IN VITRO EFFECT OF PENICILLAMINE ON NEURONAL AND GLIOMAL CELLS: POSSIBLE IMPLICATIONS ON WILSON DISEASE PATIENTS

by

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IN VITRO EFFECT OF PENICILLAMINE ON NEURONAL AND GLIOMAL CELLS: POSSIBLE IMPLICATIONS ON WILSON DISEASE PATIENTS

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In the roller coaster ride we call “life”, peaks of joy and valleys of heartache are what define our journey. Through Hope, Faith and Love, I have been able to conquer life obscurities. I have put efforts in this project; however it wouldn’t have been possible without the help of so many individuals. I would like to extend my sincerest appreciation to all of them.

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I am also greatly indebted to my sacred family for accompanying me on my path to success. I would like to thank my husband` my backbone, for his unconditional love and faith; my brother` my guardian, for his protection and tolerance; my mother` my best friend, for her tender care and patience; and my father` my angel, for his trust and inspiration.

A family is a circle of love, not broken by a loss, but made stronger by memories. I will always remember you my angel father and carry on the morals and values you have planted in me to become an achiever in your example. Thus, I dedicate this thesis, the first step towards my professional career, to your beloved memory.
Title: In vitro effect of Penicillamine on neuronal and gliomal cells: possible implications on Wilson Disease patients

Background:
Copper is an essential trace element that acts as a cofactor for many cuproenzymes. Cu is also crucial for the brain development and function. However, due to its high redox potential when present in excess, copper may damage the lipids, proteins, and DNA of the cell. Disturbance in Cu homeostasis has been implicated in many neurodegenerative disorders. Wilson’s disease is a rare autosomal recessive disorder resulting from copper accumulation in liver and brain of patients. WD is caused by mutations in ATP7B encoding a copper transporter that incorporates Cu into ceruloplasmin, a member of the multi-Cu oxidase family of enzymes. Cp maintains iron homeostasis. Copper loading into apoceruloplasmin yields the active holoceruloplasmin which has a ferroxidase activity that catalyzes iron oxidation (Fe^{2+} into Fe^{3+}) with subsequent transfer onto transferrin. D-Penicillamine, the recommended copper chelating drug for treating patients with WD, has been reported to worsen the symptoms of WD patients with neurologic manifestation.

Hypothesis and aims:
The lifelong treatment of WD patients with PA will decrease Cu loading onto apo-Cp, reduce the level of active Cp, consequently lower ferroxidase activity, iron oxidation and loading into transferrin, leading to iron deposition in the brain. This study aims to investigate the effect of treatment with D-PA alone, Cu alone, and of PA combined with copper on neuronal and glial cells viability and expression of copper and iron binding proteins.

Methods:
Neuronal (PC12) and glial (U251) cells were cultured under optimal conditions and treated with Cu and/or PA, following which we determined: viability using MTT assay and Trypan blue exclusion test, ROS level using NBT reduction assay, intracellular GSH level using fluorometric glutathione detection kit, membrane integrity using LDH release assay, mitochondrial depolarization using MitoPT-JC1 assay, distribution of cell cycle phases by flow cytometry. In addition, α-fodrin cleavage and expression levels of ceruloplasmin, ferritin, and transferrin were determined using western blotting.
Results:
Copper reduced the viability of PC12 and U251 cells in a dose-dependent manner, reaching a 50% cell death at 50 µM using MTT assay. Trypan blue exclusion test, however, showed no significant cell death. PA, at a concentration of 250 µM, exerted no effect on the viability of PC12 or U251 cells. In addition, compared to untreated cells, there was no significant variation (ROS, GSH, LDH release and % distribution in cell cycle phases) in PA or Cu treated cells. Co-treatment of cells with Cu-PA (50-250 µM) showed: a) decrease in the viability (MTT & trypan blue) of PC12 (65% & 5%) and U251 (85% & 92%) cells; b) decrease in GSH (49% & no variation) concomitant with significant increase in ROS (51% & 16%) in U251 and PC12 cells; c) complete and partial depolarization of mitochondrial membrane potential in U251 and PC12 cells, respectively; d) sub-Go increase of 99% and 12% in U251 and PC12 cells, respectively; e) LDH release (16%), the necrotic marker, in PC12 cells, but not in U251; f) α-fodrin cleavage, an apoptotic marker, in U251 cells.

Expression levels of Cu and Fe binding proteins showed: a) No variation in ferritin level in either cell line with various treatment; b) decrease of 33% & 54% in transferrin level in PC12 cells treated with PA and Cu-PA, respectively; c) decrease of 60% and increase of 54% in ceruloplasmin expression level of Cu-PA co-treated U251 and PC12 cells, respectively.

