, iron mainly exists in the ferric state, binding to transferrin as well as molecules such as citrate, ATP, ascorbic acid<sup>50</sup> and the protein lactoferrin<sup>51</sup>. Neurons express transferrin receptors and DMT1<sup>52</sup> that the expression of transferrin receptors can be response to the neuronal iron levels<sup>46,53</sup>. Some neurons also express ferritin which can store iron<sup>54</sup>.

Ferroportin works as the iron exporter in neurons, which is an essential factor regulating neuronal iron level<sup>55</sup>. Hepcidin is an inhibitor of ferroportin. Astrocytes uptake non-transferrin bound iron and export iron through lactoferrin, due to their lackage of transferrin receptor and

DMT1<sup>48</sup>. Oligodendrocytes also do not express transferrin receptors but have ferroportin<sup>55,56</sup>, so their iron uptake is similar to astrocytes and their export is by ferroportin. The major route of iron transport out the brain is via cerebrospinal fluid (CSF) reabsorption back into the blood with transferrin<sup>57</sup>.

### **1.2.3. Iron regulatory proteins**

Numerous proteins are involved in iron regulation and the following section gives a brief introduction to some iron regulatory proteins involved in this research.

#### ***Hepcidin***

Hepcidin is a hormone that plays a key role in iron regulation<sup>58</sup> and is mainly synthesized by hepatocytes<sup>59,60</sup>. Bone morphogenetic protein (BMP) and JAK2/STAT3 signaling pathways can mediate hepcidin transcription. In addition, iron can also increase hepcidin expression under nonpathological conditions<sup>58</sup>. Ferroportin is the target of hepcidin. Hepcidin can bind to ferroportin and induce its endocytosis and proteolysis in lysosomes<sup>61</sup>.

#### ***Ferroportin***

Ferroportin is a transmembrane protein that exports iron<sup>62-64</sup>. As a transporter, ferroportin mediates ferrous iron ( $\text{Fe}^{2+}$ ) efflux<sup>65,66</sup>. During cellular iron efflux, ferrous iron is oxidized into ferric iron by multicopper oxidases, either hephaestin or ceruloplasmin<sup>67-69, 70</sup>. Moreover, ferroportin is the receptor of hepcidin and can be inhibited by hepcidin.

### **1.2.4. Distribution of iron regulatory proteins in CNS**

According to existing reports, tables of iron regulatory proteins in the CNS were made to indicate the expression of iron regulatory proteins. Table 1 shows the distribution of iron regulatory proteins among different types of cells. Neurons express numerous proteins regulating

iron homeostasis; the transferrin receptor and DMT1 mediate iron import, ferritin regulates to the labile iron pool and ferroportin mediates the iron export (Table 1). Ceruloplasmin is encoded by the *CP* gene<sup>71-73</sup> and as a ferroxidase enzyme with 6 atoms of copper in its structure<sup>74</sup>. With the copper dependent oxidative activity, ceruloplasmin induces the oxidation of iron(II) into iron(III) so that the iron ions can be carried by transferrin<sup>75</sup>. Lactoferrin is a multifunctional member of the transferrin family, also known as lactotransferrin<sup>76</sup>. Lactoferrin receptors mediate lactoferrin internalization and iron absorption.

Glial cells, including astrocytes, oligodendrocytes and microglia, usually express part of these iron regulatory proteins, and all thought to play regulatory roles in maintaining iron homeostasis. Most glial cells do not express transferrin receptor and DMT1 while the expression of DMT1 in astrocytes is in debate, so glial cells lack the major method to internalize iron. However, the presence of lactoferrin receptors and ferritin as well as the expression of ferroportin in astrocytes suggests that glial cells have a capacity for iron transport, storage and export. In addition, based on the supporting functions of glial cells to neurons, glial cells might be involved in neuronal iron regulation. Moreover, in brain capillary endothelial cells, a major component of blood-brain barrier, most of the iron regulatory proteins also exist, which is consistent with the theory of iron transport across the blood-brain barrier. A major pathway of iron transport across the blood-brain barrier is via transferrin. However the other transporters such as, DMT1 and ferroportin are also involved. Generally, iron transport across the blood-brain barrier is similar to iron absorption in the intestines. Table 2 shows the distribution of iron regulatory proteins among different brain regions. Cerebral cortex and hippocampus have transferrin receptors to take iron into cells. However, there is no report showing any evidence about the expression of transferrin receptors in the corpus striatum. In addition, ferritin exists widely in different CNS components such as

cerebral cortex, hippocampus and corpus striatum as well as serum and cerebrospinal fluid, indicating that iron is stored widely throughout the brain. Also hepcidin is expressed widely, in cerebral cortex, hippocampus and corpus striatum, indicating that it is involved in iron regulation in the CNS.

In summary, different iron regulatory proteins widely exist in different regions in the brain. These proteins all contribute to maintain iron homeostasis in the CNS, forming a complicated regulation network of iron transport and storage. However, there are still some blanks in these tables and expression of these proteins in specific brain regions are still in debate. Hence, more research is needed to improve our understanding of iron regulation in the CNS.



**Table 1 Iron regulation proteins in brain cells**

Cells	Transferrin receptor	Transferrin	Divalent metal transporter 1 (DMT1)	Ferroportin	Ceruloplasmin	Lactoferrin receptor	Ferritin
<b>Neural progenitor cells (NPCs)</b>	+ <sup>45</sup>		+				
<b>Brain capillary endothelial cells (BCECs)</b>	+ <sup>46,104</sup>	+ <sup>98,99,100</sup>	+ <sup>93,94</sup> - <sup>95</sup>	+ <sup>56</sup> - <sup>55,82</sup>		+ <sup>82</sup>	+ <sup>①77</sup>
<b>Neurons</b>	+ <sup>48,90</sup>	+ <sup>②48</sup>	+ <sup>48,49,90,95</sup>	+ <sup>55,56,90</sup>	+ <sup>87</sup>	+ <sup>83-85</sup>	+ <sup>③78</sup>
<b>Astrocytes</b>	- <sup>48</sup>		+ <sup>96,97</sup> - <sup>48</sup>	- <sup>55,56</sup>	+ <sup>88,89</sup>	+ <sup>83-85</sup>	
<b>Oligodendrocytes</b>	- <sup>105</sup>	+ <sup>80,101</sup>		+ <sup>55,56</sup>		+ <sup>83-85</sup>	+ <sup>79,80</sup>
<b>Microglia<sup>④</sup></b>	- <sup>91</sup>	- <sup>91</sup>	- <sup>91</sup>	- <sup>91</sup>		+ <sup>83-85</sup>	+ <sup>⑤81</sup> + <sup>80</sup>
<b>Choroids plexuses epithelial cells</b>	+ <sup>103</sup>	+ <sup>102,103</sup>	+ <sup>95</sup>	+ <sup>92</sup>		+ <sup>⑥86</sup>	

Note: +, expressed; -, not expressed

- ① Ferritin transports across brain capillary endothelial cells.
- ② Transferrin is internalized into the neurons.
- ③ Some unique types of neurons express ferritin.
- ④ Microglia rarely expresses transferrin receptor, transferrin or DMT1. When reaching resting and fully differentiated state, microglia does not express transferrin receptor, transferrin or DMT1.
- ⑤ Migrating cells express ferritin.
- ⑥ Lactoferrin receptors are expressed in newborn calves.

**Table 2 Iron regulation in different brain regions**

Brain regions	Transferrin receptor	Ferritin	Hepcidin
<b>Cerebral cortex</b>	+ <sup>106</sup>	L +, H + <sup>106</sup>	+ <sup>106</sup>
<b>Hippocampus</b>	+ <sup>106</sup>	L +, H + <sup>106</sup>	+ <sup>106</sup>
<b>Corpus striatum</b>		L +, H - <sup>106</sup>	+ <sup>106</sup>
<b>Serum</b>		+ <sup>107,108</sup>	
<b>Cerebrospinal fluid</b>		+ <sup>107</sup>	

Note: +, expressed; -, unexpressed. L, ferritin light chain; H, ferritin heavy chain.

### 1.2.5. Iron measurements

Iron exists in its ionic forms. The following section discusses two probes applied in this research.

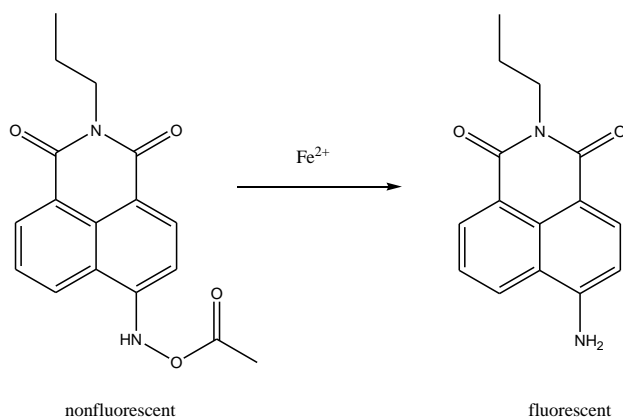
#### *Calcein-AM (calcein- acetoxymethyl ester)*

Calcein-AM is a well-established cytochemical method to measure the labile iron pool (LIP)<sup>109-112</sup>. Calcein-AM is lipophilic, so that it can easily penetrate cell membranes, after which calcein-AM is cleaved by cytosolic esterase inside the cells<sup>113</sup>. After the cleavage, calcein, one of the reaction products, quenches its green fluorescence after chelating iron in the cell. Other metal ions, such as Ca<sup>2+</sup>, Mg<sup>2+</sup> and Cu<sup>2+</sup>, hardly affect either the fluorescence or the quenching<sup>110</sup>. Dequenching will happen after addition of a highly permeant iron chelator, by which the change in fluorescence gives a reliable estimation of iron level<sup>114</sup>. However, after the reaction of cleavage, calcein becomes hydrophilic and cannot penetrate membranes any more. Therefore, calcein-AM can only detect cytosolic iron, but not the temporary localized lysosomal low-mass iron<sup>113</sup>.

#### *Hydroxylamine compounds*

N-oxide chemistry could be applied to develop fluorescent probes in order to detect ferrous ion selectively. Since Nagasawa and coworkers developed the N-oxide probe, RhoNox-1, in 2012<sup>115</sup>, several hydroxylamine compounds have been developed. Within these compounds, the N-O bond is designed for ferrous ion to react with. After the reduction and cleavage of these N-O

bonds, fluorescence will be generated to mark the presence of iron (Scheme 1). For example, a kind of N-aryl-O-acylhydroxylamine is developed, and its fluorescence could be detected through microscopy<sup>116</sup>. In addition, this probe is sensitive and hardly affected by other metal ions<sup>116</sup>, such as  $Zn^{2+}$  and  $Cu^{2+}$ .



**Scheme 1. The mechanism of ferrous ion inducing N-aryl-O-acylhydroxylamine to generate fluorescence**

### 1.3. Heat shock proteins (HSPs)

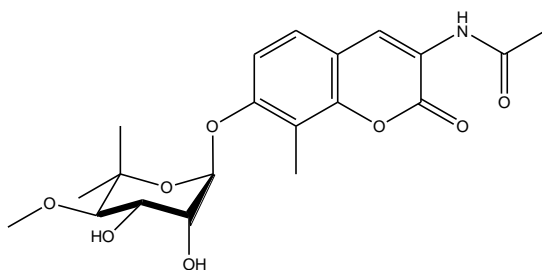
Heat shock proteins (HSP) are a family of proteins usually produced by cells in response of stressful conditions such as heat shock, inflammation, starvation and hypoxia. The function of HSP is to stabilize new proteins and help to refold damaged proteins caused by cell stress<sup>117-119</sup>.

#### 1.3.1. HSP90 and HSP70

HSP90 is a member of heat shock protein family and mediates the folding of numerous proteins into their final conformations. HSP90 can mediate tumor growth via vascular endothelial growth factor (VEGF) and nitric oxide synthase (NOS)<sup>120</sup> and cancer metastasis through matrix metalloproteinase (MMP2)<sup>121</sup>. HSP70 is also a member of heat shock protein family and has been reported as an important chaperone protein involved in oxidative stress and mitochondrial activities. At times, HSP70 work as a downstream factor of HSP90, since cell stress can lead to a

HSP90-dependent HSP70 upregulation to help to refold or clear aggregated and damaged proteins<sup>122</sup>. Moreover, upon the induction of HSP70, neuronal apoptosis and oxidative stress is often reduced<sup>123,124</sup>. According to recent research<sup>125,126</sup>, this HSP70 induction resulting from HSP90 inhibition has the potential to treat diabetic neuropathy and some neurodegenerative diseases.

HSP90 has its ATPase activity due to its N-terminal and C-terminal DNA binding domains<sup>127</sup>. KU-32 (Figure 1) was developed as a C-terminal inhibitor of HSP90<sup>128</sup>. The inhibition of C- or N-terminals will abolish the ATPase activity and prevent protein folding<sup>129</sup>. In addition, HSP90 also binds to heat shock factor 1, a transcription factor. The inhibition of HSP90 induces heat shock factor 1 dissociation from HSP90 and often its translocation to the nucleus, leads to the synthesis of various proteins such as HSP70<sup>127</sup>.



**Figure 1. Structure of KU32**

### 1.3.2. Heat shock proteins and ischemia

The function of HSP90 in the CNS under ischemia is complicated and the mechanism of HSP90 in brain is not clear<sup>130,131</sup>. However, several studies have characterized a relationship between HSP90 and hypoxia induced factor-1 (HIF-1), an important transcription factor involved in ischemia. Some reports indicate that HSP90 can interact with hypoxia induced factor-1 $\alpha$  (HIF-1 $\alpha$ ) and stabilize it<sup>132-134</sup>. A recent study indicates that the interaction between HIF-1 $\alpha$  and HSP90 is involved in the ability of N-acetylcysteine's (NAC) to protect against stroke<sup>135</sup>. While HIF-1 $\alpha$  is related to iron metabolism<sup>136,137</sup>, HIF-1 deletion will have an effect on iron regulatory

proteins<sup>136</sup>. In addition, HIF-responsive elements (HRE) are located within the promoters of iron-related genes, such as the transferrin receptor (TfR) and heme oxygenase-1 (HO-1)<sup>138</sup>. Hence, there might be delicate mechanisms between heat shock proteins, HIFs and iron regulation in ischemia.

#### **1.4. This research**

In sum, ischemic stroke is a severe disease threatening human lives, in which iron regulation and heat shock proteins might be involved. We established this research based on the hypothesis that inhibition of HSP90 reduces iron neurotoxicity and ischemia reperfusion injury in neurons. In addition, we also tested new hydroxylamine iron probe to determine cellular ferrous level.

## Chapter 2 Materials and methods

### 2.1. Materials

Agents	Catalog number	Manufacture	Address
SH-SY5Y cell line	CRL-2266	ATCC	Manassas, VA
DMEM/high glucose	SH30022.01	HyClone Laboratories, Inc.	Logan, UT
Opti-MEM	31985070	Life Technologies Corporation	Carlsbad, CA
FBS	S11150	Atlanta Biologicals, Inc.	Flowery Branch, GA
Sodium pyruvate	11360070	Life Technologies Corporation	Carlsbad, CA
Penicillin streptomycin	P0781-100ML	SIGMA-ALDRICH	Milwaukee, WI
Trypsin-EDTA	SH30042.01	HyClone Laboratories, Inc.	Logan, UT
DMSO	34869-1L	SIGMA-ALDRICH	Milwaukee, WI
Iron	307718-500G	SIGMA-ALDRICH	Milwaukee, WI
Potassium chloride	P217-500	Fisher Scientific	Fair Lawn, NJ
Calcium chloride dehydrate	C3881-500G	SIGMA-ALDRICH	Milwaukee, WI
Sodium chloride	S271-3	Fisher Scientific	Fair Lawn, NJ
Lithium chloride	L121-500	Fisher Scientific	Fair Lawn, NJ
Magnesium acetate tetrahydrate	M566-50G	SIGMA-ALDRICH	Milwaukee, WI
Zinc sulfate	8880	MALLINCKRODT, INC.	St Louis, MO
Cobalt chloride	C371-100	Fisher Scientific	Fair Lawn, NJ
Sodium thiosulfate	S-445	Fisher Scientific	Fair Lawn, NJ
Sodium sulfite	S0505	SIGMA-ALDRICH	Milwaukee, WI
L-ascorbic acid	A-4544	SIGMA-ALDRICH	Milwaukee, WI
Sodium metabisulfite	7777	MALLINCKRODT INC.	St Louis, MO
Deferoxamine	D9533-1G	SIGMA-ALDRICH	Milwaukee, WI
Calcein blue AM	564060	BD Biosciences	San Diego, CA
Dulbecco's Modified Eagle's medium	D5030-1L	SIGMA-ALDRICH	Milwaukee, WI
MTT	M6494	Life Technologies Corporation	Carlsbad, CA
Cocktail of protease inhibitors	P-8340	SIGMA-ALDRICH	Milwaukee, WI
EDTA	1858567	Thermo Scientific	Waltham, MA
Bio-Rad DC protein assay agent		Bio-Rad laboratories, Inc.	Richmond, CA
HSP70 antibody	ADI-SPA-810-D	Enzo Life Sciences, Inc.	Farmingdale, NY
Goat anti-rabbit IgG-HRP	SC2030	Santa Cruz Biotechnology	Dallas, TX
Actin (I-19)-R	SC-1616-R	Santa Cruz Biotechnology	Dallas, TX

Triton x-100	T8787-100ML	SIGMA-ALDRICH	Milwaukee, WI
Poly-D-lysine	P0899-10MG	SIGMA-ALDRICH	Milwaukee, WI

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## 2.2. Cell culture

SH-SY5Y cells were cultured in DMEM/high glucose with 20% Opti-MEM, 10% FBS, 1x sodium pyruvate and penicillin streptomycin, at 37°C, 5% CO<sub>2</sub>, 95% air. Cells were trypsinized by trypsin-EDTA and subcultured from one passage to another. The drug treatment usually started when the cells were grown up to 70% confluence. Drug treatment usually lasted for 24 hours.

## 2.3. Iron measurement

### 2.3.1. Hydroxylamine iron probe

The new hydroxylamine iron probe was provided by Dr. Wei Wang and was dissolved in DMSO at 20mM and stored at -20°C. The storage vials and the box were also kept from light. The probe was diluted in cell growth medium, prior to use.

### *In solution iron measurement*

Iron (II) was dissolved in distilled deionized water at concentration of 2mM. Other metal ion solutions were prepared at concentrations from 5mM to 100mM in distilled deionized water, K<sup>+</sup> 100mM, Ca<sup>2+</sup> 100mM, Na<sup>+</sup> 100mM, Li<sup>+</sup> 100mM, Mg<sup>2+</sup> 100mM, Zn<sup>2+</sup> 100mM, Co<sup>2+</sup> 100mM, Cu<sup>+</sup> 500μM (due to its low solubility), Cu<sup>2+</sup> 100mM, in which chloride was applied as the negative counter ion. These solutions were diluted and added into 96-well plate to measure the fluorescence at excitation wavelength 500nm and emission 550nm.

### ***In vitro* iron measurement**

Cells were cultured in multi-well plates (6-well and 24-well) and treated with metal ions in growth medium for 24 hours after reaching 50% confluence. Fluorescent images were taken by microscope. Quantification of the fluorescence signal was done with ImageJ. Deferoxamine was used as iron chelator and served as a negative control.

#### **2.3.2. Calcein blue AM**

Calcein blue AM was dissolved in DMSO at 5mM and stored in the dark at  $-20^{\circ}\text{C}$ . This stock solution was diluted into growth medium at a concentration of  $5\mu\text{M}$  and added into the cell culture wells<sup>139</sup>. After one 5-minute incubation, the fluorescent images of cells were taken by microscope. The quantification of the fluorescence was done with help of the ImageJ.

Deferoxamine was applied as iron chelator serving as a negative control.

### **2.4. Ischemia/reperfusion model**

Oxygen and glucose deprivation (OGD) was used as an *in vitro* ischemia/reperfusion model. The hypoxia chamber can maintain an environment of 95% nitrogen and 5% carbon dioxide at  $37^{\circ}\text{C}$ . Cells were transferred into the hypoxia chamber and changed to OGD medium for 2 hours to mimic the ischemic process. The cells were then placed in normal medium without FBS, transferred back to non-hypoxic conditions and kept for 24 hours to mimic the reperfusion phase. After this series of treatment, the cells were collected for further experiments.

### **2.5. MTT cell viability assay**

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution was prepared at 5mg/ml in PBS and was filtered through a  $0.2\mu\text{m}$  filter. Cells were cultured in 96-well plate at a density of 10,000 cells/well. After treatment,  $20\mu\text{l}$  of the MTT solution was added into each well.



The cells were incubated for 4 hours at 37°C with 5% carbon dioxide, 95% air. The MTT solution was removed and replaced with 200µl of DMSO. The plate was further incubated for 10-30 min, and the optical density of the wells was determined using the plate reader at 570nm and a reference wavelength of 630nm.

## **2.6. Immunoblotting**

Proteins were extracted from SH-SY5Y cells by incubating the whole lysate with RIPA (Radio Immuno Precipitation Assay) buffer in the presence of a cocktail of protease inhibitors (Thermo, Meridian, IL) and EDTA. The protein concentration was determined by Bio-Rad DC protein assay reagent (Bio-Rad, Hercules, CA). Standard Western blot procedures<sup>140</sup> were conducted by using primary antibodies against HSP70 and  $\beta$ -actin. The signal development was carried out with an enhanced chemiluminescence detection kit (Pierce, Rockford, IL). The intensity of immunoreactive bands was quantified using ImageJ. Results were normalized to  $\beta$ -actin<sup>141</sup>.

## **2.7. In vitro immunocytochemistry**

Coverslips were coated with poly-L-lysine for 12 hours at room temperature. Sterile water was applied to rinse coverslips. The coverslips were allowed to dry completely and sterilized under UV light. Cells were cultured on these coverslips and were treated at 80% confluence. After treatment, the coverslips were rinsed briefly in PBS, were fixed with methanol at room temperature (RT) for 20 min and the permeabilized with 0.3% Triton X-100 in PBS for 15min. To block nonspecific binding, cells were exposed to blocking solution (PBS containing 0.1% Triton X-100 and 1% BSA) for 30 min and incubated overnight at 4°C with primary antibody. The cells were washed with PBST (PBS containing 0.1% Triton X-100) four times for 15 minutes and incubated in secondary antibody for 90 minutes at room temperature in the dark.

After washing with PBST, the coverslips were mounted with Vectashield H1000 (temporary mounting medium, Vector Laboratories). The coverslips were sealed with nail polish.

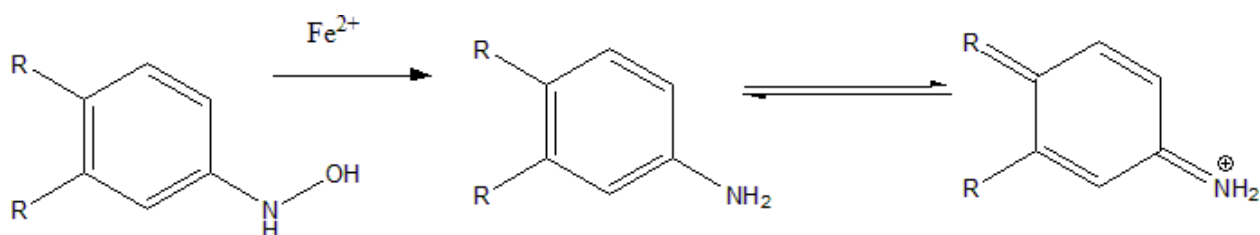
## **2.8. Statistical analysis**

Student t-test was applied to determine the significance differences between two groups of samples. One-way ANOVA was used to determine overall significance differences followed by post hoc Tukey's test for multiple comparisons. Two-way ANOVA was used to determine the interaction between pretreatments and treatments. Data were presented as means  $\pm$ SEM. Differences were considered statistically significant at  $p < 0.05$ .