Conclusion:
To sum up, we hereby show sensitivity of U251 but not PC12 to PA-Cu co-treatment. Resistance of PC12 (rat) may be attributed to species differences and to undifferentiated state of the used cells. More importantly, the induced apoptosis in Cu-PA treated U251 (human) cells may serve both protective and toxic roles. These findings may be extrapolated to WD patients with higher copper serum concentration and shed some light on one of the underlying mechanisms that worsen the manifestations of WD patients with neurological symptoms.
CONTENTS

ACKNOWLEDGMENTS ....................................................................................... v

ABSTRACT ........................................................................................................... vi

LIST OF ILLUSTRATIONS .............................................................................. xi

LIST OF TABLES ................................................................................................. xii

LIST OF ABBREVIATIONS ............................................................................... xiii

Chapter

I. INTRODUCTION ......................................................................................... 1

A. Copper, an essential trace element ........................................................... 1

B. Copper in the human brain ......................................................................... 3

1. The human brain ....................................................................................... 4
   a. Anatomy ................................................................................................. 4
   b. Cell composition .................................................................................... 6

2. Distribution of copper in the CNS ............................................................. 8

3. Copper in human neurodegenerative diseases ......................................... 9

C. Copper homeostasis in the CNS ................................................................. 11

1. Transport of copper across the CNS ......................................................... 11

2. Astrocyte-neuron coupling in copper metabolism .................................. 13

3. Intracellular metabolism of copper ......................................................... 13

D. Copper transporting P-type ATPases ......................................................... 16

1. General structure of Cu-ATPases .............................................................. 16

2. Intracellular trafficking of Cu-ATPases .................................................. 18

3. Expression pattern of Cu-ATPases in the CNS ....................................... 20

4. ATP7A ....................................................................................................... 20

5. ATP7B ....................................................................................................... 23
E. Wilson disease .................................................................................................................. 24

1. Diagnosis .......................................................................................................................... 25
2. Treatment .......................................................................................................................... 27

F. Ceruloplasmin .................................................................................................................... 29

1. Iron homeostasis in the brain ......................................................................................... 32
2. Aceruloplasminemia ......................................................................................................... 33

G. Preliminary data and objectives of study ........................................................................ 34

II. MATERIALS AND METHODS .......................................................................................... 36

A. Materials .......................................................................................................................... 36

1. Cell Lines .......................................................................................................................... 36
2. Disposable Lab Ware ...................................................................................................... 36
3. Cell Culture Reagents ..................................................................................................... 36
4. In Vitro Kits ..................................................................................................................... 37
5. Chemicals and Reagents ............................................................................................... 37
6. Antibodies ........................................................................................................................ 38
7. Buffer Preparations ......................................................................................................... 38
8. Equipments ..................................................................................................................... 40

B. Methods ........................................................................................................................... 41

1. Cell Culture ..................................................................................................................... 41
2. Viability Assays ................................................................................................................ 41
3. Intracellular ROS level determination .......................................................................... 42
4. LDH release Assay ........................................................................................................... 43
5. Immunofluorescent detection of Mitochondrial Depolarization .................................... 44
6. Cell Cycle Analysis ........................................................................................................ 44
7. Intracellular GSH level determination ........................................................................... 46
8. Western Blot Analysis ...................................................................................................... 47
9. Statistical Analysis ......................................................................................................... 48

III. RESULTS ......................................................................................................................... 49

A. Effect of CuSO$_4$ and/or PA on viability of PC12 and U251 ........................................... 49
B. Effect of CuSO$_4$ and/or PA on ROS generation in PC12 and U251 .............................. 52
C. Effect of CuSO$_4$ and/or PA on membrane integrity of PC12 and U251 ...................... 53
D. Depolarization of Mitochondrial membrane by Cu and/or PA ..................................... 55
E. Cell cycle analysis of Cu and/or PA treated PC12 and U251 cells .............................. 57
F. Effect of CuSO₄ and/or PA on intracellular GSH level............................................ 59
G. Effect of Cu and/or PA on α-Fodrin in U251 cells...................................................... 60
H. Effect of Cu and/or PA on expression of transferrin, ferritin & ceruloplasmin..60
I. Effect of PA-Cu on PC12 cells grown in the presence of NGF ................................. 64

IV. DISCUSSION .................................................................................................................. 65

BIBLIOGRAPHY .................................................................................................................. 79
ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Copper distribution in the human body</td>
<td>3</td>
</tr>
<tr>
<td>2.</td>
<td>Gross anatomy of the human brain</td>
<td>6</td>
</tr>
<tr>
<td>3.</td>
<td>Uptake and efflux of copper at brain barrier</td>
<td>12</td>
</tr>
<tr>
<td>4.</td>
<td>Cellular copper homeostasis</td>
<td>16</td>
</tr>
<tr>
<td>5.</td>
<td>Topology and organization of copper transporting ATPase</td>
<td>17</td>
</tr>
<tr>
<td>6.</td>
<td>Model of NMDA receptor mediated copper homeostasis</td>
<td>19</td>
</tr>
<tr>
<td>7.</td>
<td>Pathogenesis of Wilson disease</td>
<td>24</td>
</tr>
<tr>
<td>8.</td>
<td>Structure of D-Penicillamine</td>
<td>29</td>
</tr>
<tr>
<td>9.</td>
<td>Structure of ceruloplasmin</td>
<td>30</td>
</tr>
<tr>
<td>10.</td>
<td>Iron metabolism in the CNS</td>
<td>32</td>
</tr>
<tr>
<td>11.</td>
<td>Brain iron cycle</td>
<td>33</td>
</tr>
<tr>
<td>12.</td>
<td>Copper-catalyzed Penicillamine oxidation</td>
<td>70</td>
</tr>
<tr>
<td>13.</td>
<td>Overview of Cu-PA induced toxicity on U251 cells</td>
<td>77</td>
</tr>
</tbody>
</table>
## TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Copper dependent enzymes</td>
<td>2</td>
</tr>
<tr>
<td>2. Neurological disorders involving copper</td>
<td>10</td>
</tr>
<tr>
<td>3. Routine tests for diagnosis of Wilson disease</td>
<td>27</td>
</tr>
</tbody>
</table>
**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’ Triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BCB</td>
<td>Blood-cerebrospinal fluid barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CCO</td>
<td>Cytochrome-c oxidase</td>
</tr>
<tr>
<td>CCS</td>
<td>Copper chaperone for superoxide dismutase 1</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>Cp</td>
<td>Ceruloplasmin</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CTR1</td>
<td>Copper uptake transporter 1</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>Copper sulfate</td>
</tr>
<tr>
<td>DBH</td>
<td>Dopamine-β-hydroxylase</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemi Luminescence</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>GAPH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>Hepes</td>
<td>4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid</td>
</tr>
<tr>
<td>HepG2</td>
<td>Human Liver cancer cell line</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>JC-1</td>
<td>5, 5', 6, 6' – tetrachloro – 1, 1', 3, 3' – tetraethyl – benzamidazolocarbon cyanin iodide</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>KF</td>
<td>Kayser-Fleisher</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LTP</td>
<td>Long term potentiation</td>
</tr>
<tr>
<td>MD</td>
<td>Menkes Disease</td>
</tr>
<tr>
<td>MitoPT</td>
<td>Mitochondrial Permeability Transition</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4, 5-dimethylthiazol-2, 5-diphenylTetrazolium bromide]</td>
</tr>
<tr>
<td>MT</td>
<td>Metallothionein</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide phosphate</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium chloride</td>
</tr>
<tr>
<td>NGF</td>
<td>Neural Growth Factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>OHS</td>
<td>Occipital Horn Syndrome</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>PA</td>
<td>Penicillamine</td>
</tr>
<tr>
<td>PAM</td>
<td>Peptidyl-α-amidating monooxygenase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PC12</td>
<td>Rat pheochromocytoma</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PS</td>
<td>Penicillin-Streptomycin</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SHT</td>
<td>Sucrose Hepes Tris-Base buffer</td>
</tr>
<tr>
<td>SOD1</td>
<td>Cu-Zn dependent superoxide dismutase</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N’,N’-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans Golgi Network</td>
</tr>
<tr>
<td>Tween</td>
<td>Polyxyethylene-20-Sorbitan Monlaurate</td>
</tr>
<tr>
<td>U251</td>
<td>Human glioblastoma astrocytoma</td>
</tr>
<tr>
<td>WD</td>
<td>Wilson Disease</td>
</tr>
<tr>
<td>Abs</td>
<td>Absorbance</td>
</tr>
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</table>
To the memory of my beloved father,
Sarkis Guiragossian (1950-2015)
CHAPTER I
INTRODUCTION

A. Copper, an essential trace element

Trace elements are dietary nutrients that are required in very minute quantities by the human body to maintain proper biological functioning (Fraga 2005). The human body has a complex system for managing and regulating the in vivo amount of these essential elements in biological systems. Microelements absorbed from diet are transported into blood, incorporated into cells, or excreted depending on whether blood and cell levels are adequate or overloaded. Improper function of the system leads to abnormal level of trace elements leading to toxicity (Osredkar and Sustar 2011).

Copper (Cu) is an essential trace metal found in all living organisms in either form: oxidized cupric Cu(II) and reduced cuprous Cu(I) states (Gaetke and Chow 2003). It is present in every tissue of the body, but is mainly stored in the liver; while fewer amounts are found in the brain, heart, kidney, and muscles. This trace element serves an important role in biological processes such as respiration, iron transport, oxidative stress protection, peptide hormone production, pigmentation, blood clotting and normal cell growth and development (Puig et al 2002). Cu, as a cofactor, is essential for the catalytic and structural properties of several important enzymes (Table 1) such as cytochrome-c oxidase (respiration), hephaestin and ceruloplasmin (iron homeostasis), tyrosinase (melanin formation), dopamine-β-monooxygenase (norepinephrine synthesis), lysyl oxidase (collagen and elastin cross-linking), Cu-zinc dependent superoxide dismutase (oxygen-radical scavenging), and peptidyl-α-monooxygenase
(pituitary peptide hormone maturation) (Uauy, Olivares, and Gonzalez 1998). On the other hand, high concentration of copper has deleterious effect; it may damage the lipids, proteins and DNA of the cells through its unpaired electrons in redox reactions that result in the generation of hydroxyl radical (Halliwell and Gutteridge 1984; Fraga 2005; Osredkar and Sustar 2011).