## Chapter 3 Results

### 3.1. Iron measurement with hydroxylamine probe

A newly developed hydroxylamine by Dr. Wei Wang's lab was applied in this research to measure iron. The mechanism of this iron probe is that the iron (II)-dependent dehydroxylation of the hydroxylamine to the amine (Scheme 2). After cleavage of the N-O bond, the product, an electron-donating amine, has the feature to generate fluorescence. The intensity of fluorescence can indicate the level of ferrous.



**Scheme 2.** The structure of hydroxylamine probe and its detection mechanism

#### 3.1.1. The fluorescence on different time points and concentrations

The probe itself is non-fluorescent ( $\lambda_{max,em} = 556 \text{ nm}$ ,  $\epsilon = 4800 \text{ M}^{-1}\text{cm}^{-1}$ ). In the presence of ferrous ion, fluorescence could be generated within 10 minutes ( $\lambda_{max,em} = 542 \text{ nm}$ ,  $\epsilon = 55400 \text{ M}^{-1}\text{cm}^{-1}$ ). Curves illustrating the different fluorescent intensities under different wavelengths were made (Figure 2**Error! Reference source not found.**), from which some information about the fluorescence wavelength of this probe could be read. Through these curves, the maximum fluorescent intensity was between 550nm and 600nm, approximately around 570nm. The fluorescence below 480nm and above 630nm was weak. Based on this result and information about this compound from Dr. Wei Wang's group, we chose  $\lambda_{ex} = 500 \text{ nm}$ ,  $\lambda_{em} = 556 \text{ nm}$  for later fluorescence detection in this research. In addition, time course

analyses of fluorescent intensity ( $\lambda_{max,ex} = 500 \text{ nm}$ ,  $\lambda_{max,em} = 556 \text{ nm}$ ) (Figure 2B) was performed to define the reaction rate. During the first 10-20 minutes, the curve showed first order kinetics, meaning that during this period of time, hydroxylamine rapidly reacted with ferrous ion and generated fluorescence. After 20 minutes, rate of fluorescent increasing started to decrease, indicating zero order kinetics. Later, after 40 minutes, with the substrate depletion, the increasing of fluorescent intensity was very slow. And after about 2 hours had passed (Figure 2C), the slope of the intensity curve of fluorescence was close to zero. These results demonstrate that the fluorescence generated from the reaction between ferrous iron and hydroxylamine probe is stable and able to last for more than 2 hours.

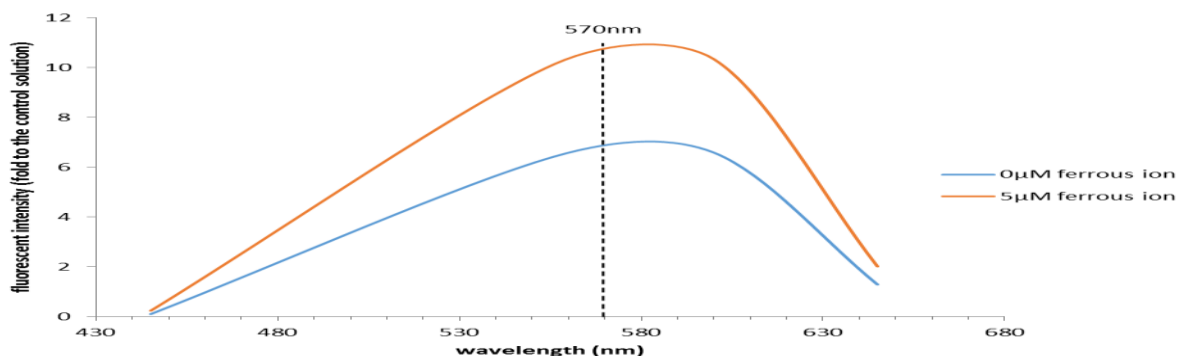
Concentration-dependent experiments were performed to analyze the relation between fluorescent intensity and iron concentration. At first  $3\mu\text{M}$  probe was added to ferrous solution at concentration ranging from  $5\mu\text{M}$  to  $25\mu\text{M}$  in 96-well plates, and the fluorescent intensity was measured by plate reader. Figure 2D shows that fluorescence was generated starting from  $5\mu\text{M}$  iron. When the iron concentration went up from  $5\mu\text{M}$  to  $10\mu\text{M}$ , the fluorescence was significantly increased. And an increase in intensity occurred when the iron concentration was added up to  $15\mu\text{M}$ . These results show that the intensity of fluorescence is positively correlated to the concentration of ferrous iron. However, the amount of increases in intensity between  $5\mu\text{M}$  and  $10\mu\text{M}$  was larger the increased amount between  $10\mu\text{M}$  and  $15\mu\text{M}$ . This suggests that the probe must be saturated at relatively higher iron concentration, and the saturation point is approximately at ratio 1 to 5-6 probe to ferrous ion. This was further substantiated by the fluorescent intensity at  $20\mu\text{M}$  and  $25\mu\text{M}$  of ferrous ion, between which no significant difference was found. Furthermore,  $5\mu\text{M}$  probe was also used to detect the fluorescence difference on different concentrations, after which similar results were achieved. In the  $5\mu\text{M}$  probe experiment

(Figure 2C), similarly, there was a significant difference in intensity between the 0 $\mu$ M ferrous ion group and 20 $\mu$ M group, but a much smaller difference between the 20 $\mu$ M group and 40 $\mu$ M group, and even no significant difference between 40 $\mu$ M and 80 $\mu$ M groups. On the other hand, this figure also shows that these differences are stable and able to last for more than 10 minutes. Similarly, this figure shows that the curve of fluorescence at 5 $\mu$ M ferrous ion is completely above the curve of 0 $\mu$ M group, indicating that the whole fluorescence generated by probe from wavelength 500nm to wavelength 600nm is completely enhanced by the presence of ferrous ion.

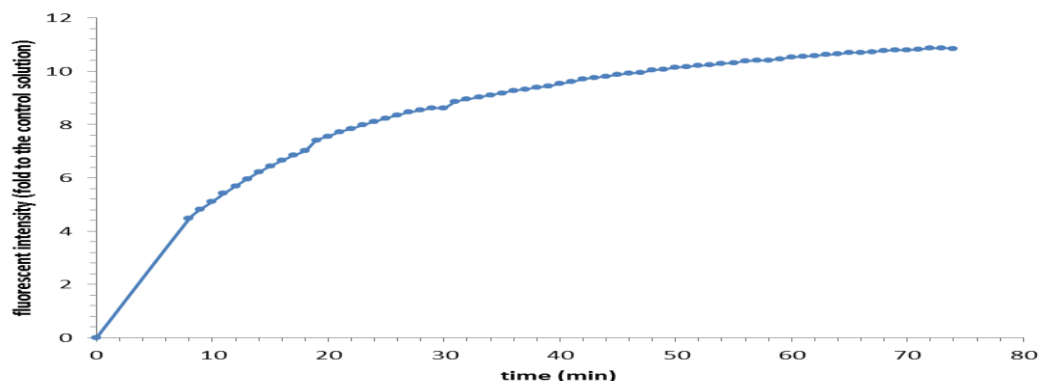
In sum, these results indicate that this hydroxylamine probe possesses a time- and concentration-dependent fluorescence, and that the maximum fluorescent intensity is around 570nm.

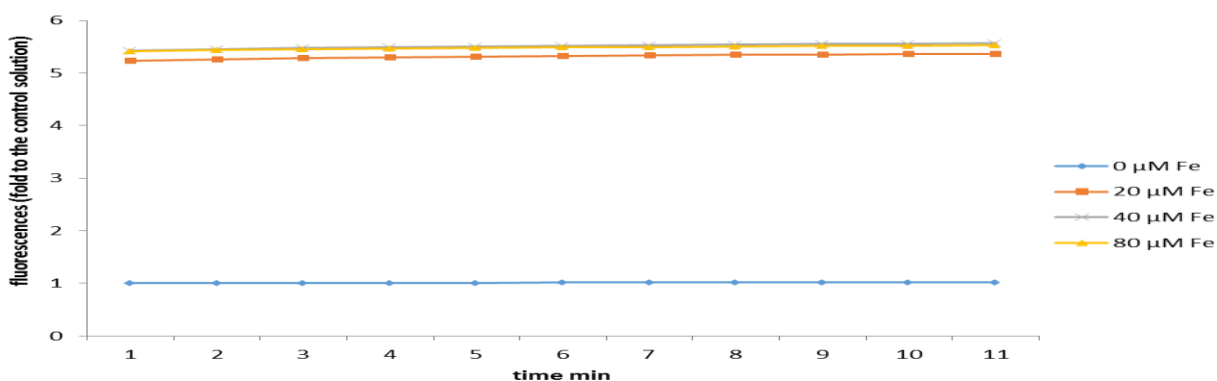
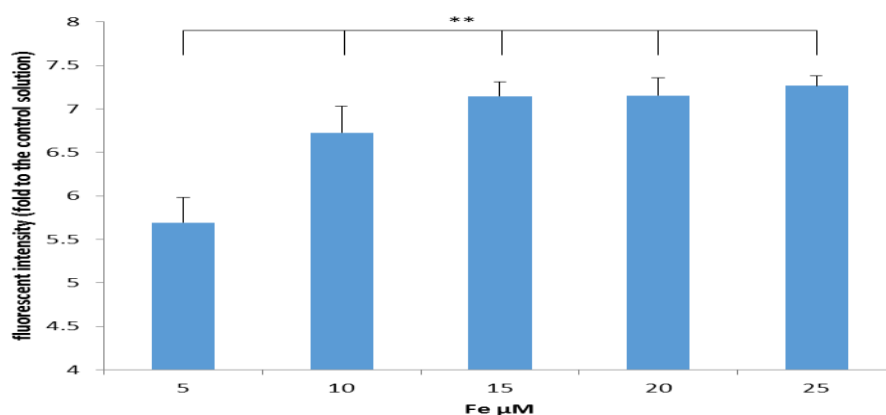
Furthermore, these results suggest that in aqueous solution, this hydroxylamine probe is a potent and sensitive ferrous iron indicator.

**A**



**B**



**C****D**

**Figure 2. Time and iron concentration dependent fluorescent intensity of the probe**

- A. The fluorescence generated from the probe reacting with 0 and 5  $\mu\text{M}$  iron under different wavelength of detection.
- B. The fluorescence generated from the probe at every minute from 8 to 74 minutes after the probe added into iron solution.
- C. The fluorescence generated from 5  $\mu\text{M}$  probe reacting with 0, 20, 40, 80  $\mu\text{M}$  iron 2 hours after the addition. This curve represents 10-minute detection.
- D. The fluorescence generated from 3  $\mu\text{M}$  probe and 5, 10, 15, 20, 25  $\mu\text{M}$  iron.

The control solution of Figure A, B and D is dd water. The control solution of Figure C is 5  $\mu\text{M}$  probe solution without ferrous ion.

\*\*  $p < 0.01$ ;  $n = 8$ , Multiple comparisons among the 5, 10, 15, 20 and 25  $\mu\text{M}$  groups

### 3.1.2. The fluorescence on other agents

Since iron can react with this hydroxylamine probe and generate fluorescence, in order to analyze its specificity to iron as a probe, other metal ions were also tested. In this research, several common metal salts were used to test the specificity of the probe. Potassium chloride, calcium chloride, sodium chloride, lithium chloride, magnesium acetate, cobalt chloride and zinc sulfate were applied to provide metal ions in solution. These ions have a relatively wide range of ion radius, with some of the ions (such as lithium ion) smaller than ferrous ion and some bigger. All these ions contain one or two charges, which is fairly common among metal ions. Also some of these metals are pretty close to iron in the periodic table, such as cobalt and zinc. More importantly, almost all of these metals have biological functions<sup>142-149</sup>. So testing of these metals on the probe will help to determine the potential biological specificity of this probe. On the other hand, negatively charged ions were not reactive, and did not influence the fluorescence significantly.

Figure 3A shows the fluorescence generated by different metal ions. In this figure, except ferrous ion, all metal ions generate relatively low level of fluorescent intensities, even though their concentration is 19 times higher than the iron concentration. Among these metal ions, the fluorescent intensities did not have significant differences. In addition, except the cobalt ion, the other ions do not have color at that concentration (100 $\mu$ M); the slight red color of cobalt ion might have an effect on the fluorescence. More importantly, the red color of cobalt ion still did not have an obvious influence in fluorescent generation by the probe.

Since iron has two forms of ions, ferrous and ferric, ferrous ion can lose one electron and turn into ferric ion. Hence, ferrous ion has reductive property. An assumption that the reductive property might be a sufficient condition of the fluorescence generation by the probe was raised.

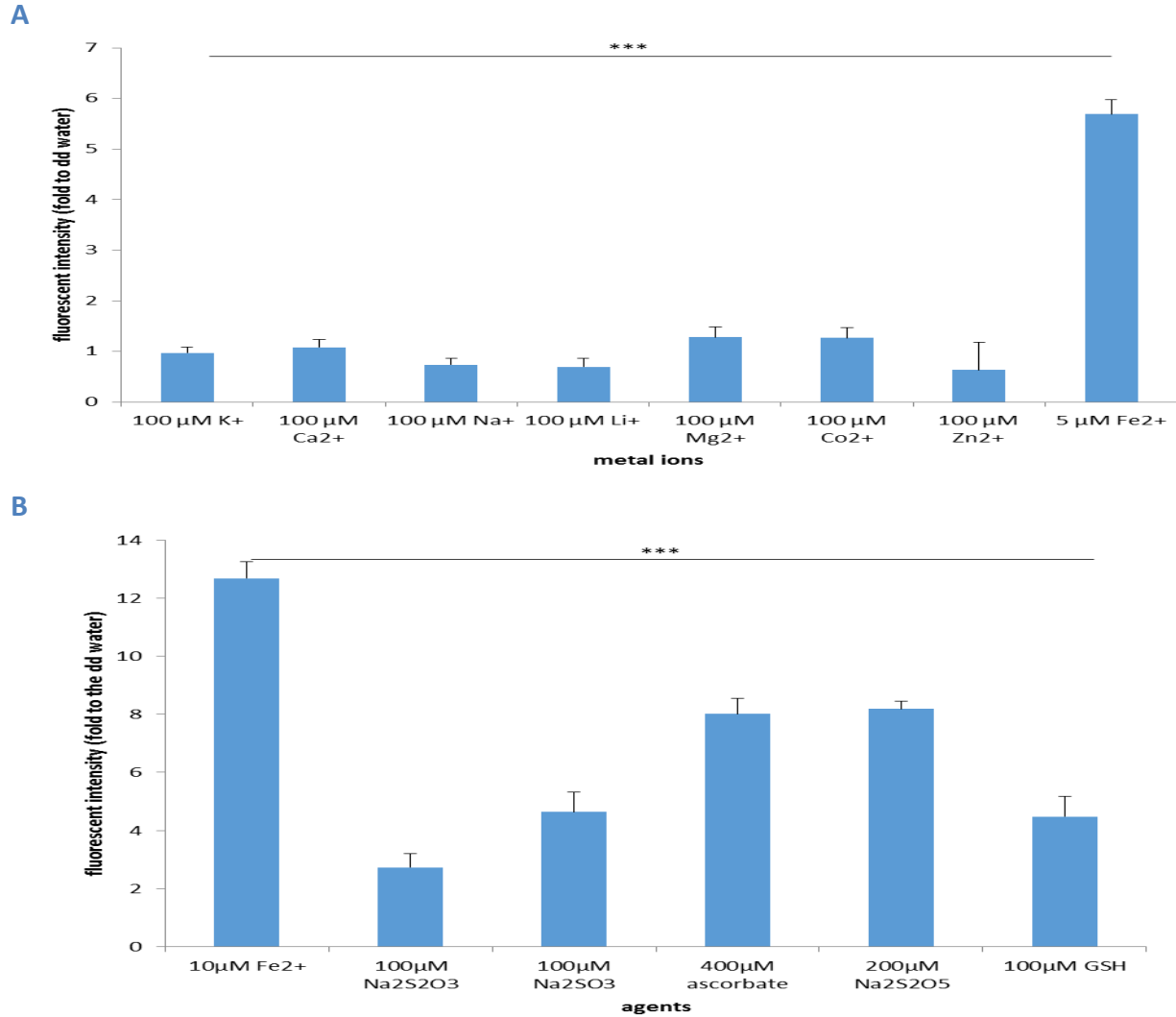
So it is necessary to test the fluorescence generation on other reducing agents. Sodium thiosulfate, sodium sulfite, ascorbic acid, glutathione (GSH) and sodium metabisulfite was used as reducing agents. Among these reducing agents, sodium sulfite and sodium metabisulfite are mostly applied for industrial use<sup>150</sup>, while sodium thiosulfate has wide medical uses<sup>151-154</sup> but seldom exists in human body. Ascorbic acid, also known as Vitamin C, has its own biological role as a highly effective antioxidant and electron donor in mammals<sup>155</sup>. GSH is also a major reductant in blood and cells with a concentration of 5-10mM. The tests of these reducing agents would help extend application of the hydroxylamine probe to industrial and biological regions. To avoid probe saturation, a relatively high concentration (50 $\mu$ M) of the probe was used. In Figure 3B, significant differences still existed between ferrous ion and other reducing agents, even though the concentration of the reducing agents is 9-39 times higher than ferrous ion. Relatively bigger differences also existed between different types of reducing agents, indicating that the fluorescent generation might depend on redox reaction and require reductive properties. But the rather significant difference between iron and these reducing agents still suggests the considerable specificity to iron (II) of this hydroxylamine probe.

Though it has been found that the hydroxylamine probe has a higher specificity to iron than glutathione, there is still one point remaining to be clarified. The cellular concentration of glutathione is much higher than the concentration we used in our experiment. And it is still unclear whether the probe is able to react with ferrous first after entering the cell.

In conclusion, neither metal ions nor reducing agents could make the probe generate fluorescence as potently as ferrous iron. In solution, this hydroxylamine probe has great sensitivity to react with ferrous iron. Even though the metal ions and reducing agents might



slightly be able to react with the probe, the rather weak intensity of fluorescence is unlikely to send an interference with the fluorescence produced by ferrous iron.



**Figure 3. The fluorescence generated by metal ions and reducing agents**

A. This bar graph displays the fluorescence generated by the probe reacting with different metal ions.

\*\*\*  $p < 0.005$ ,  $n = 8$ , comparisons with 5 μM ferrous ion

B. This bar graph displays the fluorescence generated by the probe reacting with different reducing agents at different concentrations. The control group is untreated cells.

\*\*\*  $p < 0.005$ ,  $n = 8$ , comparisons with 10 μM ferrous ion

### 3.1.3. Cellular iron measurement

Iron levels were also measured by this hydroxylamine probe at the cellular level. After the cells were treated with iron for 24 hours, the intensity of fluorescence was measured by plate reader. Figure 4A is the graph made showing the fluorescence generated at different time points. In this figure, the shapes of the curves were similar to the one obtained in the solution-level iron measured by this probe. During the first 20 minutes the intensity of fluorescence kept increasing, in zero order kinetics. At the period between 20 and 30 min the curve turned into first order kinetics. Then at about the point of 35min, the intensity of fluorescence started to decline at a slope lower than the increase period on curve. The decline implies that the probe might be degraded slowly in cells. The three lines in the graph from up to down represent  $5\mu\text{M}$  iron,  $0\mu\text{M}$  iron and  $100\mu\text{M}$  DFO. The fluorescence of the cells treated with  $100\mu\text{M}$  DFO was weak at first and disappeared within one hour ( $100\mu\text{M}$  DFO line in Figure 4A). Comparing these three lines, an increase of the intensity of fluorescence with the increase of the amount of iron treatment was showed. Through this figure, another conclusion can be made that in order to measure the cellular iron level through this probe the optimum time period for fluorescence detection locates at around 20-40 minutes after addition of the probe. And after 2 hours, the intensity of fluorescence will be too weak to detect.

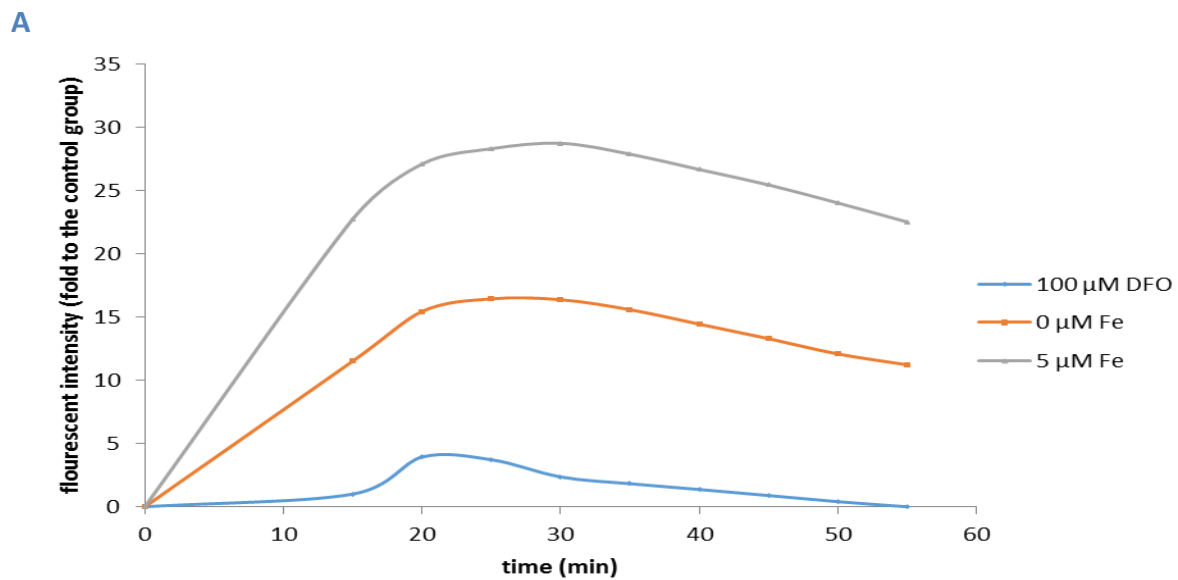
For further study, microscopy was used to detect fluorescence generation from the probe. This approach provides more information on the localization of the signals within the cells. Cultured cells were treated with different concentrations of iron for 24 hours before adding  $5\mu\text{M}$  probe. After 15 minutes the cells were washed and observed under a fluorescence microscope. Figure 4B shows the results of this microscope imagery study. According to these pictures, fluorescence increased with the augment of the concentration of iron treatment, while the blank group (no

probe used) did not generate any fluorescence. Quantification of the average intensity of each cell was made and all presented in the bar graph (Figure 4C). In the quantification, significant differences were seen between 10 and 20 $\mu$ M iron treatment, which was greater than the difference between 20 and 40 $\mu$ M. These data suggest that the probe reach the saturation point at the concentration of iron between 20 and 40 $\mu$ M. This saturation point was close to the results obtained in the solution-level of Figure 2. In addition, when the iron was chelated by DFO, the fluorescent intensity decreased significantly. These results indicate that this hydroxylamine probe can be used as a reasonable iron marker in cellular level studies.

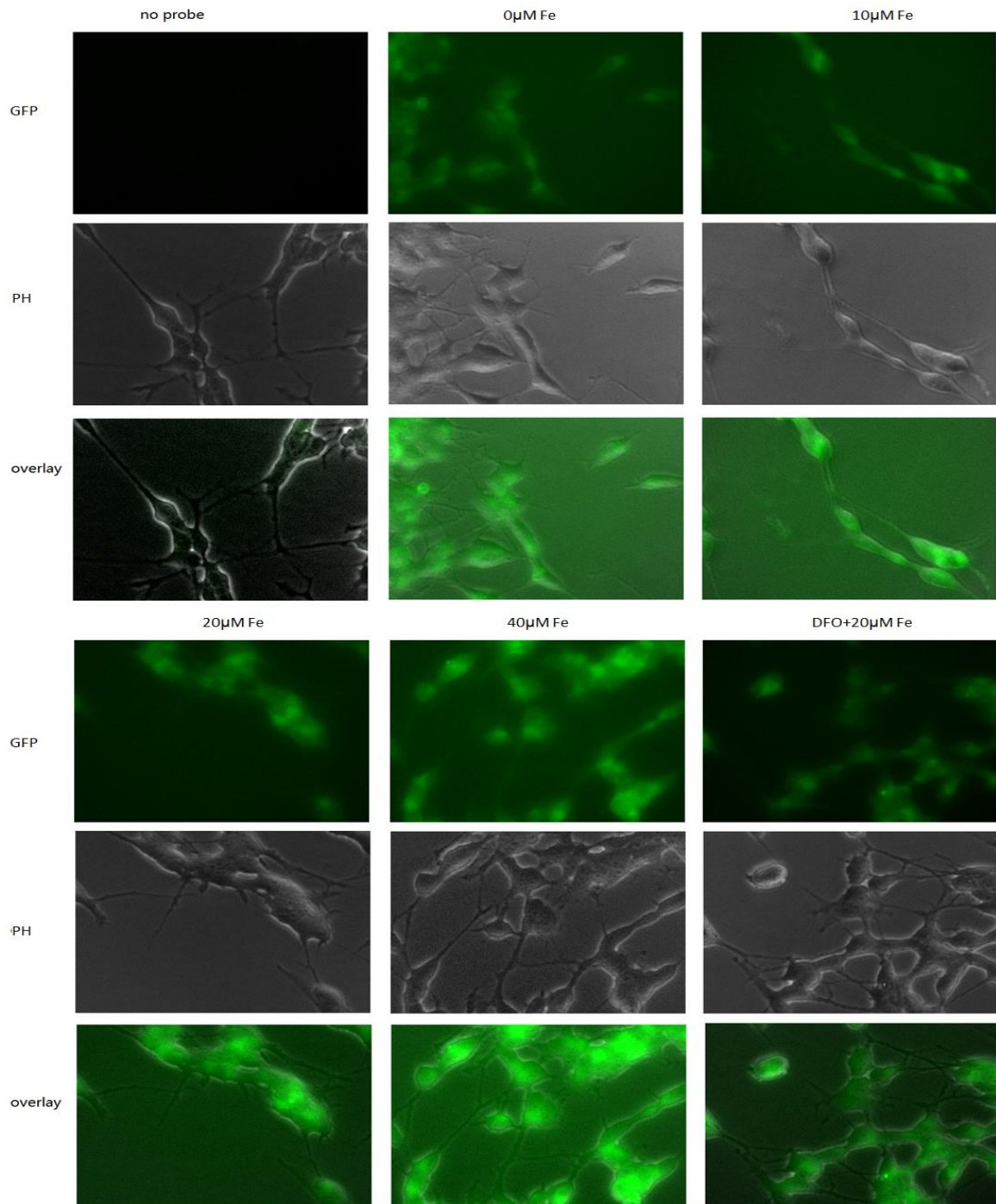
Next studies were processed to analyze the performance of the hydroxylamine probe in immunocytochemistry studies, a common and widely used study in molecular biology. During immunocytochemistry studies, proteins in cells are usually fixed and the cell membranes are permeated, which means the cells are dead and metabolism is stopped. After a 24-hour iron pretreatment of the SH-SY5Y cells, the cells were prepared following the same procedure as immunocytochemistry studies, fixed and permeated. Then 5 $\mu$ M probe was added to the cells, before the addition of DAPI as nuclear marker. After these treatments, the cells were observed under the microscope, as images of these cells captured. Figure 4D shows the results of this study. From this figure, green fluorescence generated from the probe and iron could be detected. There was no induction that the green fluorescence was affected by the nuclear stain. With the presence of iron chelator DFO, the intensity of the fluorescence was significantly decreased. Also the intensity of fluorescence was enhanced by iron treatment, which had a more directive presentation in the quantification graph, which quantified the intensity of the fluorescence and averaged to each cell (Figure 4E). Figure 4E shows a steady increase in intensity of the fluorescence with increasing iron concentration. DFO treatment abolished the fluorescence since

it is as the iron chelator. Slight recovering of the fluorescence appeared in Figure 4E in the groups with the combined treatment of DFO and iron. These results suggest that the probe is able to be applied in immunocytochemistry studies as an indicator of iron level.

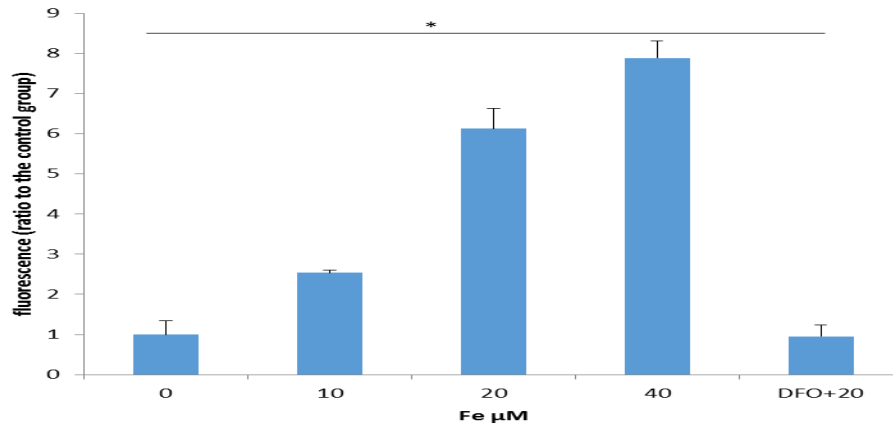
In sum, this group of studies has provided evidence supporting that this hydroxylamine probe is able to measure iron in cells. The fluorescence generated by this probe is time and concentration dependent. In addition, the fluorescence is able to be detected by microscopically to get more information about iron inside cells. Furthermore, this hydroxylamine probe could be applied to indicate iron levels both in live and dead cells.



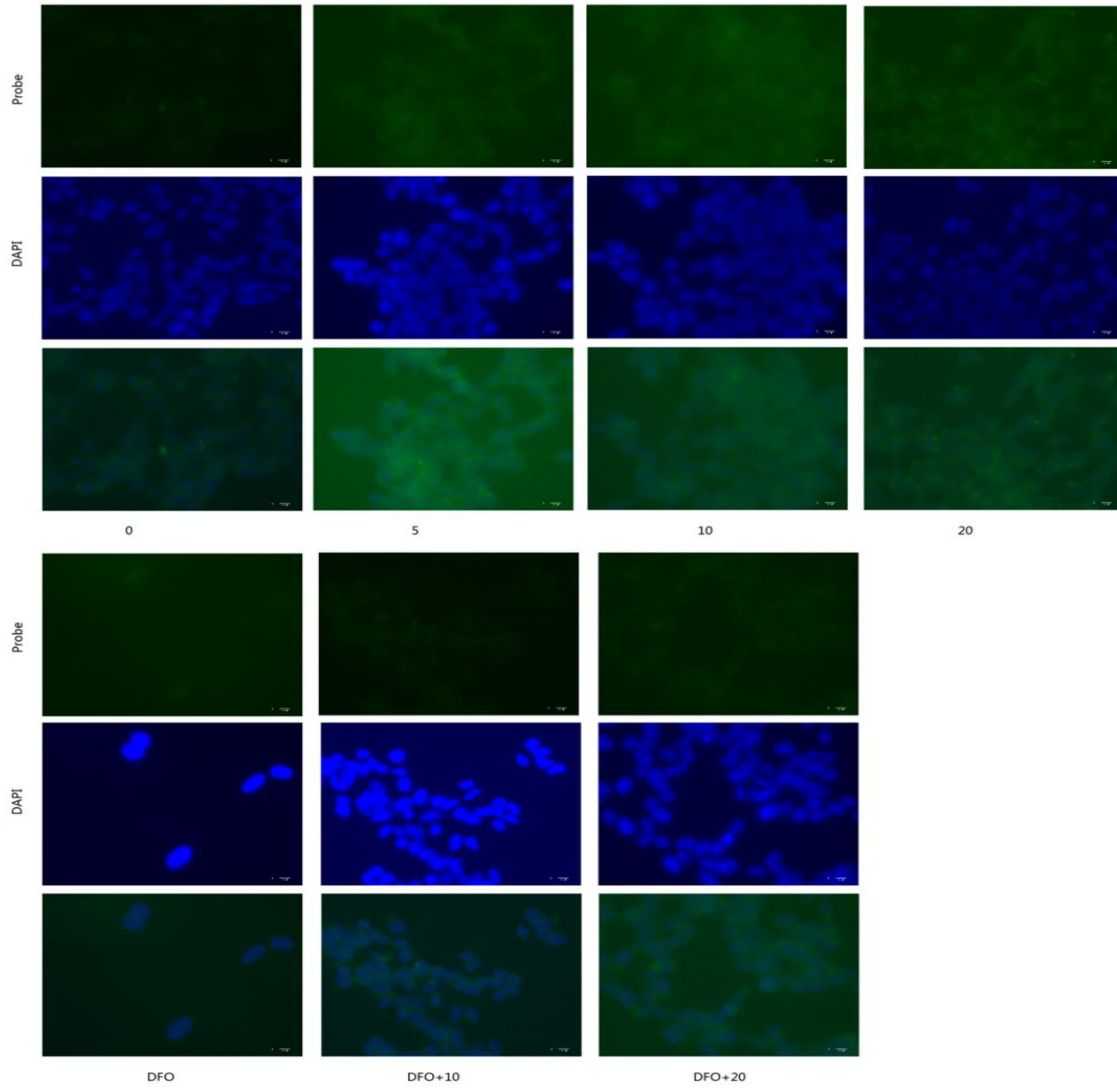
B



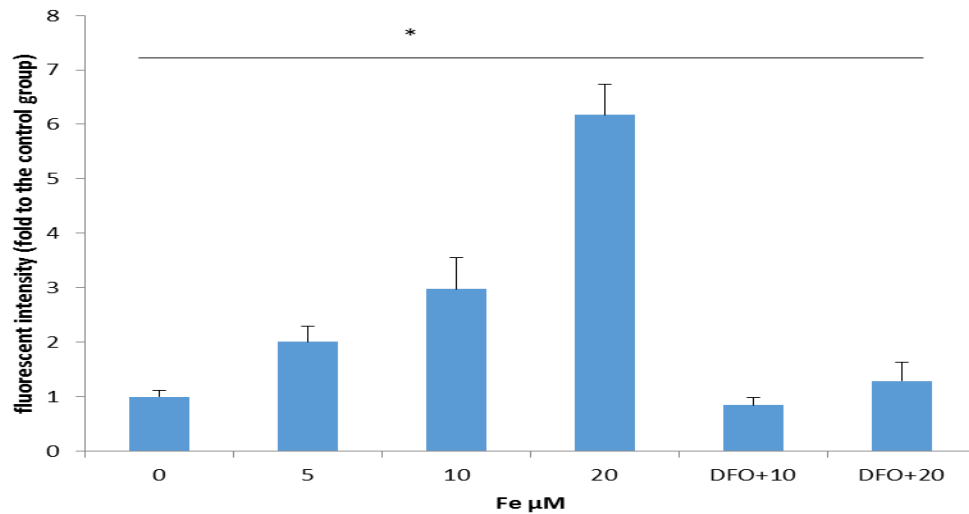
C



D



E



**Figure 4. The application of the probe in cellular iron measurement**

- A. The graph shows the fluorescence generated by the probe inside SH-SY5Y cells at different time point after addition and different iron concentrations (0μM, 5μM and 100μM DFO). n=4. The control group is the cells without treatment and probe.
- B. The images taken by microscope showing the fluorescence of the probe inside SH-SY5Y cells under different iron concentrations.
- C. The quantification of the intensities of the fluorescence in Figure B. n=4. The control group is untreated cells.
- D. The images taken by microscope showing the nucleus the fluorescence of the probe in fixed SH-SY5Y cells under different iron concentrations.
- E. The quantification of the intensities of the fluorescence in Figure D. n=4. The control group is untreated cells.

\* p<0.05, compared with non-treated (0) group

#### 3.1.4. Effects of metal ions in cellular use of the probe

In order to study the effects of metal ions on the probe measuring cellular iron levels, SH-SY5Y cells were treated for 24 hours 250 $\mu$ M calcium, lithium, magnesium, or 100 $\mu$ M zinc or 100 $\mu$ M cobalt. 5 $\mu$ M probe was added to the cells, and fluorescence was detected by a plate reader. A time and intensity of fluorescence curve was made to illustrate the fluorescence changes at different time points (Figure 5A). Based on the results of iron measurement by the probe inside cells, the time period between 20 and 30min was chosen to study the fluorescence generation under metal ions treatment. Figure 5A shows that the fluorescence did not change with time, while the intensity of the fluorescence was pretty low despite the high concentration of the metal ions. Figure 5B shows a comparison between iron and various metal ions in the intensity of fluorescence generated by the probe inside cells. This result shows that fairly small differences exist in intensity of fluorescence among the groups under different metal ion treatments, and generally all the fluorescence generated in these groups are weak even at high concentration. However, in the iron treatment group, even though the concentration of treatment was rather low, the intensity of fluorescence generated was relatively high compared to the metal ions. These results are consistent with the results from the in-solution study of the probe.

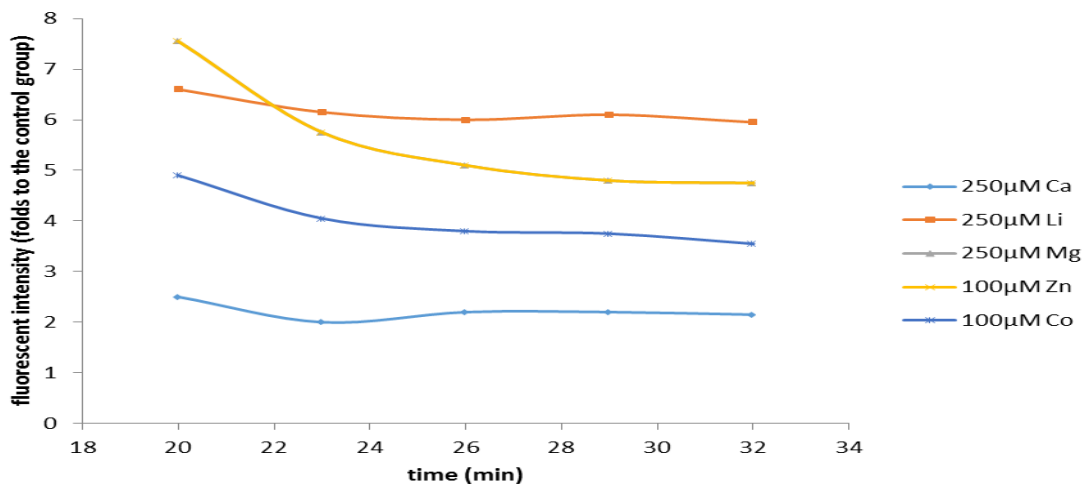
The effects of metal ions on the fluorescence were also analyzed by microscopy. Cultured SH-SY5Y cells were treated for 24 hours with high concentration of metal ions, 250 $\mu$ M potassium, calcium, sodium, lithium, magnesium, or 100 $\mu$ M zinc, 100 $\mu$ M cobalt. The probe was added to the cells 15 minutes before detection. Figure 5C shows the results of the microscopy. Though green fluorescence could be observed from the metal ions, the fluorescence is weak and more obscure compared with the iron treated group. Figure 5D shows the quantification of these results. Through this graph, a significant difference in the intensity of fluorescence between iron



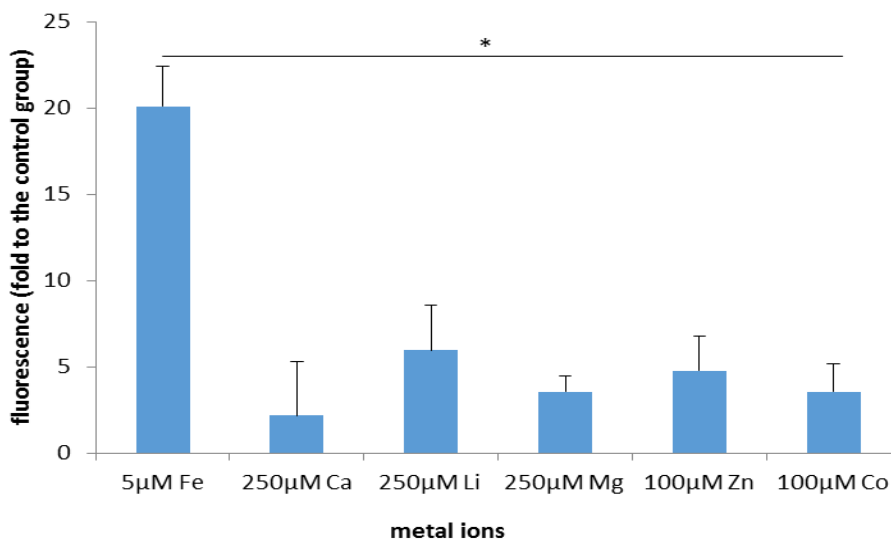
treated group and other metal ions treated group is observed. However, within the metal ions treated groups, the intensities of fluorescence did not show much difference, and was generally very low.

In summary, these studies provided evidence to support that the probe is not affected by metal ions except iron. Thus, the hydroxylamine probe is able to specifically indicate iron level even in a cellular environment with presence of other metal ions.

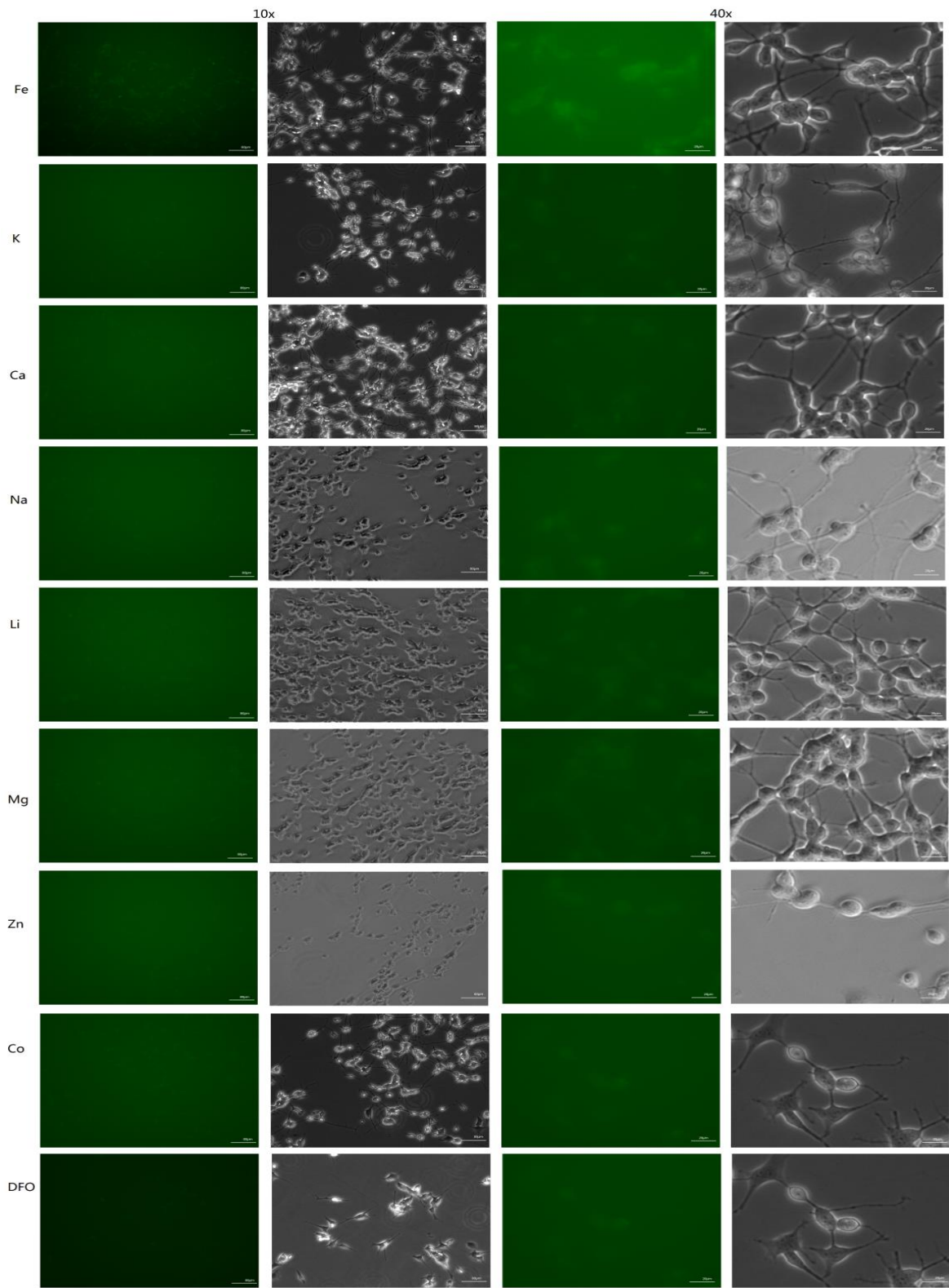
**A**

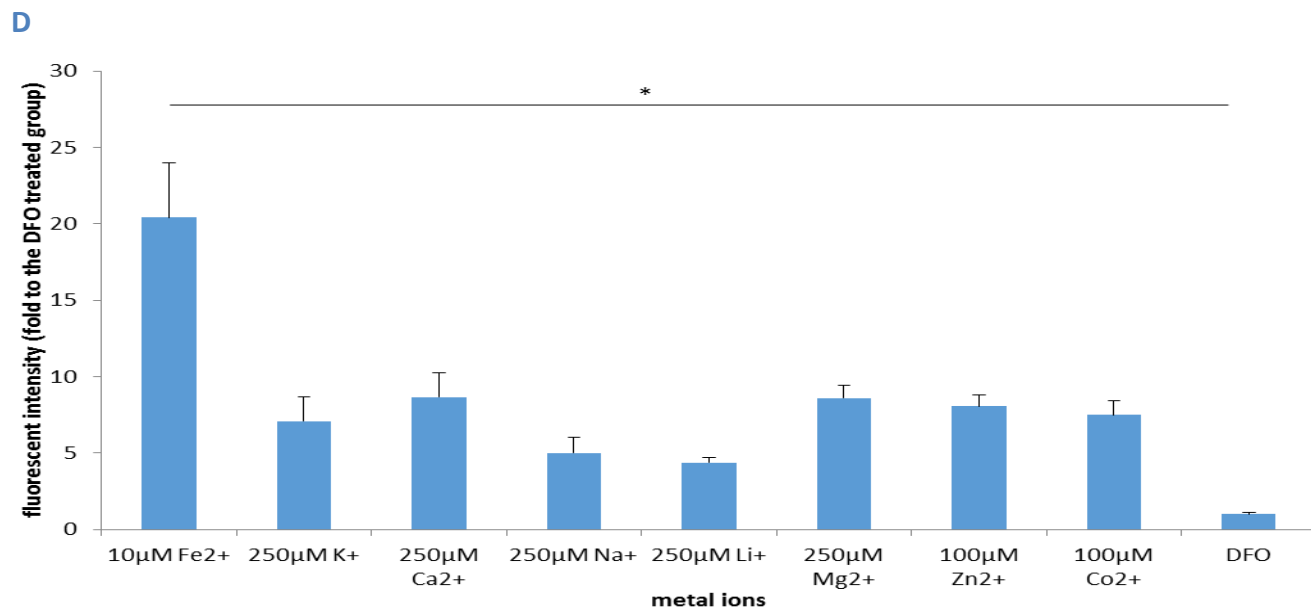


**B**



C





**Figure 5. The fluorescence generated by metal ions in cellular application of the probe**

- The time-intensity curve shows the changes of fluorescence by calcium, lithium, magnesium, zinc and cobalt in cells during the period 20-30 minutes after the addition of probe. The control group is the cells without treatment and probe.
- The bar graph shows the different intensity of fluorescence by different metal ions in cells. The control group is the cells without treatment and probe.
- The images taken by microscope under GFP showing the fluorescence of the probe inside SH-SY5Y cells under different metal ions.
- The quantification of the intensities of the fluorescence in Figure C.