The human body requires around 1 to 2 mg of copper per day (Sian et al 1999). The main dietary sources of copper are sunflower seeds, nuts, sea food, beef and organ meat (especially liver), green olives, avocados, chocolate, and black pepper (Osredkar and Sustar 2011). Dietary copper, exists in the oxidized form, enters the body through the intestinal mucosa and reaches the liver via the portal blood bound to albumin; transcuprein; or histidine. In the liver, the newly absorbed copper is incorporated into endogenous copper enzymes and copper-requiring proteins. In particular, most of it is taken up by ceruloplasmin, released into blood and delivered to different tissues (Turnlund 1998). Excess copper is excreted through bile to maintain copper homeostasis (Figure 1) (Linder et al 1998). It is important to note that the excretion of endogenous

\[
\begin{array}{|c|c|c|}
\hline
\text{Enzyme} & \text{Function} & \text{Consequences of Defective Function} \\
\hline
\text{Tyrosinase} & \text{Melanin formation} & \text{Albinism} \\
\text{Lysyl oxidase} & \text{Collagen and elastin cross link formation} & \text{Perinatal death: arterial aneurysms, diaphragmatic rupture} \\
\text{Peptidylglycine α-amidating mono-oxygenase} & \text{Activation of peptides with α terminal glycine} & \text{Embryonic lethality: cardiac failure (normal CNS)} \\
\text{Cu/Zn superoxide dismutase} & \text{Antioxidant defense (superoxide dismutation)} & \text{Impaired pulmonary defenses to paraquat; infertility} \\
\text{Ceruloplasmin} & \text{Ferrooxidase} & \text{Parenchymal iron overload, anemia diabetes, neurodegeneration} \\
\text{Hephaestin} & \text{Ferrooxidase} & \text{Impaired iron absorption, anemia} \\
\text{Dopamine β hydroyxylase} & \text{Norepinephrine synthesis} & \text{Impaired sympathetic regulation, hypotension, hypoglycemia} \\
\text{Cytochrome c oxidase} & \text{Oxidative phosphorylation} & \text{Encephalopathy, muscle weakness, cardiac failure—neonate} \\
\hline
\end{array}
\]

**Table 1.** Copper-dependent enzymes (Madsen and Gitlin 2007)
copper is strongly influenced by dietary copper intake: the higher the amount of the dietary copper, the higher the endogenous copper excretion (Turnlund et al 1989).

**B. Copper in the human brain**

The brain is the organ with the second highest copper content (8.8 mg), following the liver (10 mg) (Linder et al 1998). This trace element is essential for the normal development and function of the Central Nervous System (CNS). It contributes to the activity of cuproenzymes responsible for general as well as specialized cellular functions. Of the general processes are iron metabolism by ceruloplasmin (Cp); protection against reactive oxygen species (ROS) through Cu-Zn dependent superoxide dismutase (SOD1); cellular respiration by cytochrome-c oxidase; structural organization

---

**Figure 1.** Copper distribution in the human body (Lutsenko et al 2007)
and correct development of the tissue via lysyl oxidase. As for the more specialized actions, it is worth to list the catecholamine biosynthesis and the activation of neuroendocrine peptides and hormones (oxytocin, vasopressin, gastrin, corticotrophin-releasing factor, thyrotropin-releasing hormone) via two homologous cuproenzymes, dopamine-β-hydroxylase (DBH) and peptidyl-α-amidating monooxygenase (PAM), respectively (Lutsenko, Bhattacharjee and Hubbard 2010).

Being required in many physiological processes, copper deprivation during development would result in marked developmental defects and embryonic death. In adults, copper deficiency leads to myeloneuropathy, anemia, motor neuron disease, demyelination and microcavitation of the neuropil in the white matter of the spinal cord and brainstem as well as loss of neurons in the granular layer of the cerebellum, cortical atrophy, and extensive degeneration of gray matter. On the other hand, copper overload results in atrophy and spongy degeneration in several regions of the brain including the thalamus, subthalamic nuclei, brainstem, and frontal cortex, due to oxidative stress (Lutsenko, Bhattacharjee and Hubbard 2010). The CNS is particularly sensitive to oxidative damage because the free-radical species initiate peroxidation of the lipids that comprise neuronal cell membranes perturbing their functions (Ozcelik and Uzun 2009).