\*  $p < 0.05, n = 4$ , compared with ferrous ion

### 3.1.5. The effects from cuprous and cupric ions to this hydroxylamine probe

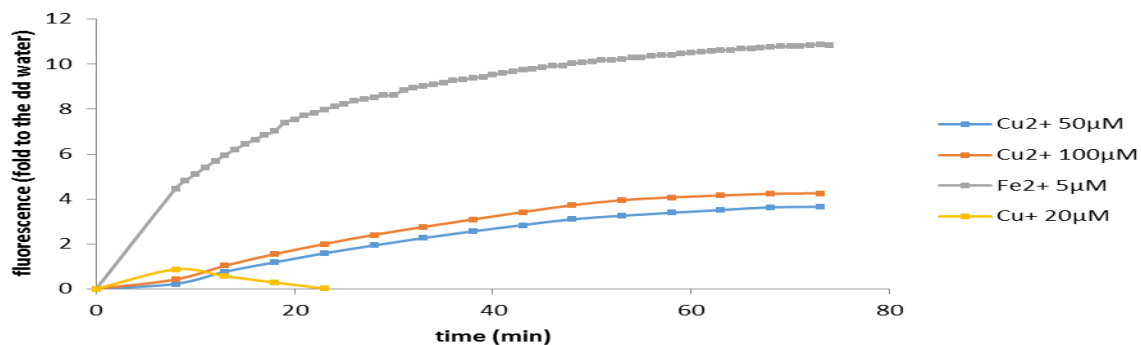
Copper is a metal element very close to iron in periodic table, which means that they have some similar physical and chemical properties, such as the similar size of copper and ions. In addition, copper also has two ionic forms copper (I) and copper (II), which means cuprous (copper (I)) ion

has a reductive property similar to ferrous iron. Moreover, copper is an important component involved in many biological reactions, which means copper widely exists in biological materials and might have an effect on the fluorescence of the probe. More importantly, copper was reported to affect an acetyl hydroxylamine probe more than other factors<sup>116</sup>. Hence, we examined the effects of copper on the hydroxylamine probe. First, the experiments were done in solution without cells with fluorescence measured by a plate reader. Figure 6A shows the curve of the fluorescence intensities at different time points during the first hour after addition of the probe. An increase in fluorescent intensity caused by cupric ion was observed during the first 50 minutes after the addition of the probe. After about one hour, the fluorescence plateaued, meaning that the increase in fluorescence is almost stopped. On the other hand, the fluorescence generated by cuprous ion was relatively potent at the first 10 minutes and started to decline and disappear by 30min, which might be because cuprous ions tend to precipitate due to their low solubility. However, even at 10-20 fold higher concentration than iron, the fluorescence generated by copper was still significantly weaker (Figure 6B and Figure 6C). Next, the study of this probe in cells also did not show a significant role of copper in fluorescence generation. In the time-intensity curve (Figure 6D), the fluorescence by both copper (I) and copper (II) had a latency in generation and declined very fast. The reason for this might be that the sensitivity of the probe to copper is low and the possible degradation mechanism of the probe in cells only provided a short period time when the concentration of probe is enough to react with copper. In addition, when the fluorescence reached into the highest point (about 35min), the intensity was still significantly lower than iron (Figure 6E). Figure 6F gives shows fluorescence images of the copper and iron reacting with the probe inside cells respectively. A bar graph indicates the quantification of the fluorescent intensity (Figure 6G). The fluorescence generated by copper

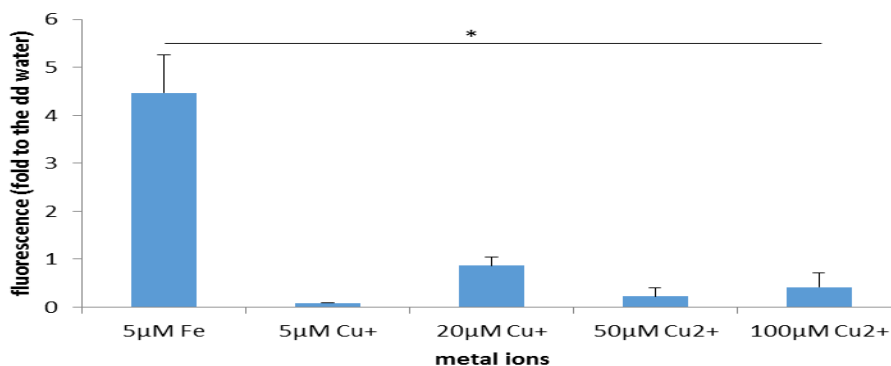
was also lower than iron, further supporting that copper does not have a potent effect to react with the probe.

Taken together, both cuprous and cupric ion poorly reacts with this probe. The fluorescence generated by the reaction is weak, and not likely to disturb iron detection. With the relatively lower level of copper (I) and copper (II) in human body<sup>156</sup>, copper showed not be a factor that interferes with the iron measurement of this hydroxylamine probe.

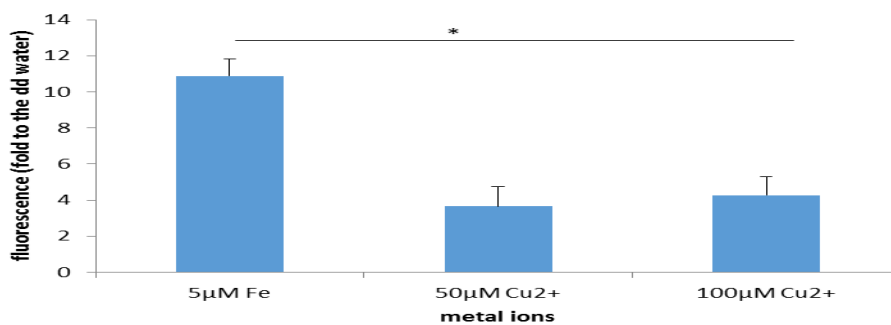
**A**

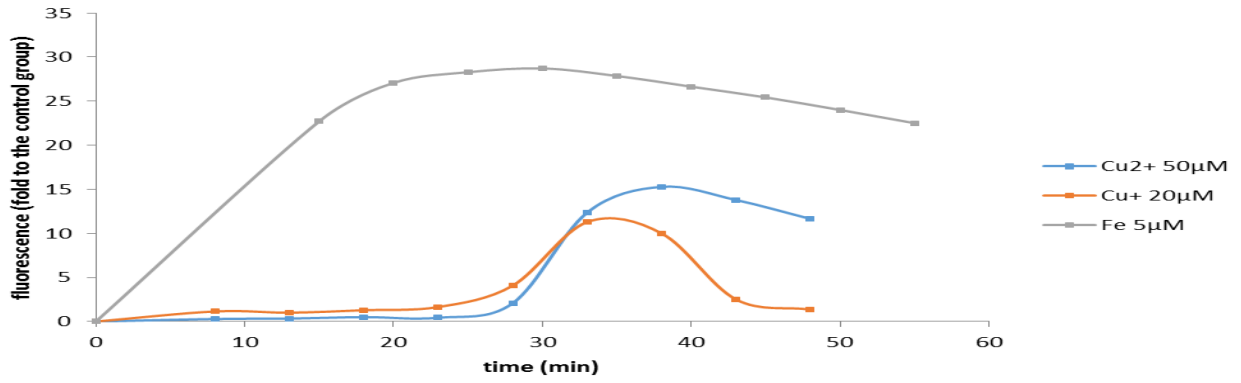
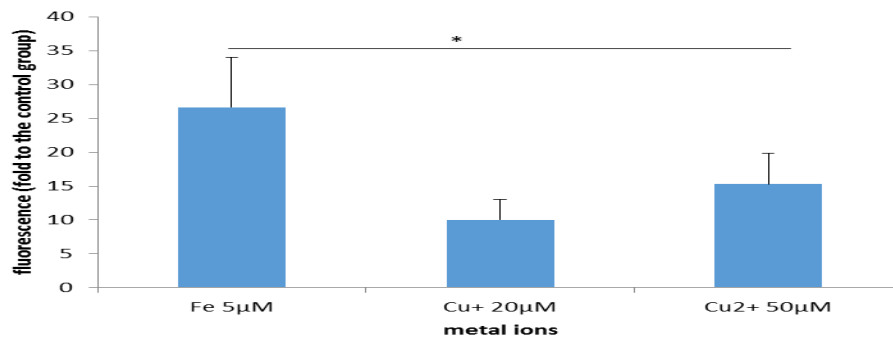
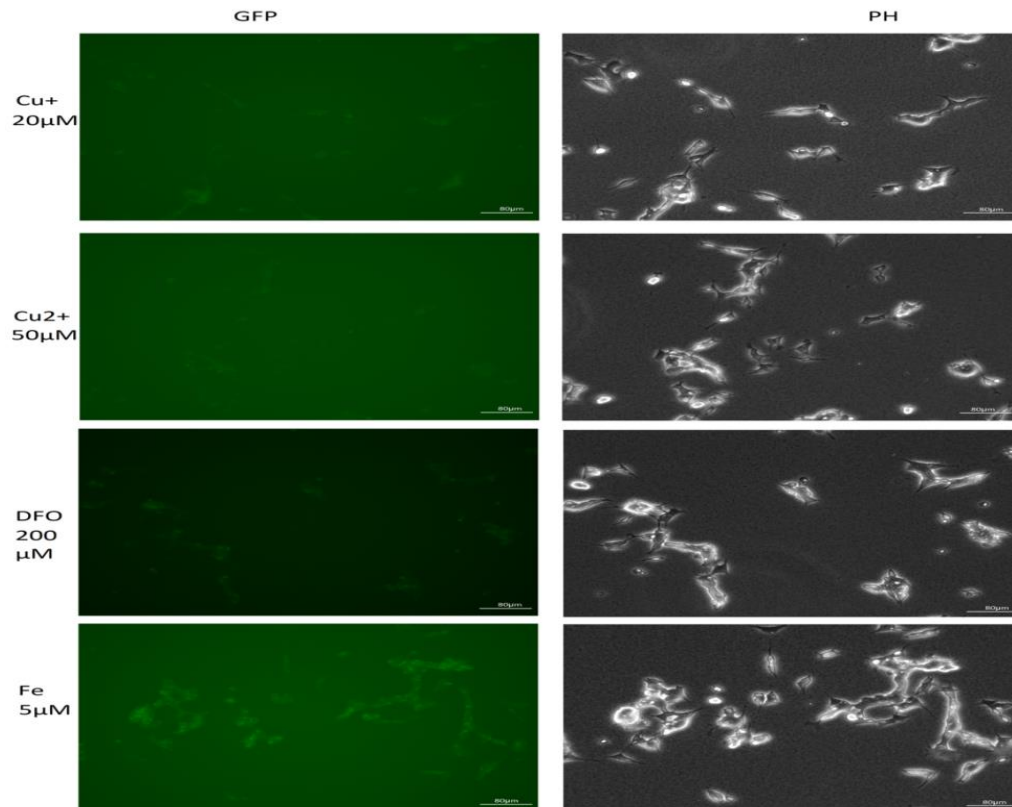


**B**

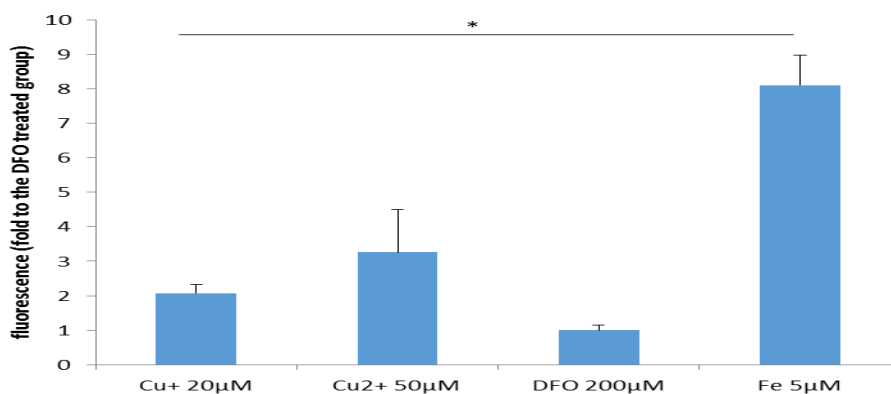


**C**



**D****E****F**

G



**Figure 6. Effects of cuprous and cupric ions on the hydroxylamine probe**

- A. The intensity-time curve shows the fluorescence by the probe in iron or copper solutions. n=8
- B. The bar graph shows the fluorescent intensity of different solutions 8 minutes after the addition of probe. n=8
- C. The bar graph shows the fluorescent intensity of different solutions 72 minutes after the addition of probe. n=8
- D. The intensity-time curve shows the fluorescence by the probe reacting with iron or copper inside cells. n=4. The control group is the cells without treatment and probe.
- E. The bar graph shows the fluorescent intensity of different treatments to cells 40 minutes after the addition of probe. n=4. The control group is the cells without treatment and probe.
- F. The images taken by microscope show the fluorescence by the probe reacting with copper or iron inside cells.
- G. The bar graph shows the quantification of the fluorescent intensity of Figure F.

\*  $p < 0.05$ , compared with treatment with iron

### 3.1.6. Summary of this hydroxylamine iron probe

In conclusion, this hydroxylamine is a newly developed, reasonable ferrous iron probe. It has good specificity to react with ferrous iron and is hardly affected by other metals ions or reducing agents. The intensity of fluorescence generated by reaction between this compound and iron is positively correlated with the concentration of ferrous iron. The fluorescence generated by this probe can be detected by either plate reader or microscopy. This probe can be widely used both in-solution and in cells.

In sum, this hydroxylamine compound is a powerful and reasonable ferrous probe and can be used as iron indicator in this research.

## 3.2. Iron measurement via calcein blue AM

Since the hydroxylamine probe can only detect ferrous ion, another iron probe is needed in this research to measure the total iron level.

Calcein-AM is a well-developed dye to measure iron in live cells. The mechanism of iron measurement by calcein blue AM (Figure 7A), is similar to calcein AM, which has been mentioned above in the introduction. Calcein blue AM can cross the double layer lipid membrane and enter the cells, with the acetoxy methyl ester group removed, generating fluorescence, while the fluorescence will be quenched with the presence of iron (Figure 7B). One difference between calcein blue AM and calcein AM is the wavelength of detection (calcein blue AM:  $\lambda_{ex} = 360 \text{ nm}$ ,  $\lambda_{em} = 445 \text{ nm}$ ). Some studies were done to confirm that calcein blue AM could be applied to measure ferric iron in our research.

Figure 7A shows the structures of calcein blue and calcein. The structures indicate that calcein blue is a monomer of calcein, so they share properties in measuring iron. At first, an in-solution

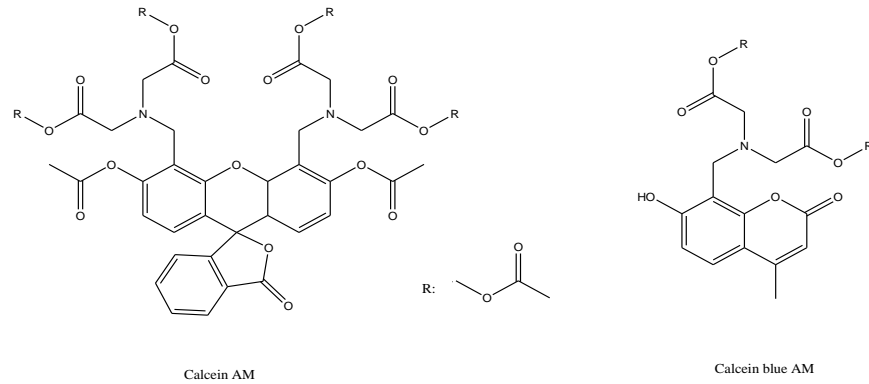


calcein blue AM assay was performed, and the results show no difference among the groups with different treatments and different concentration of iron (Figure 7C). The reason is that the acetoxymethyl ester group cannot be removed from calcein blue, resulting in no fluorescence generated. Calcein blue assay was also performed in cells with the fluorescence detected by plate reader. Cells were pretreated with different concentrations of iron, then 5 $\mu$ M calcein blue AM was added, after which the fluorescence was detected. This result shows a decrease in fluorescence intensity on the increase of iron concentration (Figure 7D). This result does not show an obvious difference between 0 and 5 $\mu$ M iron treatment. The reason for this might be because the sensitivity of the plate reader or calcein blue, which resulted in unclear results under low iron concentration. The fluorescent quenching was rather clear under higher iron concentrations. And the difference of fluorescent quenching between 20 and 40 $\mu$ M iron was approximately twice as big as the quenching difference between 10 and 20 $\mu$ M. In addition, microscope was used to observe the fluorescent quenching by iron. Figure 7E shows that as the concentration of iron treatment to the cells went up, the intensity of fluorescence went down. However, the DFO treatment significantly decreased the fluorescent quenching. A quantification of the fluorescent intensity per cell from the pictures taken by microscope was made (Figure 7F). This bar graph shows the ratio of the fluorescence to the 0 $\mu$ M iron treatment group, from which a more obvious decrease in fluorescence after the increase of iron level could be observed directly. Moreover, this bar graph also indicates the relation between iron level and fluorescence quenching is close to a linear correlation.

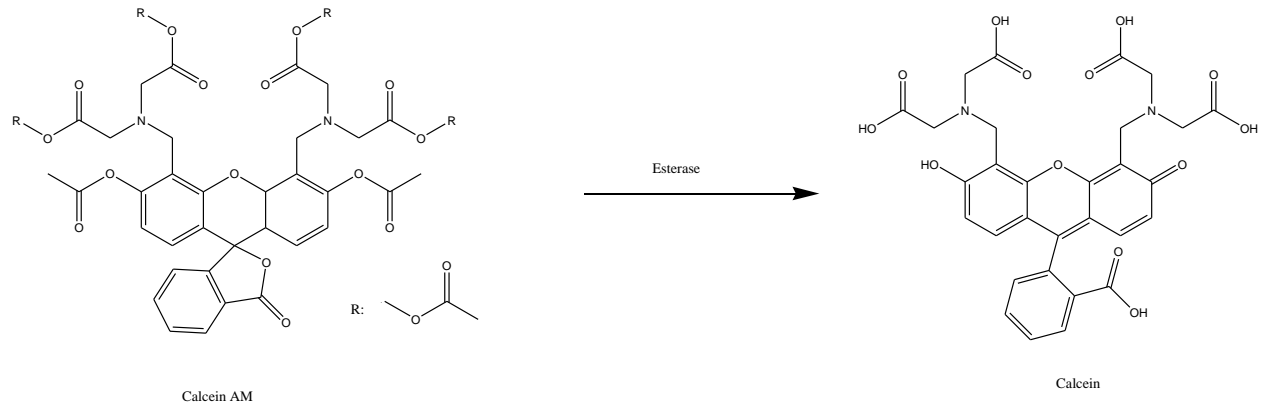
All these studies above about calcein blue AM suggested that calcein blue AM is a reasonable iron probe and suitable for our research. The fluorescence of calcein blue can both be detected by plate reader and microscope. However, the requirement of live cells by calcein blue AM will

limit its application in iron measurement. And different types of cells and the cell metabolism might also have an effect on the calcein blue fluorescence generation. But as every coin has two sides, this property limiting iron measurement also contributes calcein blue AM to be a marker of cell viability.

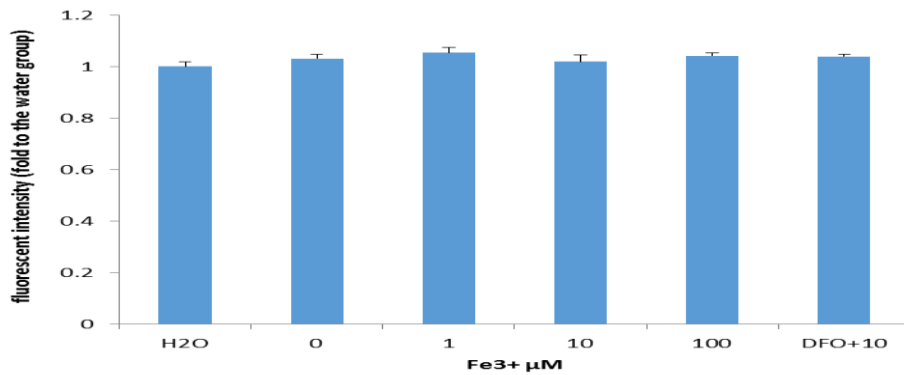
**A**



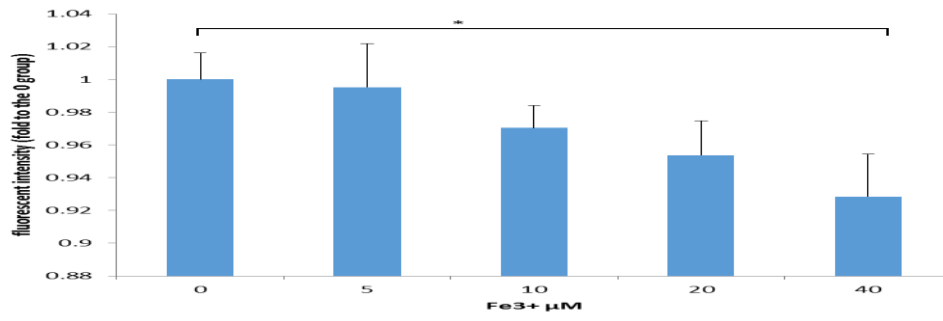
**B**



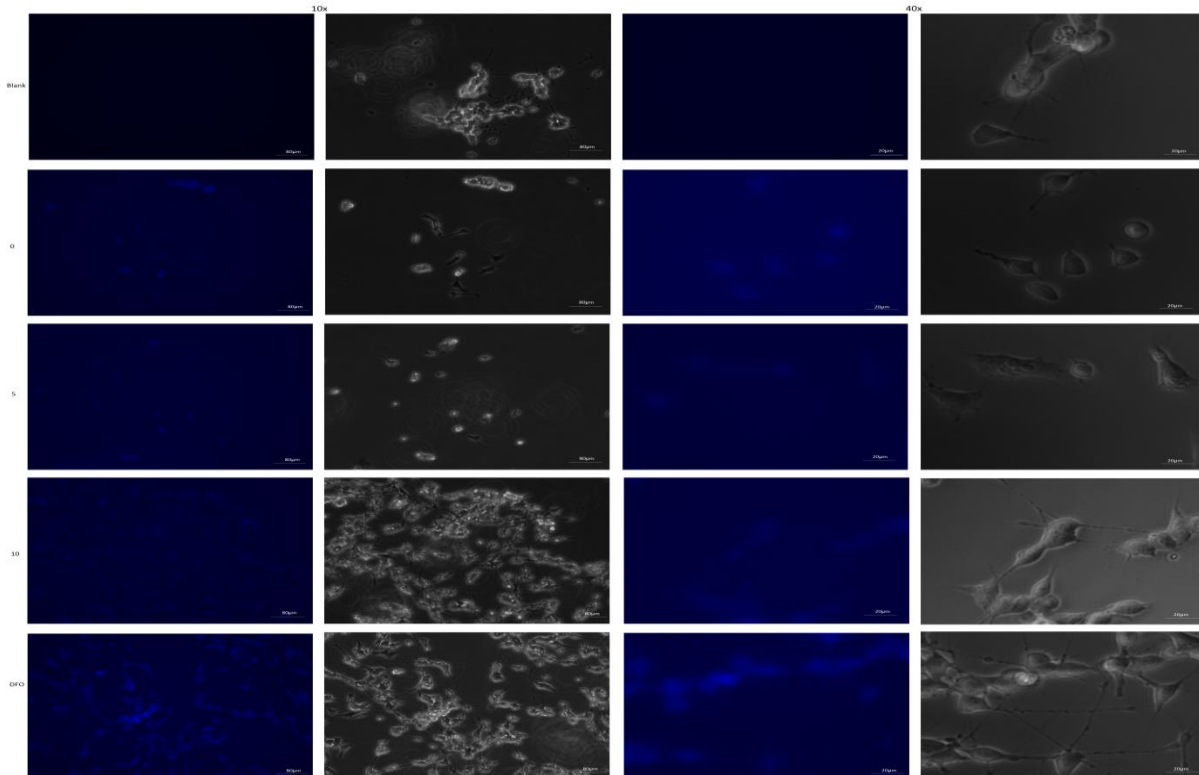
**C**



D



E



F

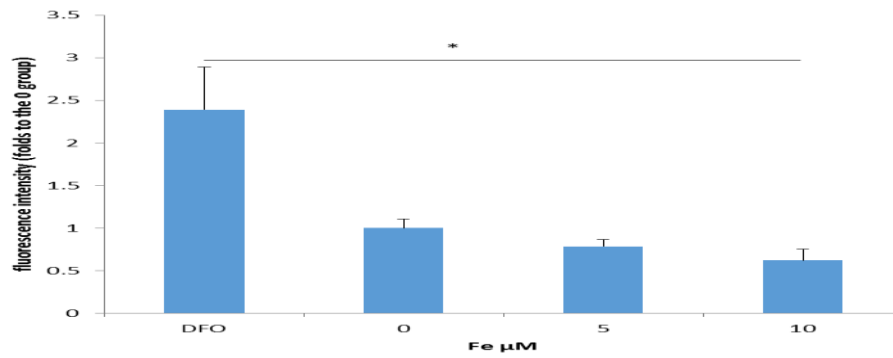


Figure 7. Iron measurement by calcein blue AM

- A. The structures of calcein AM and calcein blue AM are showed.
- B. Calcein AM is turned into calcein which can generate fluorescence by esterase.
- C. The bar graph shows the results of the application of calcein blue AM in iron solution.
- D. The bar graph shows the results of the application of calcein blue AM in cells after different concentrations of iron treatment.

\*  $p < 0.05$ ,  $n = 8$ , comparison between 0 and 40 $\mu$ M iron

- E. The images taken by microscope under DAPI showing the fluorescence of the calcein blue inside SH-SY5Y cells under different iron concentrations.

- F. The quantification of the intensities of the fluorescence in Figure E.

\*  $p < 0.05$ ,  $n = 4$ , multiple comparisons among DFO, 0, 5 and 10 $\mu$ M iron

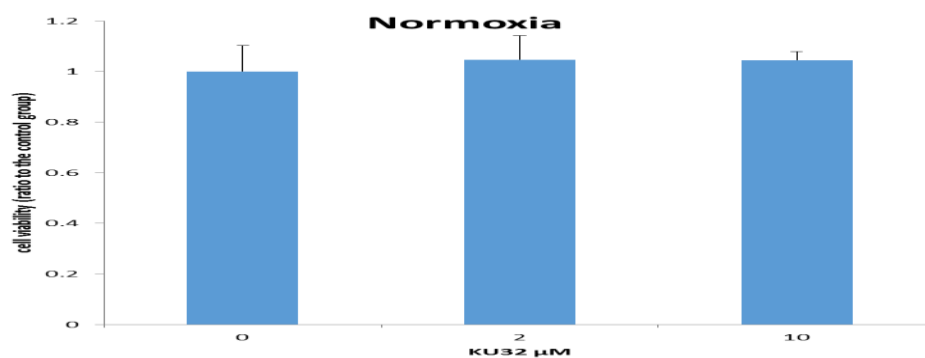
### 3.3. The effect of KU32 on cell viability

KU32 is an inhibitor of HSP90, which is a chaperone protein and involved numerous regulatory processes in the cell. To determine whether KU32 has an effect on cell viability during ischemia, two groups of SH-SY5Y cells were treated with KU32 (0 $\mu$ M, 2 $\mu$ M and 10 $\mu$ M) and then put into normoxic or OGD conditions for 2 hours. After the normoxia and OGD incubation, the MTT assay was used to determine cell viability under different conditions and concentrations of KU32. Figure 8B shows the results of the effect of KU32 under OGD condition. According to Figure 8B, the presence of KU32 did not significantly alter cell viability. In addition, under normoxia condition (Figure 8A), the cell viability hardly increased by using the MTT assay. These results indicated that KU32 might not have a potent effect on cell viability under ischemia (OGD) condition. The western blot data showed in Figure 8C also showed there was no significant change on the expression of HSP70, which has been showed to mediate some of the neuroprotective effects of KU32.

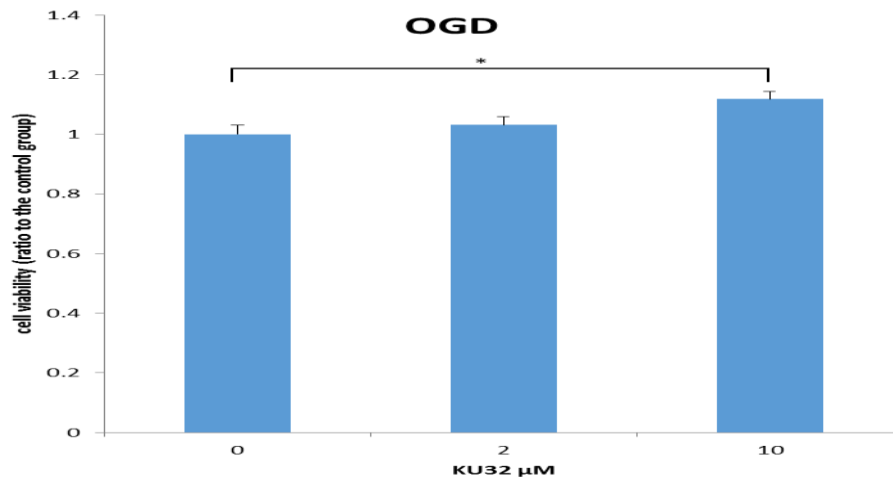
Based on the theory of reperfusion injury<sup>20-23</sup>, further experiments were designed to study the effects of KU32 treatment under ischemia and reperfusion condition. Two groups of SH-SY5Y cells were pretreated with KU32 for 24 hours and then transferred to normoxia or ischemia (OGD) condition, followed by a 24-hour reperfusion. After the ischemia/reperfusion, the MTT assay was performed. Figure 8D shows a significant increase in cell viability in the presence of 4 $\mu$ M and 10 $\mu$ M KU32. Consistent with the increase in cell viability, western blot analyzes show a significant increase in HSP70 expression by KU32.

These results suggest that HSP90 inhibitor KU32 increases HSP70 expression and has a neuro-protective effect to neurons under ischemia-reperfusion. This effect mainly happens during the reperfusion period and requires a pretreatment.

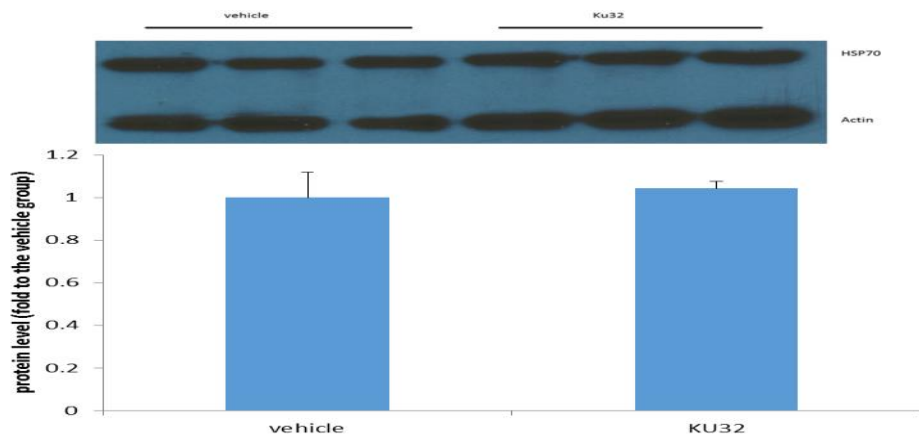
**A**



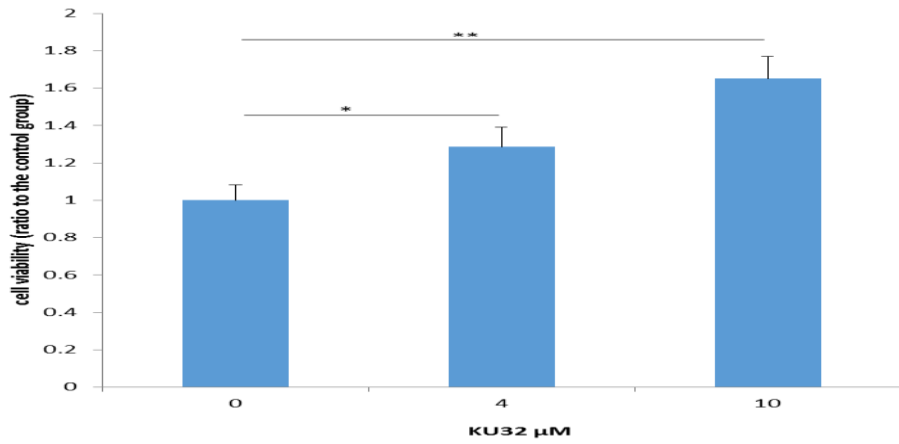
**B**



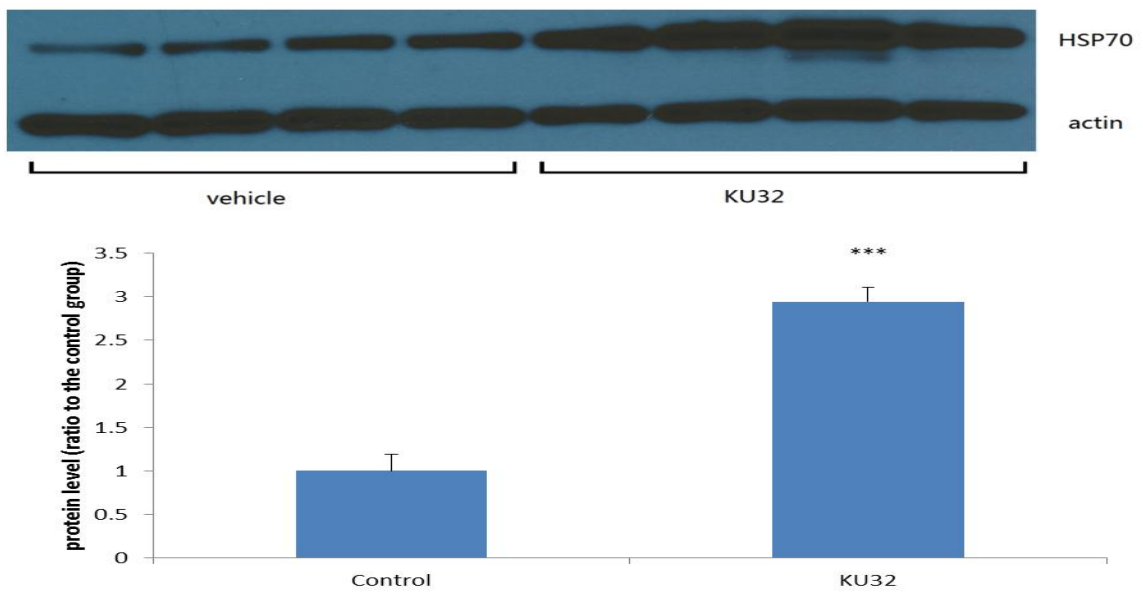
C



D



E



**Figure 8.** Effects of KU32 on cell viability and heat shock proteins

- A. This bar graph shows the cell viability of SH-SY5Y with the treatment of KU32 under normoxia condition.
- B. This bar graph shows the cell viability of SH-SY5Y with the treatment of KU32 under ischemia condition. n=8
- C. The western blot and quantification show the expression of HSP70 with the treatment of KU32 under ischemia condition.
- D. This bar graph shows the cell viability of SH-SY5Y with pretreatment and treatment of KU32 under ischemia reperfusion condition. n=7
- E. This western blot and quantification show the expression of HSP70 with pretreatment and treatment of KU32 under ischemia reperfusion condition. n=4

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.005$ ;  $n \geq 3$ , compared with the non-treated group

### **3.4. The effect of KU32 on iron levels under ischemia reperfusion**

Since a neuroprotective effect of KU32 during ischemia-reperfusion was observed and iron is widely reported as a factor inducing oxidative stress and cell apoptosis<sup>37,38,41</sup>, we hypothesized that inhibition of HSP90 by KU32 might change the iron level in neurons under ischemia-reperfusion condition.

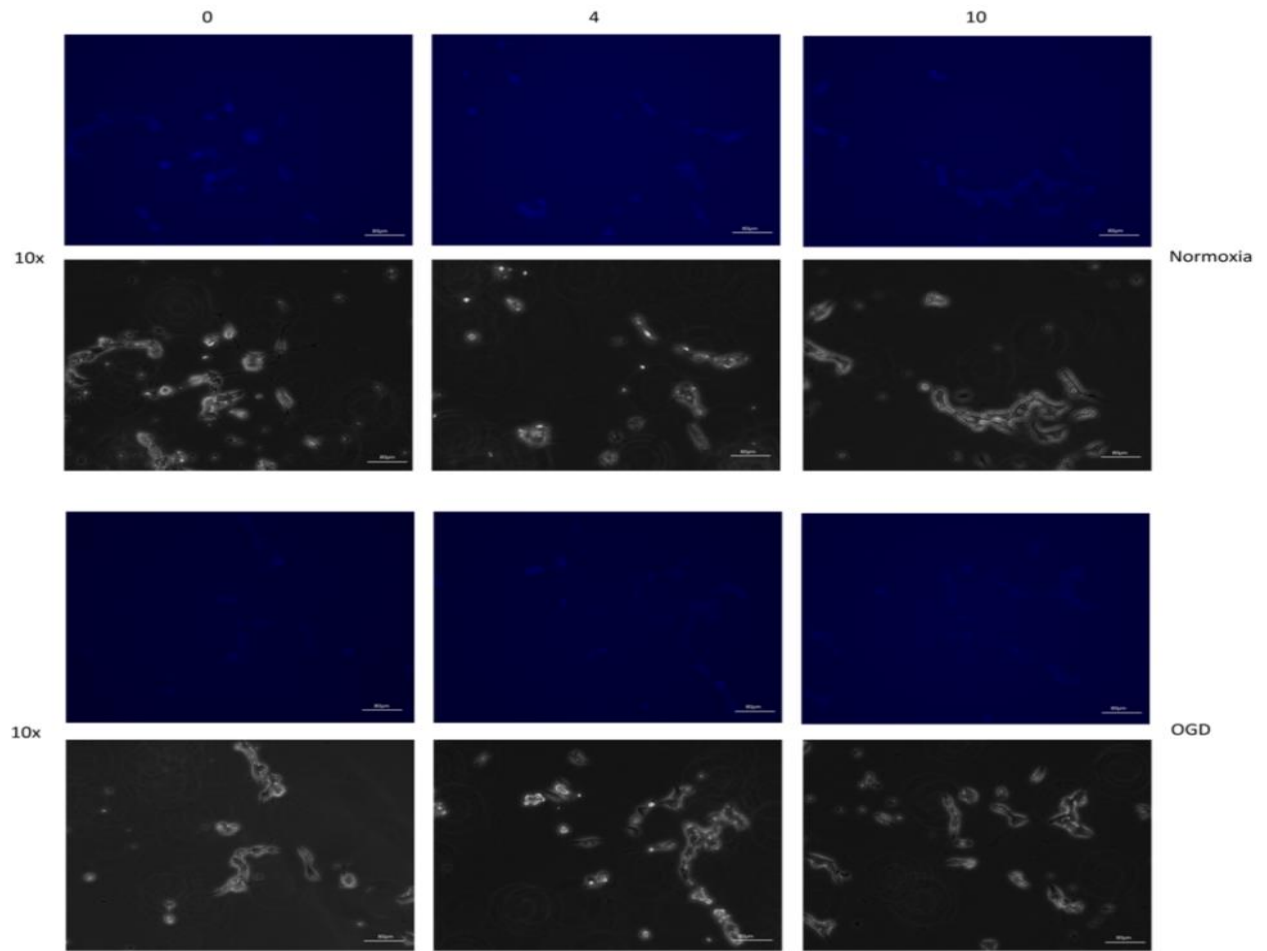
SH-SY5Y cells were cultured in 6-well plates. After reaching 80% confluence, the cells were treated with KU32 for 24 hours, which was followed by 2-hour ischemia and 24-hour reperfusion. Then both calcein blue AM and the hydroxylamine probe were utilized to measure iron levels by fluorescence microscopy. Figure 9A shows the distribution and level of iron inside cells under different conditions and KU32 treatments. Using calcein blue AM Figure 9B presents the quantification of the average fluorescence intensity. Under normoxia condition, the

two rows of images on the top of Figure 9A, the intensities of fluorescence from calcein blue did not show much difference with different concentrations of KU32. On the other hand, under ischemia condition, the two rows of images on the bottom of Figure 9A, a decrease of the intensities of fluorescence was detected comparing with the groups under normoxia, indicating an increase of iron occurred under ischemia. However, with the presence of KU32, the fluorescence of calcein blue was enhanced, suggesting the amount of iron inside cells was decreased by KU32. Figure 9B is a bar graph generated after the quantification of the intensities of fluorescence, giving a more direct presentation of these results. The newly developed hydroxylamine probe was also used in this study to indicate iron level. Figure 9C shows the results of iron level in cells under ischemia reperfusion, in which the green fluorescence represents the level of iron. According to Figure 9C and the quantification of the intensity of fluorescence (Figure 9C), the iron level was decreased with the increase of the concentrations of KU32 treatment, which is consistent with the results obtained with calcein blue AM.

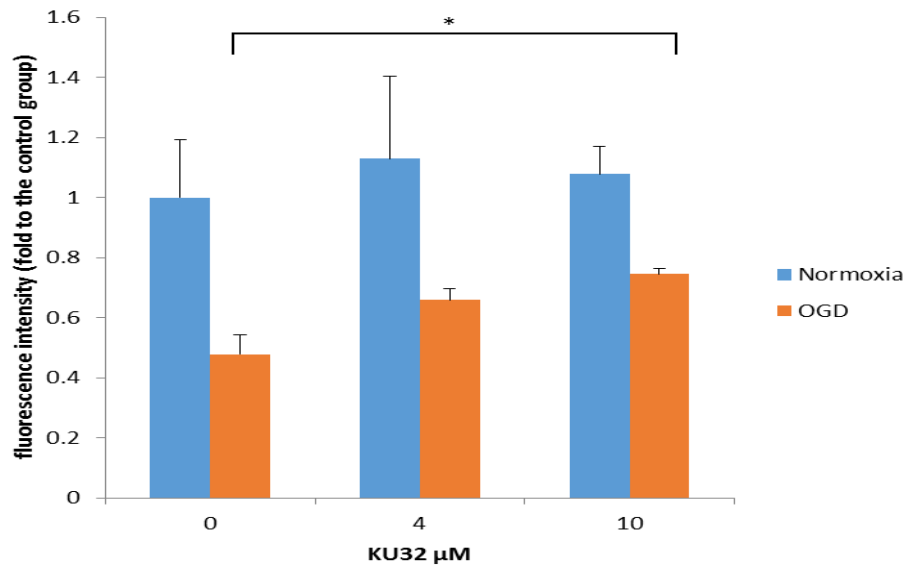
Overall, this group of experiments suggests that the inhibition of HSP90 by KU32 has a negative effect on iron accumulation in SH-SY5Y cells during ischemia-reperfusion. This effect on iron regulation might contribute to the neuroprotective effect of HSP90 inhibition by KU32.



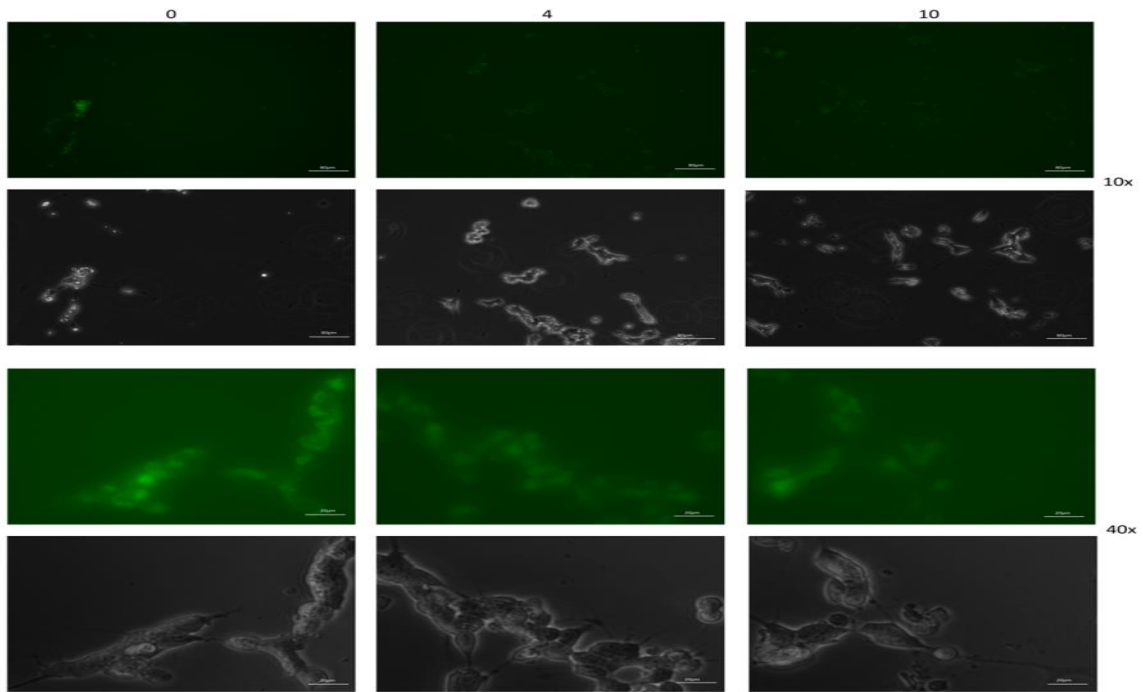
A



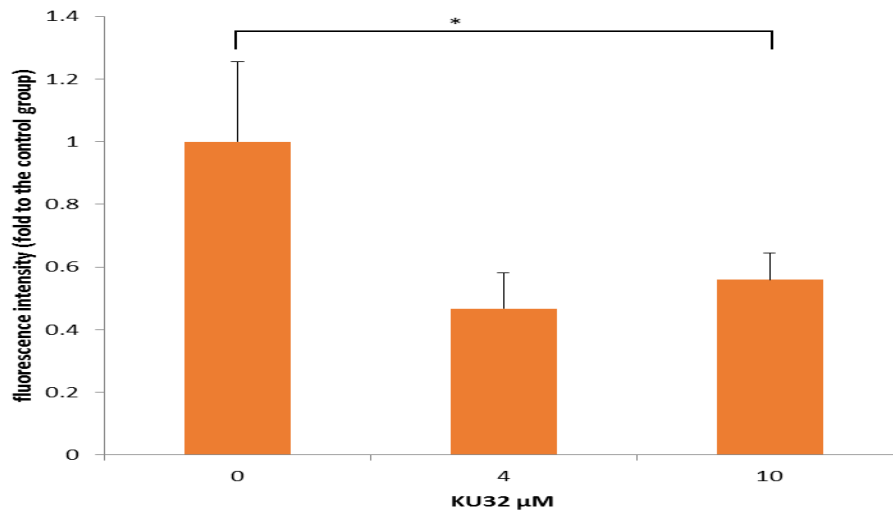
B



C



D



**Figure 9. Effects of KU32 on the iron level in SH-SY5Y cells exposed to ischemia-reperfusion**

- A. The images taken by microscope under DAPI showing the fluorescence of calcein blue inside SH-SY5Y cells with the treatment of KU32.
- B. The quantification of the intensities of the fluorescence in Figure A. The control group is the untreated cell under normoxia.

- C. The images taken by microscope under GFP showing the fluorescence of the probe inside SH-SY5Y cells with the treatment of KU32 under ischemia reperfusion.
- D. The quantification of the intensities of the fluorescence in Figure C. The control group is the untreated cell under normoxia.