1. **The human brain**

a. **Anatomy**

The brain is composed of the cerebrum, brainstem, and cerebellum and is classified into 6 postembryonic divisions: telencephalon, diencephalon, mesencephalon, pons, medulla, and cerebellum (Figure 2) (Fix 2008).
Telencephalon consists of the cerebral hemispheres in addition to many subcortical structures such as the subcortical nuclei basal ganglia essential for motor control, hippocampus imperative for long term memory formation, and olfactory bulb responsible for sense of smell (Greenstein 2000). The cerebral hemispheres are the largest part of the brain. They are separated by the longitudinal cerebral fissure. At the base of this groove lies a thick bundle of nerve fibres, called the corpus callosum, which interconnects the hemispheres and acts as a relay center between them. The cerebral cortex is a thin layer of gray matter that covers the surface of the two hemispheres and consists of 4 lobes (frontal lobe, parietal lobe, temporal lobe, occipital lobe) (Fix 2008).

Diencephalon is located between the telencephalon and mesencephalon and functions as a relay system between sensory input neurons and other parts of the brain. It consists of epithalamus, thalamus, hypothalamus and subthalamus, and forms the central core of the brain (Moore, Agur and Dalley 2015).

Mesencephalon (midbrain) is the rostral part of the brainstem and lies between the diencephalon and pons (Moore, Agur and Dalley 2015). An important structure located in this part is the substantia nigra that produces the neurotransmitter dopamine (Nissbrandt et al 1989).

Pons is the part of the brainstem located between the midbrain and medulla and includes the locus ceruleus which contains the largest number of norepinephrinergic neurons in the CNS (Fix 2008).

Medulla oblongata is located towards the caudal part of the brainstem and is continuous with the spinal cord. It houses several autonomic centers, such as the respiratory, cardiac, and vasomotor centers (Moore, Agur and Dalley 2015).
The Cerebellum lies posterior to the pons and medulla and inferior to the posterior part of the cerebrum, and coordinates voluntary movements such as posture, balance, and speech. It consists of two cerebellar hemispheres interlinked by a narrow middle part, the vermis (Moore, Agur and Dalley 2015).

**Figure 2.** Gross anatomy of the human brain (Donner 2002)

b. **Cell composition**

The nervous tissue of the brain consists of populations of nerve cells and neuroglia. There are 4 types of glial cells in the central nervous system: astrocytes, oligodendrocytes, microglial cells, and ependymal cells. These cells collectively outnumber the neurons by a ratio of 3 to 1 (Purves et al 2001).

Neurons are the main information processing units in the brain. They receive an input of information from other neurons or sensory receptors, process the received information and send an output signal as a response. They play a role in motor activity, sensory perception, behavior, and maintenance of homeostasis (Stufflebeam 2008).
Astrocytes surround nearly all synapses and ensure proper synaptic transmission by maintaining the extracellular fluid, ion, pH, and transmitter homeostasis in the synaptic cleft. Moreover, they act to regulate blood flow in the brain through the dilation and constriction of blood vessels. They surround capillary endothelial cells and contribute to the blood brain barrier (Sofroniew and Vinters 2010).

Oligodendrocytes are the Schwann cell equivalents in the CNS: they wrap axons with the lipid-rich myelin sheath, providing insulation. This increases the speed of propagation of action potentials and prevents short-circuiting of nerve impulses (Purves 2001).

Microglial cells can be derived from either hematopoietic or neural stem cells. They play the role of macrophages in the CNS and remove cellular debris. They are activated following neuronal and/or synaptic degeneration and mount an immune response (Gehrmann, Matsumoto, and Kreutzberg 1995).

Ependymal cells form an epithelial layering around the cerebrospinal fluid (CSF)-filled ventricles. They function as a barrier between the parenchyma and cerebrospinal fluid, and act a major role in cerebral fluid balance, toxin metabolism, and secretion. They also synthesize proteins that modulate neurogenesis and stem cell renewal (Genzen et al 2009).
2. **Distribution of copper in the CNS**

Warren et al investigated the distribution of copper in the different areas of the human brain and reported that the copper content in gray matter is 2 to 3 times higher than that of white matter. Particularly, the substantia nigra and the locus caeruleus of the gray matter were shown to have the highest copper content (Warren, Earl, and Thompson 1960). Copper is present in most regions of the brain and is most abundant in the basal ganglia. It is detected in the cell bodies of cortical pyramidal and cerebellar granular neurons, in the neuropil of the cerebral cortex, in the hippocampus and in the cerebellum (Madsen and Gitlin 2007).

Moreover, the axonal terminals and secretory granules were also shown to be highly enriched with copper consistent with the fact that dopamine-beta-hydroxylase and peptide alpha-amidating enzymes, present in the secretory apparatus of the neuronal tissue, are cuproenzymes and so require copper to function. At these sites, copper can thus be incorporated in these cuproenzymes and also released out by synaptic activity (Hartter and Barnea 1988).