\*  $p < 0.05$ ,  $n=4$ , comparison between 0 and  $10\mu\text{M}$  KU32

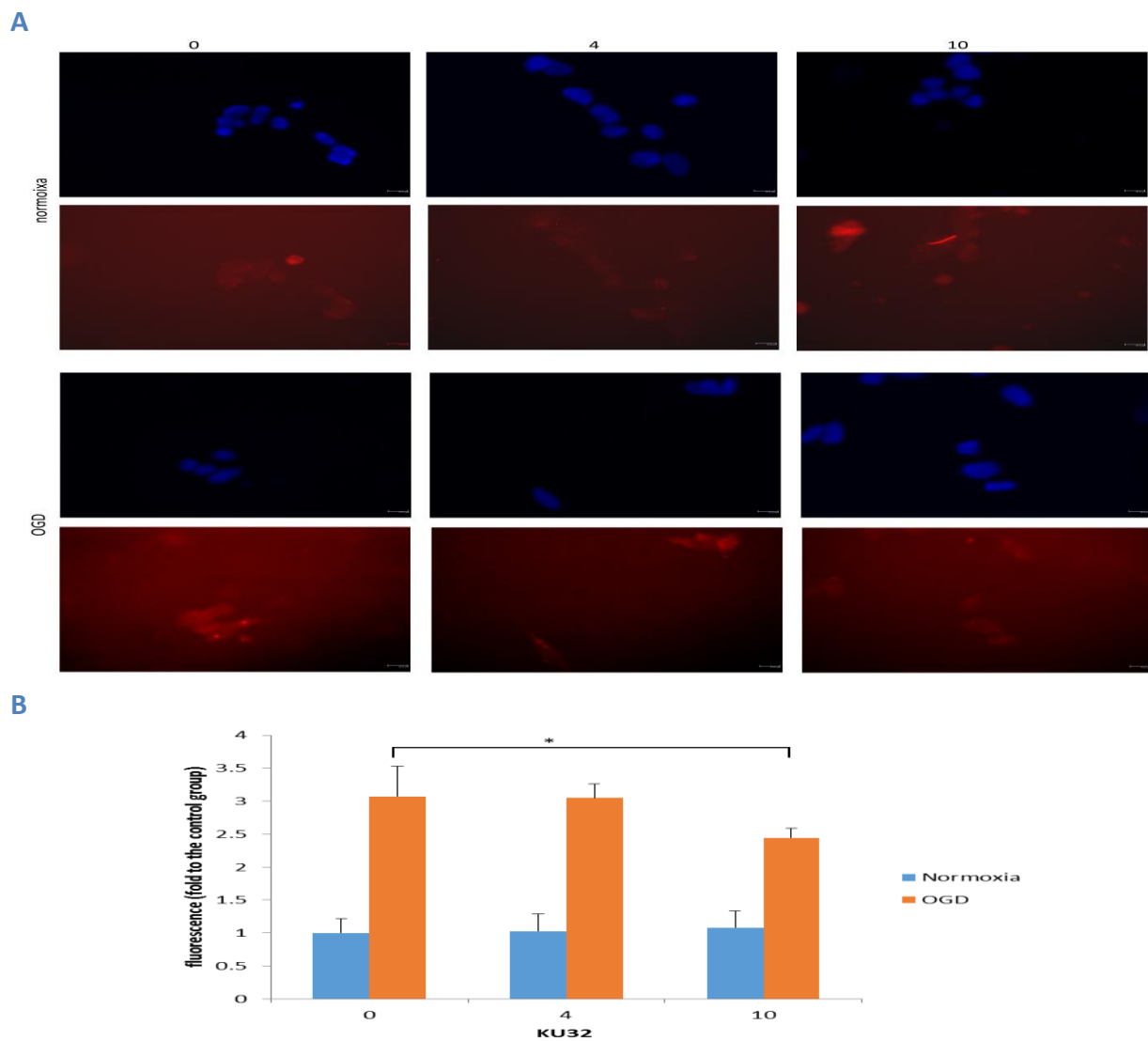
### 3.5. The effect of KU32 on hepcidin under ischemia

Iron homeostasis is closely regulated by iron regulatory proteins. Since the inhibition of HSP90 by KU32 decreases cellular iron levels during ischemia-reperfusion, there may be a relation between the KU32 treatment and iron regulatory proteins. A hypothesis was raised that the inhibition of HSP90 by KU32 might have an effect on expression of hepcidin (approximate to  $2.5\text{kDa}^{157}$ ).

SH-SY5Y cells were cultured on coated coverslips. After reaching 80% confluence, the cells were treated with KU32 for 24 hours, which was followed by 2-hour ischemia and 24-hour reperfusion. Then specific antibodies were used to detect the amount and distribution of hepcidin. The images and the quantification of the intensities of fluorescence are showed in Figures 10A and B. Through the images and the quantification, an increase in the expression in hepcidin occurred under ischemia condition comparing with the normoxia groups, which could support the theory that cellular iron level is increased during ischemia. Moreover, under normoxia condition, the expression of hepcidin did not change with different concentrations of KU32 treatment, while under the ischemia conditions treatment of KU32 did make a reduction of the expression of hepcidin, especially at higher concentration. Lower hepcidin levels mean higher activity of ferroportin resulting in more iron exportation, which contributes to lower level

of cellular iron. Hence, these results are support the findings that the KU32 treatment can reduce cellular iron level during ischemia.

In conclusion, this study of the role of HSP90 inhibition by KU32 on the expression of hepcidin implies that KU32 treatment is able to negatively influence the expression of hepcidin during ischemia-reperfusion injury, which might be related to the effects of KU32 on cellular iron regulation and neuro-protective activity.



**Figure 10.** Effects of KU32 on iron level in SH-SY5Y cells under ischemia-reperfusion

- A. The images taken by microscope under TXRD showing the expression of hepcidin inside SH-SY5Y cells with the treatment of KU32 under ischemia reperfusion.
- B. The bar graph shows quantification of the intensities of the fluorescence in Figure A. The control group is the untreated cell under normoxia.

\*  $p < 0.05$ ,  $n = 3$ , comparison between 0 and  $10 \mu\text{M}$  KU32

### **3.6. The neuro-protective activity of KU32, iron level and hepcidin**

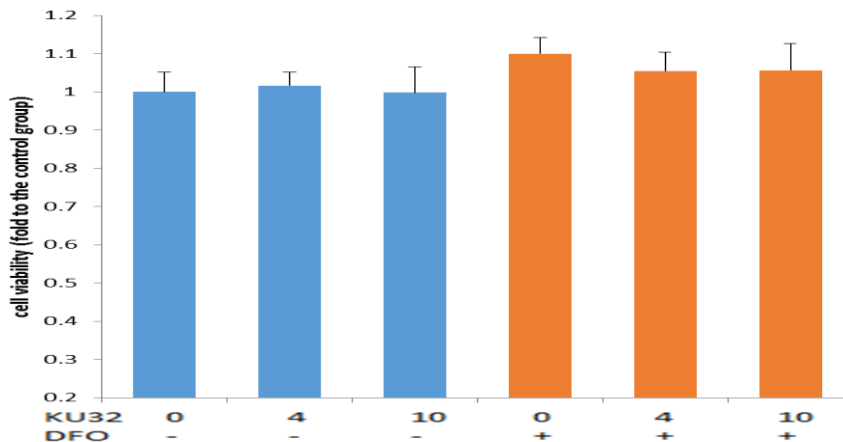
#### **3.6.1. The relation between the effects of KU32 on iron and its neuro-protective activity**

Since KU32 treatment was neuroprotective and able to down regulate cellular iron level, it is necessary to determine whether there are any relations between them. In order to clarify this hypothesis,  $200 \mu\text{M}$  deferoxamine (DFO) was used to chelate iron in order to exclude the iron activities. After SH-SY5Y cells reached 90% confluence in the 96-well cell culture plate, DFO as well as KU32 was added to the cell culture medium 24 hours before the 2-hour ischemia incubation and the 24-hour reperfusion. A MTT assay was performed to test cell viability under the different treatments. Figure 11 shows the results of the MTT cell viability assay. According to these results, normoxia groups (Figure 11A) did not show a significant difference among either the groups treated with KU32 or the groups treated with both KU32 and DFO. However, in the ischemia (OGD) groups, the KU32 treatment showed a neuroprotective effect (Figure 11B). However, with the cellular iron chelated by DFO, the addition of KU32 did not further increase the cell viability in ischemia-reperfusion. However, a difference existed between the non-treated group and DFO alone treated group, showing the protective of DFO. But with the increase of the level of KU32 treatment, no further increase in cell viability was showed. It indicates that the neuroprotection of KU32 was decreased with iron chelation. A two-way

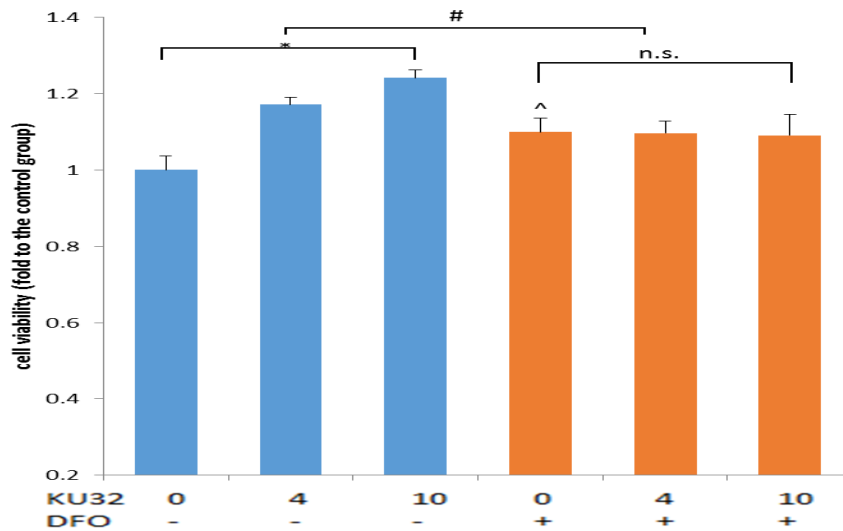
ANOVA (Figure 11B) revealed a highly possible interaction between the treatment of KU32 and treatment of DFO, which suggests a relation between the iron chelation by DFO and the neuroprotective effect of KU32. This result provides indirect evidence that iron chelation prevents the neuroprotective activity of KU32. In fact, DFO has proved beneficial in ischemic brains. In our results, the cell viability with DFO treatment is higher than the control group. Hence, in Figure 11B, iron chelation by DFO reduced the neuroprotective effects of KU32. Taken together, it is possible that these results suggest that iron regulation is related to the neuroprotective activity of KU32.

In conclusion, these results suggest that the effect on iron regulation made by KU32 during ischemia reperfusion is related to its neuroprotection and is possible to contribute to enhancing cell viability.

A



B



**Figure 11. Effects of KU32 on iron cell survival**

A. This bar graph shows the cell viability with the treatment of KU32 and combined treatment with both KU32 and DFO under normoxia. The control group is the untreated cells.

B. This bar graph shows the cell viability with the treatment of KU32 and combined treatment with both KU32 and DFO under ischemia-reperfusion condition. The control group is the untreated cells.

\*  $p < 0.05$ ,  $n = 4$ , Multiple comparisons among the 0, 4 and 10 $\mu$ M groups

^  $p < 0.05$ ,  $n = 4$ , comparisons between non-DFO pretreated group and DFO pretreated group

#  $F(1, 2) = 4.87$ ,  $p < 0.05$ , two-way ANOVA revealed an interaction between treatment of KU32 and treatment of DFO.

### 3.6.2. The relation between the effects of KU32 on hepcidin and its neuro-protective activity

A treatment with KU32 was neuroprotective and correlated with the down regulation of hepcidin expression during ischemia reperfusion, it is necessary to find out whether there is any relation between the neuroprotection and hepcidin.

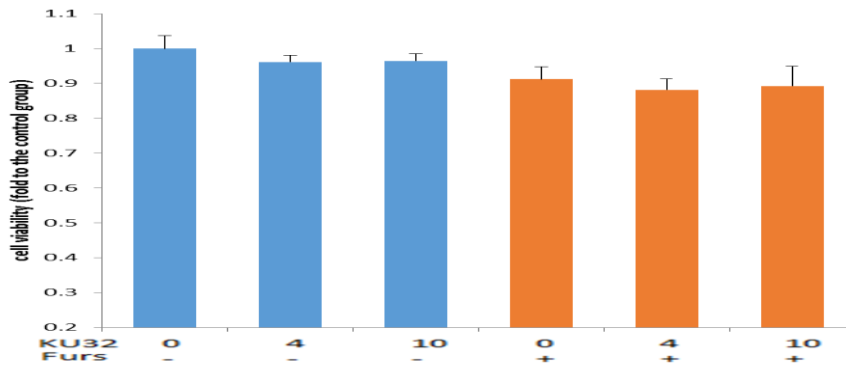
Fursultiamine can bind to ferroportin, the receptor of hepcidin. This disturbs the interaction between hepcidin and ferroportin and antagonizes the role of hepcidin in iron regulation<sup>158</sup>. Hence, 20 $\mu$ M fursultiamine was used in this study as the hepcidin antagonist to exclude the actions of hepcidin. After SH-SY5Y cell reached 90% confluence in the 96-well cell culture plate, fursultiamine as well as KU32 was added to the cell culture medium 24 hours before the 2-hour ischemia incubation and the 24-hour reperfusion. An MTT assay was performed to test cell viability under different treatments. Figure 12 shows the results of the MTT cell viability assay. According to these results, normoxia groups did not show significant difference among either the groups treated with KU32 only or the groups treated with both KU32 and fursultiamine, slight decreases occurred with the presence of fursultiamine (Figure 12A). However, in the ischemia (OGD) groups (Figure 12B), the KU32 treatment showed its neuroprotective activities, KU32 plus fursultiamine did not show a significant difference among the groups, with a two-way ANOVA showing an interaction between KU32 and fursultiamine, indicating that fursultiamine might block the neuro-protective activity of KU32 by acting as a hepcidin antagonist. Similarly, difference between the control group and fursultiamine alone treated group shows the protective of fursultiamine. But with the increase of the level of KU32 treatment, no further increase in cell viability was showed among the groups with both fursultiamine and KU32 treatment. It indicates that the neuroprotection of KU32 was decreased with the function of hepcidin disrupted. This



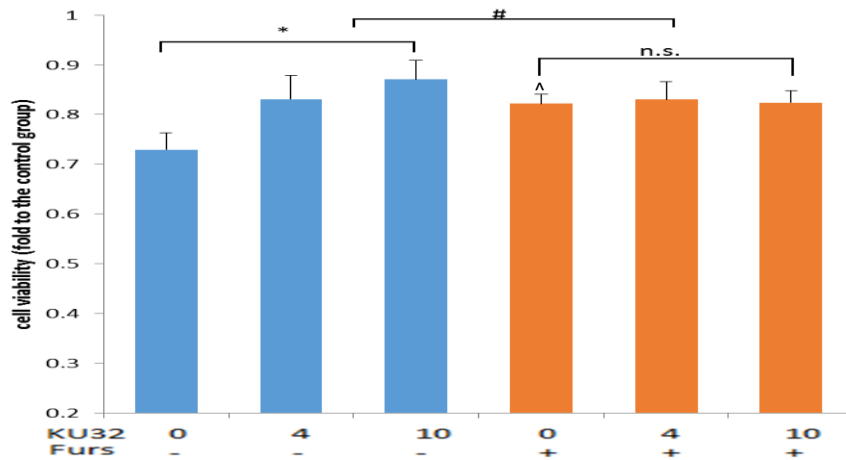
result suggests that it is possible that the interrupting the function of hepcidin in iron export might reduce the neuroprotective effect of KU32. These results could be a clue indicating that hepcidin also is involved in the neuroprotectivity of KU32.

In conclusion, these results suggest that the effect on the expression of hepcidin made by KU32 during ischemia reperfusion is related to the neuroprotection of KU32 and is possible to contribute to enhancing cell viability.

A



B



**Figure 12. The effects of KU32 on hepcidin cell survival**

A. This bar graph shows the cell viability with the treatment of KU32 or combined treatment of KU32 and fursultiamine under normoxia.

B. This bar graph shows the cell viability with the treatment of KU32 or combined treatment of KU32 and fursultiamine under ischemia reperfusion. The vertical axis shows the folds of cell viabilities to the control group under normoxia (Figure A).

\*  $p < 0.05, n = 7$ , Multiple comparisons among the 0, 4 and 10 $\mu$ M groups

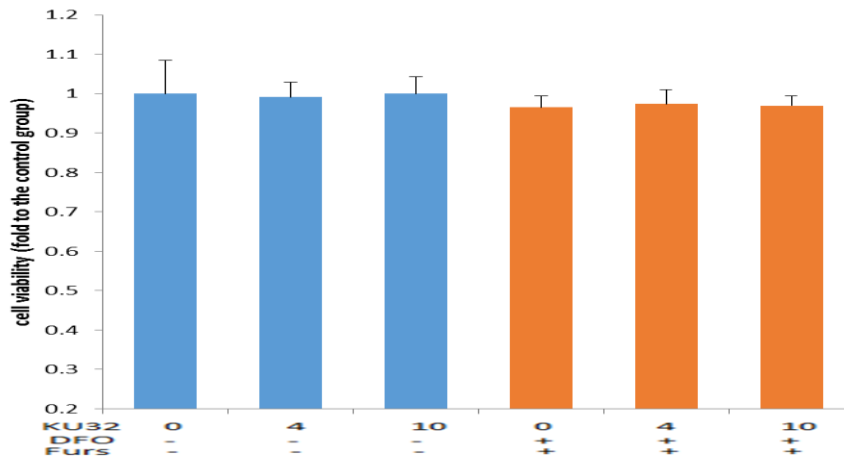
^  $p < 0.05, n = 4$ , comparisons between non-fursultiamine pretreated group and fursultiamine pretreated group

#  $F(1, 2) = 13.21, p < 0.05$ , two-way ANOVA revealed an interaction between treatment of KU32 and treatment of fursultiamine.

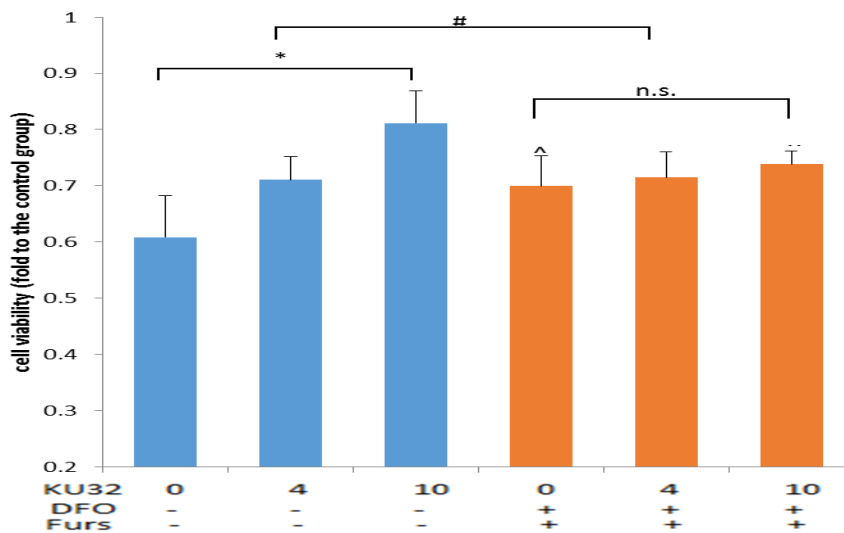
### 3.6.3. The neuro-protective activity of KU32, iron level and hepcidin

Since the relation between KU32 on iron level and neuro-protective activity as well as the relation between KU32 on hepcidin and neuro-protective activity has been supported by the evidence above, a further experiment with both DFO and fursultiamine was performed to confirm the relation among neuroprotective effect of KU32, hepcidin and iron level. The results showed in Figure 13 are pretty similar to the ones above, that no significant difference occurred under normoxia, and the increase in cell viability by KU32 is prevented by the addition of iron chelator and fursultiamine, showing a relatively higher level of cell viability with smaller differences. Also the combined treatment increased the cell viability without KU32. In the groups with 10 $\mu$ M KU32 plus DFO and fursultiamine, the cell viability is also lower than the 10 $\mu$ M KU32 group. A two-way ANOVA was also done and shows a highly possible interaction between the treatment of KU32 and the combined pretreatment DFO and fursultiamine in cell viability under ischemia-reperfusion. Similar to the results above, this result provides a possibility that the neuroprotective effect of KU32 is related to the iron regulation.

A



B



**Figure 13. The neuro-protective activity of KU32, iron level and hepcidin**

A. This bar graph shows the cell viability with the treatment of KU32 or combined treatment of KU32, DFO and fursultiamine under normoxia.

B. This bar graph shows the cell viability with the treatment of KU32 or combined treatment of KU32, DFO and fursultiamine under ischemia reperfusion. The vertical axis shows the folds of cell viabilities to the control group under normoxia (Figure A).

\*  $p < 0.05$ ,  $n = 10$ , multiple comparisons among the 0, 4 and 10 $\mu$ M groups

$\wedge p < 0.05, n = 4$ , comparisons between non-DFO-fursultiamine pretreated group and DFO-fursultiamine pretreated group

#  $F(1, 2) = 4.31, p < 0.05$ , two-way ANOVA revealed an interaction between treatment of KU32 and treatment of combination of DFO and fursultiamine.

All these results suggest the relation between the neuro-protective activity of KU32 and the effect of KU32 on iron levels and hepcidin expression. Based on this study, we can conclude that the inhibition of HSP90 by KU32 might have a neuroprotective effect during ischemia reperfusion, which is related to the decrease of cellular iron level that possibly caused by the down regulation of hepcidin.

## Chapter 4 Discussions and future directions

Iron homeostasis plays an essential role in the health of CNS<sup>26-28</sup>, especially with regards to free radical generation and enhancing oxidative stress<sup>30,32</sup>. A general study of literature about iron regulation within CNS is necessary to form a basic understanding of iron regulation. Table 1 and Table 2 show the distribution of iron regulatory proteins in central nervous system. In general, neurons are well studied and a brief map of the iron transport in neurons has been established. In neurons, transferrin with iron is taken up by the transferrin receptor and after endocytosis, the iron is released and enters the cytosol through DMT1, forming labile iron pool that binds with ferritin, which is exported by ferroportin<sup>159</sup>. On the other hand, all of these proteins do not necessarily exist in the other types of cells, such as glia and endothelial cells, suggesting that these types of cells may assist in neuronal iron regulation. Moreover, table 2 also tells that hepcidin is widely expressed throughout different brain regions, suggesting a key role of hepcidin in iron regulation in brain. However, these two tables are not able to cover all the information related to iron regulation proteins in CNS. There are still some debatable points, such as whether DMT1 and ferroportin are present in brain capillary endothelial cells. It is unidentified whether other types of cells in CNS share the same pathways of iron regulation with neuron or whether glial and endothelial cells have their own pathways regulating iron. Some proteins related to iron regulation are not listed in these tables, such as hephaestin<sup>160,161</sup>. In sum, iron regulation in the CNS is complicated and more research is needed to establish a comprehensive and detailed model to explain the regulation and transportation of iron in the brain.

After the review of iron expression in brain, several studies on iron were performed. According to the Fenton reaction<sup>39,40</sup>, ferrous iron is one of the most dangerous forms, but its measurement is not easy. Hence, studies to test a newly developed hydroxylamine iron probe were performed.

In order to test the properties of fluorescence of the hydroxylamine probe, in-solution experiments were performed, getting the intensity-time, intensity-wavelength and intensity-concentration graphs. Through these graphs, the properties of the fluorescence generated by the probe were determined. The  $\lambda_{em,max}$  is close to 570nm. The intensity of fluorescence increases during an hour and the time point for reasonable detection was 40 minutes. Fluorescence has a linear relation with ferrous ion concentration. The  $\lambda_{em,max}$  from our research is a little different from the one reported by Dr. Wei Wang's group which is 556nm. The reason might be due to the different fluorometers. Besides, according to the intensity-wavelength curve, 15nm difference in wavelength around 560nm does not show much difference in intensity. All these results support the efficacy of this probe in measuring iron level in solution.

Several factors that might affect the fluorescence of the probe in iron detection were tested<sup>116</sup>. Both metal ions and reducing agents were used in these duties, and no significant effects on the fluorescent generation from these ions and agents were found, implying high specificity of this probe to ferrous iron.

One weakness of our results is about the glutathione reacting with the hydroxylamine probe, even though it has been found that the probe has higher specificity to ferrous than glutathione. The concentration of glutathione we chose is much lower than the concentration of glutathione in cells. That experiment was not well designed to mimic the biological conditions. Now it still leaves blank how the probe reacts in the condition of ferrous and much higher level of

glutathione. It is not determined whether the probe can react with ferrous properly with rather larger amount of glutathione (up to 5 mM in cells). Even though our cellular studies showing a positive correlation between ferrous and fluorescence have provided possibilities that glutathione might not have a strong effect on the ferrous detection by the probe in cells, direct evidence is still missing to prove whether the effects of glutathione on iron measure by the probe can be neglected.

Additionally, hydroxylamine groups have been found that they can react with aldehyde or ketone. But in our study, neither aldehyde nor ketone was used to test the probe. It is still unknown whether the presence of aldehyde or ketone has an effect on the iron detection of the probe. This aspect is also a weakness in our research.

Cellular-level studies on the efficacy of this probe were also performed. After the addition of probe, the fluorescence generated by this probe reached a maximum at around 20 minutes after addition and slowly declined over 35 minutes. These kinetic properties showed a little difference from the in-solution studies. One possible explanation is that the cells might have degraded the probe. The degradation by cells starts when the probe enters the cells and its rate is related to the concentration of the probe inside the cells. When the rate of degradation goes up and equal to the rate of the probe entering and reacting with ferrous ions, the fluorescence reaches the maximum. This assumption can also explain why the fluorescence reaches maximum in cells earlier than in solution and the decline of the fluorescence in cells. The results also show that the in-cell use of this probe is able to be detected by fluorescent microscope. Quantification of the fluorescence in the images also showed a linear relation between ferrous concentration and intensity. On the other hand, the effects of other metal ions on the fluorescence generation were without any considerable effect. The fluorescence generated by non-iron metal ions and the probe is weak

and hardly detectable by microscopy. Furthermore, the application of this probe can also extend to dead cells, such as in immunocytochemistry. All the studies done on this hydroxylamine probe suggested that it is a reasonable and reliable iron probe and could be utilized in our further study.

As a major iron probe currently in wide use, calcein blue AM was also tested to identify its efficacy to measure iron. Calcein blue showed a decent quenching in fluorescence in the presence of iron, and this loss of fluorescent intensity could be detected both by a plate reader and by microscopy. According to the quantification, the quenching of fluorescence correlated with the concentration of iron, indicating that calcein blue AM is a decent iron probe that is compatible with our research model. Furthermore, unlike the hydroxylamine iron probe, the detection of calcein blue AM is not limited to the ferrous iron, while ferric iron can also cause a decent quenching in the fluorescence of calcein blue, which can surely extend the application of calcein blue AM. However, the requirement of using live cells with calcein blue AM was also confirmed by our results, which means calcein blue AM is not able to be used in solution assay and more importantly calcein blue AM is theoretically not likely able to be used in immunocytochemistry studies. The reason for needing live cells to use calcein blue AM is that the cleavage between calcein blue group and AM group which is the prerequisite process for fluorescence generation is enzyme dependent. Interestingly, this property ensures another major application of calcein blue AM as a cell viability marker in flow cytometry. Generally, calcein blue AM is a reasonable iron probe and compatible with our research model, so that it can be used in our studies.

As for the comparison between the two probes in this research, calcein blue AM and hydroxylamine probe, two points have been already mentioned above that calcein blue AM requires live cells but the hydroxylamine probe does not. Second calcein blue AM can detect



both ferrous and ferric iron while the hydroxylamine probe can only detect ferrous iron. In addition, another important point is that the hydroxylamine probe is an “off-on” fluorescent probe, which means the fluorescent intensity is positively correlated to the iron level, and has a relatively higher sensitivity than calcein blue AM. Hence, we can conclude that this hydroxylamine compound is an efficient and reasonable iron probe with high sensitivity.

As an inhibitor of heat shock protein 90, KU32 enhanced cell viability under ischemia-reperfusion conditions in SH-SY5Y cells. The inhibition to HSP90 by KU32 was confirmed by western blot showing an up regulation of HSP70. Furthermore, treatment with KU32 was also correlated with a down regulation the cellular iron level under ischemia-reperfusion.

Additionally the expression of hepcidin was also decreased by treatment with KU32. Further experiments found that the effect of KU32 in enhancing cell viability under ischemia reperfusion was diminished by the iron chelator deferoxamine, the hepcidin antagonist fursultiamine and the combination of deferoxamine and fursultiamine.

In general, these results show that KU32 has an inhibitory effect on heat shock protein 90 and a neuroprotective effect in SH-SY5Y cells under ischemia-reperfusion. Condition neuro-protective effect depends on the downregulation of hepcidin and a decrease in iron levels. These results provide a clue that KU32 might have a therapeutic effect on ischemic stroke, which might set up a new way in the therapy to ischemic stroke. Also these results put some light on the relation between heat shock protein inhibition and iron regulation, which might lead new discoveries into heat shock proteins regulation of iron homeostasis. Before this research, few studies analyzed the relation between heat shock proteins and iron regulation, which means that these findings are new in the area of iron and HSP studies. In sum, our findings suggest that KU32 leads to a possible new route to regulate hepcidin expression and cellular iron level, which might help cells

to survive under ischemia reperfusion conditions. Generally, a possible new route helping cell survive has been found and this route is related to heat shock protein and iron regulation.

In specific, these results suggest heat shock protein 90 regulates the expression of hepcidin, which is an inhibitor of the iron exporter, ferroportin. That means the KU32 contributes to the cellular iron export in ischemia-reperfusion, which plays a role in enhancing cell viability. This pathway is able to explain all the results obtained in our studies. However, there are still several points about this regulation pathway remaining unidentified. Because of the inhibitory role of KU32, the amount of HSP90 is actually not changed with the presence of KU32. So the downstream factor of HSP90, HSP70 was chosen to indicate the activity of HSP90. So it is necessary to add direct evidence that the function of HSP90 was inhibited by KU32. Otherwise, there is the possibility that KU32 might increase the level of HSP70 directly. More importantly, since the level of HSP70 was chosen to indicate the activity of HSP90, and HSP70 also plays considerable roles in different pathways, it is also highly likely that HSP70 mediates the changes in the expression of hepcidin and iron regulation. In other words, HSP70 was involved in this research with its roles still being unidentified. Moreover, the link between heat shock proteins and iron regulation is weakly supported by our results, where considerable questions could be raised. For example, whether the iron level is moderated by HSP90, HSP70 or KU32 itself still remains unclear, and also what factors make the direct contribution to the down regulation of hepcidin remains unidentified. On the other hand, iron regulation is complicated and regulated by many factors. Based on our results, a conclusion cannot be made, that all of the other iron regulatory proteins are not involved in this process. However, on the contrary, it is highly possible that more than one iron regulatory proteins, such as transferrin and ferritin, are involved in this process after the treatment of KU32. Furthermore, there is no direct evidence that the

downregulation of cellular iron level has a cell protective effect. Hence, in order to fully prove this regulatory pathway, all of the questions raised above should be studied.

In order to link the neuroprotective effect of KU32 and the functions in iron regulation of KU32, our results show that KU32 alone was able to increase cell viability, and when the iron was chelated or hepcidin was disrupted, the addition of KU32 cannot increase cell viability. Though the two-way ANOVAs in these results indicate that it is possible that iron regulation is involved in the neuroprotective effects of KU32, the cause and effect relation between neuroprotection and iron downregulation by KU32 cannot be surely established. Because some factors, such as ferroportin, involved in this process were not tested in our studies, we cannot certainly conclude that the neuroprotection of KU32 depends on iron regulation. More evidence should be added to support this pathway. Since downregulation of hepcidin can induce iron export, the upregulation of ferroportin and more iron released from cells by treatment of KU32 can be evidence to support the relation between KU32's neuroprotection and downregulation of iron. Moreover, since iron overload can produce free radicals in cells, the downregulation of free radicals by KU32 treatment can also be evidence to support the relation. In addition, knocking down or inhibiting ferroportin can be an approach to further confirm the relation between the KU32's neuroprotection and iron downregulation. On the other hand, we did not test if the drugs (KU32, DFO and fursultiamine) can react with each other in our research model, so it is also possible that the decreasing of neuroprotection is due to the interaction between drugs but not iron regulation. In sum, our result about KU32's neuroprotection and iron downregulation proved an interaction between the treatment with KU32 and iron regulation in cell viability under ischemia-reperfusion, which can be a clue indicating that the neuroprotection and iron downregulation links to each other. Numerous further studies are necessary to confirm the whole pathway and

cause effect relations of the neuroprotective and iron-regulative effects of HSP90 inhibition by KU32.

In addition to the questions listed above, there is also something needed to clarify in this research on KU32 functions. First, and the most important, KU32 actually did not showed a huge neuroprotective effect on the SH-SY5Y cell line, though the difference in cell viability by KU32 treatment was significant. Several reasons might explain to this issue. The MTT assay was the only cell viability assay used, and some oxidative materials might have been released after the death of the cells during ischemia-reperfusion, which might cause false positive results by dead cells in MTT assay. In addition, the immunochemistry approach we used is not a perfect method to analyze hepcidin level. As a result of the small size of hepcidin (2.5kDa), it is difficult to find a both sensitive and accurate method to measure its level. Due to the relatively low accuracy of immunostaining, the standard error of the results was high and the difference between the groups was not very obvious. This weakness reduces the persuasiveness of our conclusions. If possible, more method should be added in determine the level of hepcidin, such as Tricine-SDS-PAGE, ELISA and real time PCR. Moreover, another type of the inhibitor of HSP90, iron chelator and hepcidin antagonist, might be used to increase the reliability of the results and conclusions.

In summary, though there are some weaknesses and some areas needed to be further proved, a conclusion can still be presented that treatment with KU32 before and during ischemia-reperfusion plays a role in decreasing hepcidin expression and cellular iron homeostasis and further has an iron- and hepcidin- dependent neuroprotective activity in SH-SY5Y cells.

## Future directions

Iron is an essential element in human body and its homeostasis is regulated by numerous iron regulatory proteins. In the CNS, the distribution and expression of these proteins are not clear. In the future, one long-term goal is to establish a detailed iron regulation model to analyze the iron regulation and its roles in neurodegenerative diseases. Additionally, improving our understanding of the role of glial cells in iron regulation in the CNS could be another direction of future studies. Another area is to focus on the iron transportation and regulation across the blood brain barrier, since iron transported across the blood brain barrier is a major source of iron in the CNS.

As for the iron probe, there are also various studies to do in future. First, further research is necessary to determine whether the high level of glutathione in cells can have a strong interfere to probe in iron measure. And more studies analyzing the mechanism of the chemical reactions between glutathione and the probe should be performed, in order to obtain a better understanding of the possible interfering effects of glutathione. Moreover, it is also necessary to determine whether aldehyde and ketone can react with the probe in solutions and cells. Since the probe specifically reacts with ferrous iron, its application is limited more or less. So to develop a probe that can detect both ferrous and ferric iron will be greatly helpful for biological iron study. In addition, according to the results of this probe in measuring cellular iron, there might be a degradation mechanism to this probe inside cell, which will limit the iron detection in a small period time. In order to solve this, future studies could be done to analyze the degradation mechanism and increase the stability of the probe in cells. Furthermore, according to our results, the probe reacts with cobalt ions, and it seems that the color of the solution might have a slight effect on the fluorescence of the probe.

For the studies on the heat shock protein 90 inhibition, cell viability and cellular iron regulation, there are a lot more areas needed to be improved. All the unclear areas listed in the section above as well as the weaknesses of the studies require further work to provide more insight into how heat shock proteins affect iron regulation and cell viability. For example, it is a reasonable direction for future studies to determine a direct interaction exists between HSP90 and hepcidin. It is also important to determine whether up regulation of HSP70 by HSP90 inhibition has a further effect on cell viability. Since our work on HSP90 inhibition was performed using the SH-SY5Y cell line, in order to get better understanding and far more reliable findings, primary cell cultures and even in vivo research should be applied to future studies. Also as a long-term goal, there might also be effects of the heat shock proteins on the other metal ions regulation, such as potassium and calcium, which also play significant roles in cell viability and activities. Since the study of the relationship between heat shock proteins and iron metabolisms is a relatively new area of research, there are countless areas could be determined and studied in future.

## References

- 1 Bonita, R. Epidemiology of stroke. *Lancet* **339**, 342-344 (1992).
- 2 Murray, C. J. & Lopez, A. D. Mortality by cause for eight regions of the world: Global Burden of Disease Study. *Lancet* **349**, 1269-1276, doi:10.1016/S0140-6736(96)07493-4 (1997).
- 3 Donnan, G. A., Fisher, M., Macleod, M. & Davis, S. M. Stroke. *Lancet* **371**, 1612-1623, doi:10.1016/S0140-6736(08)60694-7 (2008).
- 4 Murray, C. J. & Lopez, A. D. Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. *Lancet* **349**, 1436-1442, doi:10.1016/S0140-6736(96)07495-8 (1997).
- 5 Lopez, A. D., Mathers, C. D., Ezzati, M., Jamison, D. T. & Murray, C. J. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *Lancet* **367**, 1747-1757, doi:10.1016/S0140-6736(06)68770-9 (2006).
- 6 WHO Collaborating Centres for Research and Training in Neurosciences., National Institute of Neurological and Communicative Disorders and Stroke. Committee on Cerebrovascular Diseases. & World Health Organization. *Cerebrovascular disorders : a clinical and research classification*. (World Health Organization, 1978).
- 7 Wang, J. Preclinical and clinical research on inflammation after intracerebral hemorrhage. *Progress in neurobiology* **92**, 463-477, doi:10.1016/j.pneurobio.2010.08.001 (2010).
- 8 Shuaib, A. & Hachinski, V. C. Mechanisms and management of stroke in the elderly. *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne* **145**, 433-443 (1991).
- 9 Stam, J. Thrombosis of the cerebral veins and sinuses. *The New England journal of medicine* **352**, 1791-1798, doi:10.1056/NEJMra042354 (2005).
- 10 Pullicino, P. & Homma, S. Stroke in heart failure: atrial fibrillation revisited? *Journal of stroke and cerebrovascular diseases : the official journal of National Stroke Association* **19**, 1-2, doi:10.1016/j.jstrokecerebrovasdis.2009.09.002 (2010).
- 11 Deb, P., Sharma, S. & Hassan, K. M. Pathophysiologic mechanisms of acute ischemic stroke: An overview with emphasis on therapeutic significance beyond thrombolysis. *Pathophysiology : the official journal of the International Society for Pathophysiology / ISP* **17**, 197-218, doi:10.1016/j.pathophys.2009.12.001 (2010).
- 12 Koh, J. Y. & Choi, D. W. Vulnerability of cultured cortical neurons to damage by excitotoxins: differential susceptibility of neurons containing NADPH-diaphorase. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **8**, 2153-2163 (1988).
- 13 Kristian, T. & Siesjo, B. K. Calcium-related damage in ischemia. *Life sciences* **59**, 357-367 (1996).
- 14 Chan, P. H. Reactive oxygen radicals in signaling and damage in the ischemic brain. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **21**, 2-14, doi:10.1097/00004647-200101000-00002 (2001).
- 15 Hall, E. D. & Braughler, J. M. Central nervous system trauma and stroke. II. Physiological and pharmacological evidence for involvement of oxygen radicals and lipid peroxidation. *Free radical biology & medicine* **6**, 303-313 (1989).

- 16 Chan, P. H. Oxygen radicals in focal cerebral ischemia. *Brain pathology* **4**, 59-65 (1994).
- 17 Chan, P. H. Role of oxidants in ischemic brain damage. *Stroke; a journal of cerebral circulation* **27**, 1124-1129 (1996).
- 18 Fujimura, M., Morita-Fujimura, Y., Murakami, K., Kawase, M. & Chan, P. H. Cytosolic redistribution of cytochrome c after transient focal cerebral ischemia in rats. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **18**, 1239-1247, doi:10.1097/00004647-199811000-00010 (1998).
- 19 Fujimura, M. *et al.* Copper-zinc superoxide dismutase prevents the early decrease of apurinic/apyrimidinic endonuclease and subsequent DNA fragmentation after transient focal cerebral ischemia in mice. *Stroke; a journal of cerebral circulation* **30**, 2408-2415 (1999).
- 20 O'Donnell, C. J. & Nabel, E. G. Genomics of cardiovascular disease. *The New England journal of medicine* **365**, 2098-2109, doi:10.1056/NEJMra1105239 (2011).
- 21 Yellon, D. M. & Hausenloy, D. J. Myocardial reperfusion injury. *The New England journal of medicine* **357**, 1121-1135, doi:10.1056/NEJMra071667 (2007).
- 22 Lee, J. M., Grabb, M. C., Zipfel, G. J. & Choi, D. W. Brain tissue responses to ischemia. *The Journal of clinical investigation* **106**, 723-731, doi:10.1172/JCI11003 (2000).
- 23 Kristian, T. Metabolic stages, mitochondria and calcium in hypoxic/ischemic brain damage. *Cell calcium* **36**, 221-233, doi:10.1016/j.ceca.2004.02.016 (2004).
- 24 Adibhatla, R. M. & Hatcher, J. F. Lipid oxidation and peroxidation in CNS health and disease: from molecular mechanisms to therapeutic opportunities. *Antioxidants & redox signaling* **12**, 125-169, doi:10.1089/ARS.2009.2668 (2010).
- 25 Damle, S. S. *et al.* Hemoglobin-based oxygen carrier induces heme oxygenase-1 in the heart and lung but not brain. *Journal of the American College of Surgeons* **208**, 592-598, doi:10.1016/j.jamcollsurg.2009.01.015 (2009).
- 26 Zecca, L., Youdim, M. B., Riederer, P., Connor, J. R. & Crichton, R. R. Iron, brain ageing and neurodegenerative disorders. *Nature reviews. Neuroscience* **5**, 863-873, doi:10.1038/nrn1537 (2004).
- 27 Lozoff, B. *et al.* Long-lasting neural and behavioral effects of iron deficiency in infancy. *Nutrition reviews* **64**, S34-43; discussion S72-91 (2006).
- 28 Beard, J. L. & Connor, J. R. Iron status and neural functioning. *Annual review of nutrition* **23**, 41-58, doi:10.1146/annurev.nutr.23.020102.075739 (2003).
- 29 Liu, G. *et al.* Nanoparticle and other metal chelation therapeutics in Alzheimer disease. *Biochimica et biophysica acta* **1741**, 246-252, doi:10.1016/j.bbadis.2005.06.006 (2005).
- 30 Sian-Hulsmann, J., Mandel, S., Youdim, M. B. & Riederer, P. The relevance of iron in the pathogenesis of Parkinson's disease. *Journal of neurochemistry* **118**, 939-957, doi:10.1111/j.1471-4159.2010.07132.x (2011).
- 31 Boddaert, N. *et al.* Selective iron chelation in Friedreich ataxia: biologic and clinical implications. *Blood* **110**, 401-408, doi:10.1182/blood-2006-12-065433 (2007).
- 32 Weinreb, O., Amit, T., Mandel, S., Kupersmidt, L. & Youdim, M. B. Neuroprotective multifunctional iron chelators: from redox-sensitive process to novel therapeutic opportunities. *Antioxidants & redox signaling* **13**, 919-949, doi:10.1089/ars.2009.2929 (2010).
- 33 Youdim, M. B., Stephenson, G. & Ben Shachar, D. Ironing iron out in Parkinson's disease and other neurodegenerative diseases with iron chelators: a lesson from 6-



- hydroxydopamine and iron chelators, desferal and VK-28. *Annals of the New York Academy of Sciences* **1012**, 306-325 (2004).
- 34 Carbonell, T. & Rama, R. Iron, oxidative stress and early neurological deterioration in ischemic stroke. *Current medicinal chemistry* **14**, 857-874 (2007).
- 35 Selim, M. H. & Ratan, R. R. The role of iron neurotoxicity in ischemic stroke. *Ageing research reviews* **3**, 345-353, doi:10.1016/j.arr.2004.04.001 (2004).
- 36 Palmer, C., Menzies, S. L., Roberts, R. L., Pavlick, G. & Connor, J. R. Changes in iron histochemistry after hypoxic-ischemic brain injury in the neonatal rat. *Journal of neuroscience research* **56**, 60-71 (1999).
- 37 Lipscomb, D. C., Gorman, L. G., Traystman, R. J. & Hurn, P. D. Low molecular weight iron in cerebral ischemic acidosis in vivo. *Stroke; a journal of cerebral circulation* **29**, 487-492; discussion 493 (1998).
- 38 Qi, Y., Jamindar, T. M. & Dawson, G. Hypoxia alters iron homeostasis and induces ferritin synthesis in oligodendrocytes. *Journal of neurochemistry* **64**, 2458-2464 (1995).
- 39 Koppenol, W. H. The Haber-Weiss cycle--70 years later. *Redox report : communications in free radical research* **6**, 229-234, doi:10.1179/135100001101536373 (2001).
- 40 Winterbourn, C. C. Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicology letters* **82-83**, 969-974 (1995).
- 41 Ishimaru, H. *et al.* Activation of iron handling system within the gerbil hippocampus after cerebral ischemia. *Brain research* **726**, 23-30 (1996).
- 42 Pratico, D. *et al.* Iron-dependent human platelet activation and hydroxyl radical formation: involvement of protein kinase C. *Circulation* **99**, 3118-3124 (1999).
- 43 Duffy, S. J. *et al.* Iron chelation improves endothelial function in patients with coronary artery disease. *Circulation* **103**, 2799-2804 (2001).
- 44 Kiechl, S., Willeit, J., Egger, G., Poewe, W. & Oberhollenzer, F. Body iron stores and the risk of carotid atherosclerosis: prospective results from the Bruneck study. *Circulation* **96**, 3300-3307 (1997).
- 45 Copp, A. J., Estibeiro, J. P., Brook, F. A. & Downs, K. M. Exogenous transferrin is taken up and localized by the neurulation-stage mouse embryo in vitro. *Developmental biology* **153**, 312-323 (1992).
- 46 Moos, T., Oates, P. S. & Morgan, E. H. Expression of the neuronal transferrin receptor is age dependent and susceptible to iron deficiency. *The Journal of comparative neurology* **398**, 420-430 (1998).
- 47 Simpson, I. A. *et al.* A novel model for brain iron uptake: introducing the concept of regulation. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **35**, 48-57, doi:10.1038/jcbfm.2014.168 (2015).
- 48 Moos, T. & Morgan, E. H. The significance of the mutated divalent metal transporter (DMT1) on iron transport into the Belgrade rat brain. *Journal of neurochemistry* **88**, 233-245 (2004).
- 49 Moos, T., Skjoerringe, T., Gosk, S. & Morgan, E. H. Brain capillary endothelial cells mediate iron transport into the brain by segregating iron from transferrin without the involvement of divalent metal transporter 1. *Journal of neurochemistry* **98**, 1946-1958, doi:10.1111/j.1471-4159.2006.04023.x (2006).
- 50 Bradbury, M. W. Transport of iron in the blood-brain-cerebrospinal fluid system. *Journal of neurochemistry* **69**, 443-454 (1997).