Copper is released during neuronal activity rising the level from few millimolar to as high as few hundred micromolar in the synaptic cleft (Marchetti, Bosiacka and Gavazzo 2013). Previous studies have reported that exogenous Cu mediates inhibition of neurotransmitter-activated channels, including GABA and glutamate receptor channels, as well as voltage-gated ion channels. Cu is a well-known antagonist for N-methyl-D-aspartate (NMDA) and inhibits NMDA-dependent long term potentiation (LTP) in the hippocampus (Marchetti, Bosiacka and Gavazzo 2013). NMDA receptors are one of the main classes of ionotropic glutamate receptors in the CNS. They are activated by the major excitatory neurotransmitter of the brain, glutamate, or co-agonist glycine. This
leads to the opening of channels permeable to Na\(^+\) and Ca\(^{2+}\), which contribute to postsynaptic depolarization and intracellular physiological effects, respectively. These receptors are fundamental for the brain as they mediate synaptic strength and connectivity, important in the formation of memory and learning (Hardingham and Bading 2003; Stys, You and Zamponi 2012).

3. *Copper in human neurodegenerative diseases*

Recent approaches into the molecular physiology of copper metabolism have facilitated the understanding of the role of copper in normal brain development and function (Waggoner, Bartnikas and Gitlin 1999). Consequently, studies have investigated the influential role of redox-active copper in neurodegenerative disorders and evidence is accumulating to show that metallochemical reactions involving copper might have a crucial role in the progression of Alzheimer’s disease, prion diseases, amyotrophic lateral sclerosis, Parkinson’s disease, and Huntington disease (Table 2). In these disorders, copper, like iron, abnormally interact with proteins and induce oxidative stress through ROS generation (Bush 2000).
Table 2. Neurological disorders involving copper

<table>
<thead>
<tr>
<th>Disease</th>
<th>Cause</th>
<th>Phenotype</th>
<th>Copper Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer’s disease (AD)</td>
<td>Abnormal proteolytic cleavage of amyloid precursor protein</td>
<td>Progressive dementia, loss of memory and other cognitive abilities (Holtzman, John and Goate 2011)</td>
<td>APP possesses 2 copper binding domains, located in the N-terminal and C-terminal regions within the AD(Ceresa et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>accumulation of amyloid-β (Aβ) peptide</td>
<td></td>
<td>Copper is highly concentrated in AD plaques and are directly bound to the Aβ fragments of these deposits (Ceresa et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>formation of amyloid plaque in the brain (Ceresa et al. 2005).</td>
<td></td>
<td>Cu²⁺ ions modulate the oxidative stress mechanisms of Aβ toxicity (Barnham and Bush 2008)</td>
</tr>
<tr>
<td>Spongiform encephalopathies (prion diseases)</td>
<td>misfolding of soluble prion protein (PrPc)</td>
<td>Neuroanatomical formation and dementia, like AD, but more aggressive and contagious (Bush 2000)</td>
<td>PrPC, expressed in neurons and other cells, is a Cu-dependent SOD-like protein (Tapiero, Townsend, and Tew 2003)</td>
</tr>
<tr>
<td></td>
<td>accumulation of abnormal infectious insoluble PrPSc (Tapiero, Townsend, and Tew 2003)</td>
<td></td>
<td>Copper stimulates the endocytosis of this prion protein and facilitates the renaturation and stabilization of PrPSc molecules (Tapiero, Townsend, and Tew 2003)</td>
</tr>
<tr>
<td>Familial amyotrophic lateral sclerosis</td>
<td>an autosomal dominant mutation in the gene coding for Cu/Zn SOD (Wagner, Bartsch, and Giffin 1999)</td>
<td>Fatal neurodegeneration of the human motor system, muscle weakness and respiratory problems (Desai and Kaler 2008)</td>
<td>Structural alteration of mutant enzyme results in a more open Cu²⁺ containing active site, leading to oxidation of normally inaccessible biomolecules and an increase in hydroxyl radical production and lipid oxidation-derived radicals (Sayre, Perry, and Smith 1999)</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>misfolded and aggregated α-synuclein, responsible for the modulation of dopamine activity</td>
<td>Rest tremors, bradykinesia, rigidity, and postural instability, Degeneration of dopaminergic neurons in the substantia nigra of the midbrain (Jin et al. 2011)</td>
<td>Copper is decreased in the substantia nigra, whereas its concentration in cerebrospinal fluid is increased (Bush 2000)</td>
</tr>
<tr>
<td></td>
<td>Lewy bodies in dopaminergic neurons (Tavaresko et al. 2014)</td>
<td></td>
<td>α-synuclein can interact with many metals, including Cu(II), leading to protein aggregation and crosslinking (Barnham and Bush 2008)</td>
</tr>
<tr>
<td>Huntington disease</td>
<td>Triplet repeat expansion of the gene coding for huntingtin</td>
<td>Motor, psychiatric and cognitive symptoms due to oxidative stress, energetic insufficiency, and neuronal degeneration</td>
<td>Copper concentration in the brain was found to be elevated promoting oxidative stress and neurodegeneration via binding to low affinity sites in huntingtin protein and stimulating neurotoxic aggregation of this protein (Desai and Kaler 2006, Schepers, Morcos, and Dirnagl 2014).</td>
</tr>
</tbody>
</table>
C. Copper homeostasis in the CNS