- 51 Brock, J. Lactoferrin: a multifunctional immunoregulatory protein? *Immunology today* **16**, 417-419 (1995).
- 52 Moos, T. & Morgan, E. H. Evidence for low molecular weight, non-transferrin-bound iron in rat brain and cerebrospinal fluid. *Journal of neuroscience research* **54**, 486-494 (1998).
- 53 Ke, Y. *et al.* Age-dependent and iron-independent expression of two mRNA isoforms of divalent metal transporter 1 in rat brain. *Neurobiology of aging* **26**, 739-748, doi:10.1016/j.neurobiolaging.2004.06.002 (2005).
- 54 Hansen, T. M., Nielsen, H., Bernth, N. & Moos, T. Expression of ferritin protein and subunit mRNAs in normal and iron deficient rat brain. *Brain research. Molecular brain research* **65**, 186-197 (1999).
- 55 Moos, T. & Rosengren Nielsen, T. Ferroportin in the postnatal rat brain: implications for axonal transport and neuronal export of iron. *Seminars in pediatric neurology* **13**, 149-157, doi:10.1016/j.spen.2006.08.003 (2006).
- 56 Wu, L. J. *et al.* Expression of the iron transporter ferroportin in synaptic vesicles and the blood-brain barrier. *Brain research* **1001**, 108-117, doi:10.1016/j.brainres.2003.10.066 (2004).
- 57 Moos, T. & Morgan, E. H. Kinetics and distribution of [<sup>59</sup>Fe-125I]transferrin injected into the ventricular system of the rat. *Brain research* **790**, 115-128 (1998).
- 58 Zhao, N., Zhang, A. S. & Enns, C. A. Iron regulation by hepcidin. *The Journal of clinical investigation* **123**, 2337-2343, doi:10.1172/JCI67225 (2013).
- 59 Ganz, T. Hepcidin and iron regulation, 10 years later. *Blood* **117**, 4425-4433, doi:10.1182/blood-2011-01-258467 (2011).
- 60 De Domenico, I., McVey Ward, D. & Kaplan, J. Regulation of iron acquisition and storage: consequences for iron-linked disorders. *Nature reviews. Molecular cell biology* **9**, 72-81, doi:10.1038/nrm2295 (2008).
- 61 Nemeth, E. *et al.* Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* **306**, 2090-2093, doi:10.1126/science.1104742 (2004).
- 62 Abboud, S. & Haile, D. J. A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *The Journal of biological chemistry* **275**, 19906-19912, doi:10.1074/jbc.M000713200 (2000).
- 63 McKie, A. T. *et al.* A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. *Molecular cell* **5**, 299-309 (2000).
- 64 Donovan, A. *et al.* The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. *Cell metabolism* **1**, 191-200, doi:10.1016/j.cmet.2005.01.003 (2005).
- 65 Ganz, T. Cellular iron: ferroportin is the only way out. *Cell metabolism* **1**, 155-157, doi:10.1016/j.cmet.2005.02.005 (2005).
- 66 Donovan, A. *et al.* Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature* **403**, 776-781, doi:10.1038/35001596 (2000).
- 67 Jeong, S. Y. & David, S. Glycosylphosphatidylinositol-anchored ceruloplasmin is required for iron efflux from cells in the central nervous system. *The Journal of biological chemistry* **278**, 27144-27148, doi:10.1074/jbc.M301988200 (2003).
- 68 De Domenico, I. *et al.* Ferroxidase activity is required for the stability of cell surface ferroportin in cells expressing GPI-ceruloplasmin. *The EMBO journal* **26**, 2823-2831, doi:10.1038/sj.emboj.7601735 (2007).

- 69 Vulpe, C. D. *et al.* Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. *Nature genetics* **21**, 195-199, doi:10.1038/5979 (1999).
- 70 McCarthy, R. C. & Kosman, D. J. Ferroportin and exocytosomal ferroxidase activity are required for brain microvascular endothelial cell iron efflux. *The Journal of biological chemistry* **288**, 17932-17940, doi:10.1074/jbc.M113.455428 (2013).
- 71 Takahashi, N., Ortel, T. L. & Putnam, F. W. Single-chain structure of human ceruloplasmin: the complete amino acid sequence of the whole molecule. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 390-394 (1984).
- 72 Koschinsky, M. L., Funk, W. D., van Oost, B. A. & MacGillivray, R. T. Complete cDNA sequence of human preceruloplasmin. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 5086-5090 (1986).
- 73 Royle, N. J., Irwin, D. M., Koschinsky, M. L., MacGillivray, R. T. & Hamerton, J. L. Human genes encoding prothrombin and ceruloplasmin map to 11p11-q12 and 3q21-24, respectively. *Somatic cell and molecular genetics* **13**, 285-292 (1987).
- 74 O'Brien, P. J., Bruce, W. R. & Wiley InterScience (Online service). p (Wiley-VCH ; John Wiley distributor ., Weinheim Chichester, 2010).
- 75 Song, D. & Dunaief, J. L. Retinal iron homeostasis in health and disease. *Frontiers in aging neuroscience* **5**, 24, doi:10.3389/fnagi.2013.00024 (2013).
- 76 Sanchez, L., Calvo, M. & Brock, J. H. Biological role of lactoferrin. *Archives of disease in childhood* **67**, 657-661 (1992).
- 77 Fisher, J. *et al.* Ferritin: a novel mechanism for delivery of iron to the brain and other organs. *American journal of physiology. Cell physiology* **293**, C641-649, doi:10.1152/ajpcell.00599.2006 (2007).
- 78 Rouault, T. A. Iron metabolism in the CNS: implications for neurodegenerative diseases. *Nature reviews. Neuroscience* **14**, 551-564, doi:10.1038/nrn3453 (2013).
- 79 Hulet, S. W. *et al.* Characterization and distribution of ferritin binding sites in the adult mouse brain. *Journal of neurochemistry* **72**, 868-874 (1999).
- 80 Benkovic, S. A. & Connor, J. R. Ferritin, transferrin, and iron in selected regions of the adult and aged rat brain. *The Journal of comparative neurology* **338**, 97-113, doi:10.1002/cne.903380108 (1993).
- 81 Moos, T. Increased accumulation of transferrin by motor neurons of the mouse mutant progressive motor neuronopathy (pnm/pnm). *Journal of neurocytology* **24**, 389-398 (1995).
- 82 Fillebeen, C. *et al.* Receptor-mediated transcytosis of lactoferrin through the blood-brain barrier. *The Journal of biological chemistry* **274**, 7011-7017 (1999).
- 83 Kawamata, T., Tooyama, I., Yamada, T., Walker, D. G. & McGeer, P. L. Lactotransferrin immunocytochemistry in Alzheimer and normal human brain. *The American journal of pathology* **142**, 1574-1585 (1993).
- 84 Faucheux, B. A. *et al.* Expression of lactoferrin receptors is increased in the mesencephalon of patients with Parkinson disease. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 9603-9607 (1995).
- 85 Leveugle, B. *et al.* The iron-binding protein lactotransferrin is present in pathologic lesions in a variety of neurodegenerative disorders: a comparative immunohistochemical analysis. *Brain research* **650**, 20-31 (1994).

- 86 Talukder, M. J. & Harada, E. Binding characteristics and distribution of lactoferrin receptors in the gut and choroid plexus in newborn calves. *Indian journal of experimental biology* **44**, 783-790 (2006).
- 87 Jeong, S. Y. & David, S. Age-related changes in iron homeostasis and cell death in the cerebellum of ceruloplasmin-deficient mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **26**, 9810-9819, doi:10.1523/JNEUROSCI.2922-06.2006 (2006).
- 88 Klomp, L. W., Farhangrazi, Z. S., Dugan, L. L. & Gitlin, J. D. Ceruloplasmin gene expression in the murine central nervous system. *The Journal of clinical investigation* **98**, 207-215, doi:10.1172/JCI118768 (1996).
- 89 Patel, B. N. & David, S. A novel glycosylphosphatidylinositol-anchored form of ceruloplasmin is expressed by mammalian astrocytes. *The Journal of biological chemistry* **272**, 20185-20190 (1997).
- 90 Burdo, J. R. *et al.* Distribution of divalent metal transporter 1 and metal transport protein 1 in the normal and Belgrade rat. *Journal of neuroscience research* **66**, 1198-1207 (2001).
- 91 Moos, T., Rosengren Nielsen, T., Skjorringe, T. & Morgan, E. H. Iron trafficking inside the brain. *Journal of neurochemistry* **103**, 1730-1740, doi:10.1111/j.1471-4159.2007.04976.x (2007).
- 92 Zheng, W. & Monnot, A. D. Regulation of brain iron and copper homeostasis by brain barrier systems: implication in neurodegenerative diseases. *Pharmacology & therapeutics* **133**, 177-188, doi:10.1016/j.pharmthera.2011.10.006 (2012).
- 93 Siddappa, A. J. *et al.* Developmental changes in the expression of iron regulatory proteins and iron transport proteins in the perinatal rat brain. *Journal of neuroscience research* **68**, 761-775, doi:10.1002/jnr.10246 (2002).
- 94 Siddappa, A. J. *et al.* Iron deficiency alters iron regulatory protein and iron transport protein expression in the perinatal rat brain. *Pediatric research* **53**, 800-807, doi:10.1203/01.PDR.0000058922.67035.D5 (2003).
- 95 Gunshin, H. *et al.* Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* **388**, 482-488, doi:10.1038/41343 (1997).
- 96 Huang, E., Ong, W. Y. & Connor, J. R. Distribution of divalent metal transporter-1 in the monkey basal ganglia. *Neuroscience* **128**, 487-496, doi:10.1016/j.neuroscience.2004.06.055 (2004).
- 97 Huang, E., Ong, W. Y., Go, M. L. & Connor, J. R. Upregulation of iron regulatory proteins and divalent metal transporter-1 isoforms in the rat hippocampus after kainate induced neuronal injury. *Experimental brain research* **170**, 376-386, doi:10.1007/s00221-005-0220-x (2006).
- 98 Roberts, R. L., Fine, R. E. & Sandra, A. Receptor-mediated endocytosis of transferrin at the blood-brain barrier. *Journal of cell science* **104 ( Pt 2)**, 521-532 (1993).
- 99 Bickel, U., Kang, Y. S., Yoshikawa, T. & Pardridge, W. M. In vivo demonstration of subcellular localization of anti-transferrin receptor monoclonal antibody-colloidal gold conjugate in brain capillary endothelium. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* **42**, 1493-1497 (1994).
- 100 Gosk, S., Vermehren, C., Storm, G. & Moos, T. Targeting anti-transferrin receptor antibody (OX26) and OX26-conjugated liposomes to brain capillary endothelial cells using in situ perfusion. *Journal of cerebral blood flow and metabolism : official journal*

- of the *International Society of Cerebral Blood Flow and Metabolism* **24**, 1193-1204, doi:10.1097/01.WCB.0000135592.28823.47 (2004).
- 101 de Arriba Zerpa, G. A. *et al.* Alternative splicing prevents transferrin secretion during differentiation of a human oligodendrocyte cell line. *Journal of neuroscience research* **61**, 388-395 (2000).
- 102 Aldred, A. R., Dickson, P. W., Marley, P. D. & Schreiber, G. Distribution of transferrin synthesis in brain and other tissues in the rat. *The Journal of biological chemistry* **262**, 5293-5297 (1987).
- 103 Tsutsumi, M., Skinner, M. K. & Sanders-Bush, E. Transferrin gene expression and synthesis by cultured choroid plexus epithelial cells. Regulation by serotonin and cyclic adenosine 3',5'-monophosphate. *The Journal of biological chemistry* **264**, 9626-9631 (1989).
- 104 Simpson, I. A. *et al.* A novel model for brain iron uptake: introducing the concept of regulation. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, doi:10.1038/jcbfm.2014.168 (2014).
- 105 Bloch, B., Popovici, T., Levin, M. J., Tuil, D. & Kahn, A. Transferrin gene expression visualized in oligodendrocytes of the rat brain by using in situ hybridization and immunohistochemistry. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 6706-6710 (1985).
- 106 Ding, H. *et al.* Hcpidin is involved in iron regulation in the ischemic brain. *PloS one* **6**, e25324, doi:10.1371/journal.pone.0025324 (2011).
- 107 Hallgren, R., Terent, A., Wide, L., Bergstrom, K. & Birgegard, G. Cerebrospinal fluid ferritin in patients with cerebral infarction or bleeding. *Acta neurologica Scandinavica* **61**, 384-392 (1980).
- 108 Davalos, A. *et al.* Iron-related damage in acute ischemic stroke. *Stroke; a journal of cerebral circulation* **25**, 1543-1546 (1994).
- 109 Cabantchik, Z. I., Glickstein, H., Milgram, P. & Breuer, W. A fluorescence assay for assessing chelation of intracellular iron in a membrane model system and in mammalian cells. *Analytical biochemistry* **233**, 221-227, doi:10.1006/abio.1996.0032 (1996).
- 110 Kakhlon, O. & Cabantchik, Z. I. The labile iron pool: characterization, measurement, and participation in cellular processes(1). *Free radical biology & medicine* **33**, 1037-1046 (2002).
- 111 Esposito, B. P., Epsztejn, S., Breuer, W. & Cabantchik, Z. I. A review of fluorescence methods for assessing labile iron in cells and biological fluids. *Analytical biochemistry* **304**, 1-18, doi:10.1006/abio.2002.5611 (2002).
- 112 Glickstein, H., El, R. B., Shvartsman, M. & Cabantchik, Z. I. Intracellular labile iron pools as direct targets of iron chelators: a fluorescence study of chelator action in living cells. *Blood* **106**, 3242-3250, doi:10.1182/blood-2005-02-0460 (2005).
- 113 Tenopoulou, M., Kurz, T., Doulias, P. T., Galaris, D. & Brunk, U. T. Does the calcein-AM method assay the total cellular 'labile iron pool' or only a fraction of it? *The Biochemical journal* **403**, 261-266, doi:10.1042/BJ20061840 (2007).
- 114 Epsztejn, S., Kakhlon, O., Glickstein, H., Breuer, W. & Cabantchik, I. Fluorescence analysis of the labile iron pool of mammalian cells. *Analytical biochemistry* **248**, 31-40 (1997).

- 115 Hirayama, T., Okuda, K. & Nagasawa, H. A highly selective turn-on fluorescent probe for iron(II) to visualize labile iron in living cells. *Chem Sci* **4**, 1250-1256, doi:10.1039/c2sc21649c (2013).
- 116 Xuan, W. *et al.* Reaction-Based "Off-On" Fluorescent Probe Enabling Detection of Endogenous Labile Fe(2+) and Imaging of Zn(2+)-induced Fe(2+) Flux in Living Cells and Elevated Fe(2+) in Ischemic Stroke. *Bioconjugate chemistry* **27**, 302-308, doi:10.1021/acs.bioconjchem.5b00259 (2016).
- 117 De Maio, A. Heat shock proteins: facts, thoughts, and dreams. *Shock* **11**, 1-12 (1999).
- 118 Cao, Y. *et al.* TGF-beta1 mediates 70-kDa heat shock protein induction due to ultraviolet irradiation in human skin fibroblasts. *Pflugers Archiv : European journal of physiology* **438**, 239-244 (1999).
- 119 Laplante, A. F. *et al.* Expression of heat shock proteins in mouse skin during wound healing. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* **46**, 1291-1301 (1998).
- 120 Fontana, J. *et al.* Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release. *Circulation research* **90**, 866-873 (2002).
- 121 Eustace, B. K. *et al.* Functional proteomic screens reveal an essential extracellular role for hsp90 alpha in cancer cell invasiveness. *Nature cell biology* **6**, 507-514, doi:10.1038/ncb1131 (2004).
- 122 Pratt, W. B., Morishima, Y., Peng, H. M. & Osawa, Y. Proposal for a role of the Hsp90/Hsp70-based chaperone machinery in making triage decisions when proteins undergo oxidative and toxic damage. *Experimental biology and medicine* **235**, 278-289, doi:10.1258/ebm.2009.009250 (2010).
- 123 Bienemann, A. S., Lee, Y. B., Howarth, J. & Uney, J. B. Hsp70 suppresses apoptosis in sympathetic neurones by preventing the activation of c-Jun. *Journal of neurochemistry* **104**, 271-278, doi:10.1111/j.1471-4159.2007.05006.x (2008).
- 124 Chaudhury, S., Welch, T. R. & Blagg, B. S. Hsp90 as a target for drug development. *ChemMedChem* **1**, 1331-1340, doi:10.1002/cmdc.200600112 (2006).
- 125 Urban, M. J. *et al.* Inhibiting heat-shock protein 90 reverses sensory hypoalgesia in diabetic mice. *ASN neuro* **2**, e00040, doi:10.1042/AN20100015 (2010).
- 126 Luo, W. *et al.* Roles of heat-shock protein 90 in maintaining and facilitating the neurodegenerative phenotype in tauopathies. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 9511-9516, doi:10.1073/pnas.0701055104 (2007).
- 127 Ma, J. *et al.* Heat shock protein 70 is necessary to improve mitochondrial bioenergetics and reverse diabetic sensory neuropathy following KU-32 therapy. *The Journal of pharmacology and experimental therapeutics* **348**, 281-292, doi:10.1124/jpet.113.210435 (2014).
- 128 Matts, R. L. *et al.* A systematic protocol for the characterization of Hsp90 modulators. *Bioorganic & medicinal chemistry* **19**, 684-692, doi:10.1016/j.bmc.2010.10.029 (2011).
- 129 Peterson, L. B. & Blagg, B. S. To fold or not to fold: modulation and consequences of Hsp90 inhibition. *Future medicinal chemistry* **1**, 267-283, doi:10.4155/fmc.09.17 (2009).
- 130 Li, J. *et al.* 17-AAG post-treatment ameliorates memory impairment and hippocampal CA1 neuronal autophagic death induced by transient global cerebral ischemia. *Brain research* **1610**, 80-88, doi:10.1016/j.brainres.2015.03.051 (2015).

- 131 He, D., Song, X. & Li, L. Geranylgeranylacetone protects against cerebral ischemia and  
reperfusion injury: HSP90 and eNOS phosphorylation involved. *Brain research* **1599**,  
150-157, doi:10.1016/j.brainres.2014.12.019 (2015).
- 132 Isaacs, J. S. *et al.* Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible  
factor-1 alpha-degradative pathway. *The Journal of biological chemistry* **277**, 29936-  
29944, doi:10.1074/jbc.M204733200 (2002).
- 133 Isaacs, J. S., Jung, Y. J. & Neckers, L. Aryl hydrocarbon nuclear translocator (ARNT)  
promotes oxygen-independent stabilization of hypoxia-inducible factor-1alpha by  
modulating an Hsp90-dependent regulatory pathway. *The Journal of biological chemistry*  
**279**, 16128-16135, doi:10.1074/jbc.M313342200 (2004).
- 134 Mabjeesh, N. J. *et al.* Geldanamycin induces degradation of hypoxia-inducible factor  
1alpha protein via the proteosome pathway in prostate cancer cells. *Cancer research* **62**,  
2478-2482 (2002).
- 135 Zhang, Z., Yan, J., Taheri, S., Liu, K. J. & Shi, H. Hypoxia-inducible factor 1 contributes  
to N-acetylcysteine's protection in stroke. *Free radical biology & medicine* **68**, 8-21,  
doi:10.1016/j.freeradbiomed.2013.11.007 (2014).
- 136 Peyssonnaud, C. *et al.* Regulation of iron homeostasis by the hypoxia-inducible  
transcription factors (HIFs). *The Journal of clinical investigation* **117**, 1926-1932,  
doi:10.1172/JCI31370 (2007).
- 137 Peyssonnaud, C., Nizet, V. & Johnson, R. S. Role of the hypoxia inducible factors HIF in  
iron metabolism. *Cell cycle* **7**, 28-32 (2008).
- 138 Lee, D. W. & Andersen, J. K. Role of HIF-1 in iron regulation: potential therapeutic  
strategy for neurodegenerative disorders. *Current molecular medicine* **6**, 883-893 (2006).
- 139 Giordano, G., Hong, S., Faustman, E. M. & Costa, L. G. Measurements of cell death in  
neuronal and glial cells. *Methods in molecular biology* **758**, 171-178, doi:10.1007/978-1-  
61779-170-3\_11 (2011).
- 140 Mahmood, T. & Yang, P. C. Western blot: technique, theory, and trouble shooting. *North  
American journal of medical sciences* **4**, 429-434, doi:10.4103/1947-2714.100998  
(2012).
- 141 Yan, J., Zhang, Z. & Shi, H. HIF-1 is involved in high glucose-induced paracellular  
permeability of brain endothelial cells. *Cellular and molecular life sciences : CMLS* **69**,  
115-128, doi:10.1007/s00018-011-0731-5 (2012).
- 142 Schrauzer, G. N. Lithium: occurrence, dietary intakes, nutritional essentiality. *Journal of  
the American College of Nutrition* **21**, 14-21 (2002).
- 143 Lusk, J. E., Williams, R. J. & Kennedy, E. P. Magnesium and the growth of *Escherichia  
coli*. *The Journal of biological chemistry* **243**, 2618-2624 (1968).
- 144 Marschner, H. *Mineral nutrition of higher plants*. 2nd edn, (Academic Press, 1995).
- 145 Yamada, K. Cobalt: its role in health and disease. *Metal ions in life sciences* **13**, 295-320,  
doi:10.1007/978-94-007-7500-8\_9 (2013).
- 146 Cracan, V. & Banerjee, R. Cobalt and corrinoid transport and biochemistry. *Metal ions in  
life sciences* **12**, 333-374, doi:10.1007/978-94-007-5561-1\_10 (2013).
- 147 Maret, W. Zinc and human disease. *Metal ions in life sciences* **13**, 389-414,  
doi:10.1007/978-94-007-7500-8\_12 (2013).
- 148 Prasad, A. S. Zinc in human health: effect of zinc on immune cells. *Molecular medicine*  
**14**, 353-357, doi:10.2119/2008-00033.Prasad (2008).
- 149 Sugarman, B. Zinc and infection. *Reviews of infectious diseases* **5**, 137-147 (1983).

- 150 Milne, G. W. A. *Gardner's commercially important chemicals : synonyms, trade names, and properties*. (Wiley-Interscience, 2005).
- 151 Cicone, J. S., Petronis, J. B., Embert, C. D. & Spector, D. A. Successful treatment of calciphylaxis with intravenous sodium thiosulfate. *American journal of kidney diseases : the official journal of the National Kidney Foundation* **43**, 1104-1108 (2004).
- 152 Selk, N. & Rodby, R. A. Unexpectedly severe metabolic acidosis associated with sodium thiosulfate therapy in a patient with calcific uremic arteriolopathy. *Seminars in dialysis* **24**, 85-88, doi:10.1111/j.1525-139X.2011.00848.x (2011).
- 153 Hall, A. H., Dart, R. & Bogdan, G. Sodium thiosulfate or hydroxocobalamin for the empiric treatment of cyanide poisoning? *Annals of emergency medicine* **49**, 806-813, doi:10.1016/j.annemergmed.2006.09.021 (2007).
- 154 Dorland, W. A. N. 1 online resource (xxvii, 2147 p (Elsevier/Saunders,, Philadelphia, Pa., 2011).
- 155 Stipanuk, M. H. *Biochemical and physiological aspects of human nutrition*. (W.B. Saunders, 2000).
- 156 Xuan, W., Pan, R., Cao, Y., Liu, K. & Wang, W. A fluorescent probe capable of detecting H<sub>2</sub>S at submicromolar concentrations in cells. *Chemical communications* **48**, 10669-10671, doi:10.1039/c2cc35602c (2012).
- 157 Wang, S. M. *et al.* Role of hepcidin in murine brain iron metabolism. *Cellular and molecular life sciences : CMLS* **67**, 123-133, doi:10.1007/s00018-009-0167-3 (2010).
- 158 Fung, E. *et al.* High-throughput screening of small molecules identifies hepcidin antagonists. *Molecular pharmacology* **83**, 681-690, doi:10.1124/mol.112.083428 (2013).
- 159 Hare, D., Ayton, S., Bush, A. & Lei, P. A delicate balance: Iron metabolism and diseases of the brain. *Frontiers in aging neuroscience* **5**, 34, doi:10.3389/fnagi.2013.00034 (2013).
- 160 Petrak, J. & Vyoral, D. Hephaestin--a ferroxidase of cellular iron export. *The international journal of biochemistry & cell biology* **37**, 1173-1178, doi:10.1016/j.biocel.2004.12.007 (2005).
- 161 Anderson, G. J., Frazer, D. M., McKie, A. T. & Vulpe, C. D. The ceruloplasmin homolog hephaestin and the control of intestinal iron absorption. *Blood cells, molecules & diseases* **29**, 367-375 (2002).