The exchange of copper between the periphery and the brain is highly regulated by blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCB). The molecular mechanism by which copper is handled within the different cells of the brain is rudimentary (Telianidis et al 2013). Yet, all of the key copper handling proteins that participate in copper homeostasis of peripheral organs are also present in the brain, suggesting that the copper metabolism in the brain is similar to peripheral organs. These proteins can be sorted into 3 major classes: copper uptake transporters (CTR1) that transport copper into the cytosol; copper chaperones (CCS, Cox17, Cox11, Sco1, Sco2, Atox1) that mediate copper distribution to intracellular protein targets; and Cu-transporting ATPases (ATP7A and ATP7B) that transfer copper from cytosol to the lumen of secretory pathway and small endocytic vesicles for the delivery of copper to copper-dependent enzymes and export, respectively (Lutsenko, Bhattacharjee, and Hubbard 2010).

1. Transport of copper across the CNS

The cerebrospinal fluid, produced by the cells of choroid plexus, supports the central nervous system against injury, transports hormones and hormone-releasing factors, and removes metabolic waste products through absorption (Fix 2008). Although the cells of choroid plexus present in the BCB are highly enriched with copper, they tightly regulate the movement of copper into CSF, suggesting that the copper efflux from the BCB cells to CSF, rather than uptake from blood, is limiting copper delivery to brain parenchyma through CSF (Choi and Zheng 2009). However, BCB is responsible
for the export of copper from the CSF to the blood (Scheiber, Mercer, and Dringen 2014; Kuo et al 2006).

The brain endothelial cells, which comprise the BBB, take up copper from blood by the high affinity copper transporter 1 (CTR1) located at the apical side. This transporter specifically transports Cu(I) but not Cu(II) (Edward Harris 2000) and is uniformly expressed in all the brain cells but higher in the ependymal cells of the choroid plexus (Platonova et al 2005). It is essential for organism growth and development of the neural tissue, because cell death in the brain and spinal cord as well as impairment of neural tube and neuroepithelial layering were reported in response to defective CTR1 (Lee, Prohaska, and Theile 2001; Hopt et al 2003). Copper is released from the BBB into the brain parenchyma by the copper-transporter ATP7A and then transported in the cytosol of the parenchymal cells via CTR1 with the help of reductases. After copper is utilized in these cells, its excess is consequently released to CSF via the brain interstitial fluid. Copper in the CSF can then be taken up by epithelial cells of the choroid plexus of BCB through CTR1, to be stored for transport to CSF by ATP7B or exported into blood by ATP7A (Figure 3) (Scheiber, Mercer, and Dringen 2014; Kuo et al 2006).

![Figure 3. Uptake and efflux of copper at brain barriers (Scheiber, Mercer and Dringer2014)](image-url)
2. *Astrocyte-neuron coupling in copper metabolism*

Astrocytes, which contribute the main class of neuroglia, are distributed abundantly throughout the entire brain and perform many important functions, such as nervous system repair, modulation of synaptic transmission and physical structuring. Due to their highly antioxidative potential and capacity to accumulate extracellular copper, it has also been proposed that astrocytes control the metabolism of copper in the brain to provide metabolic stability for neurons. Astrocytes, being the first brain parenchyma cells to come across copper crossing the BBB, store copper as metallothioneins (MT) or glutathione (GSH) complexes; supplying thus the neurons as needed. In fact, *in vitro* studies have shown that astrocytes take up copper more efficiently than cultured neurons and protect the latter from copper toxicity. The protection of neurons from copper-induced toxicity also involves the release of antioxidants or copper-chelating substances from the astrocytes to prevent the copper-mediated inactivation or degradation of extracellular GSH (Scheiber, Mercer, and Dringen 2014).

3. *Intracellular metabolism of copper*

Cu(I) cannot exist as a free ion in the cytosol of the cells because it can participate in reactions whose products damage cell membranes, proteins and nucleic acids. Thus, once copper enters the cells, its distribution takes place with the help of copper chaperones (Penä, Lee, and Thiele 1999). The latter are small molecular weight proteins that bind and deliver copper ions to their final destination at intracellular compartments and/or incorporate copper into the active sites of specific copper-dependent enzymes (Markossian and Kurganov 2003).
Copper Chaperone for Superoxide dismutase 1 (CCS) is the largest copper chaperone and has two major functions: formation of a crucial disulfide bond in its target protein SOD1 and copper delivery to the catalytic site of SOD1. It has three distinct domains: the N-terminal Domain I with a remarkable homology with chaperone Atox1 including the MxCxxC copper binding motif to maximize CCS function; the central Domain II is homologous to the target protein SOD1 to allow heterodimerization of CCS and SOD1 during copper transfer; and the C-terminal Domain III contains the highly conserved CxC motif to participate in both disulfide bond formation and copper transfer to the catalytic site of SOD1. The latter is a soluble zinc and copper-requiring enzyme that protects the cells against oxidative stress by scavenging toxic superoxide anion radicals through redox reactions at the bound copper ion (O’Halloran and Culotta 2000).

Cox17 is an essential cytosolic chaperone, as it delivers Cu to the mitochondria. Unlike CCS and Atox1, it does not contain the MxCxxC consensus motif, but rather tandem cysteine residues near the N terminus as Cu binding sites. This chaperone binds to Cu as a binuclear cluster on two consecutive cysteine residues (Harris 2000). Copper delivery to the mitochondria is fundamental because cytochrome-c oxidase (CCO), the terminal enzyme of the electron transport chain (ETC), is copper dependent.

Cox11, Sco1 and Sco2 are three mitochondrial membrane bound chaperones that have a crucial role in CCO assembly: Cox 11 heterodimerizes with Cox17 and mediates copper transfer to CCO (Ferguson-Miller and Babcock 1996), whereas Sco1 and Sco2 do not function as copper carriers but rather as redox signaling molecules. The
functional CCO, in its turn, catalyzes the transfer of electron to molecular oxygen and plays a role in the synthesis of ATP through its contribution in the formation of the electrochemical potential (Lutsenko, Bhattacharjee and Hubbard 2010).

**Atox1** targets copper to intracellular copper-transporting P-type ATPases, ATP7A or ATP7B, located in the Golgi compartment of the secretory pathway (Schlief and Gitlin 2006). Copper binds to this metallochaperone within the surface exposed loop that contains the MxCxxC conserved motif. This binding is coordinated by two cysteine residues to stabilize the protein structure (Robinson and Winge 2010). Then, Cu-Atox1 complex physically interacts with the P-type ATPases through the Atox1-like MxCxxC motif. Also, an electrostatic interaction seems to occur between the negatively charged residues of the copper transporters and the positively charged residues of Atox1. The docking of the Atox1 to its target protein leads to the replacement of the coordinated cysteines of Atox1 with cysteine residues of the copper transporter. Finally, these Cu-transporting ATPases, present in the membranes of the Trans Golgi network and endocytic vesicles, translocate copper from the cytosol to the lumen of the Golgi and small vesicles for the delivery of copper to newly synthesized cuproenzymes (lysyl oxidase for ATP7A, and Ceruloplasmin for ATP7B) and export them into extracellular fluids, respectively (Suzuki and Gitlin 1999).

As for the excess copper, **metallothionein**, which is a cystein-rich cytoplasmic protein, chelates it and protects the cells from toxicity (Kelly and Palmiter 1996). In addition, low molecular mass **glutathione**, important for detoxification of peroxides and ROS, forms stable complexes with Cu(I) and transfers copper to MT and copper chaperones or sequesters it to further protect the cells from oxidative damage (Figure 4) (Scheiber, Mercer, and Dringen 2014).
D. Copper transporting P-type ATPases

The P-type ATPase functions in ATP-dependent transport of specific ions and molecules across cell membranes and is characterized by the formation of a covalently bound aspartate phosphorylated intermediate from ATP during the transport cycle (Moller, Juul and le Maire 1995). The copper transporting P-type ATPases are crucial for the regulation and maintenance of copper homeostasis (La Fontaine and Mercer 2007). In addition, these two ATPases play an essential role in the biosynthesis of cuproenzymes such as tyrosinase, lysyl oxidase, peptidyl-α-monooxygenase and ceruloplasmin (Linz and Lutsenko. 2007).

1. General structure of Cu-ATPases

ATP7A and ATP7B have a high similarity in amino acid structure (60%) and function. Their general structure consists of eight transmembrane segments that form a
passage through cell membranes for copper transport. The highly conserved CPC motif in the 6th transmembrane is required for copper translocation through the membrane. The N-terminus is made of six metal binding domains (MBDs) with each of these copper binding domains with a highly conserved metal-binding motif GMxCxxC. Other highly conserved domains include the N (nucleotide binding)-domain containing the ATP-binding site; P (phosphorylation)-domain with the conserved aspartic acid residue; and the A-domain containing the catalytic phosphatase domain. As for the COOH-terminus, it is 80-100 residues long, and contains conserved dileucine (in ATP7A) or trileucine (in ATP7B) motifs, which are required for the trafficking of these transporters (Figure 5). Copper-binding, together with other N- and C-terminal signals, regulate the activity, the intracellular location as well as the copper-induced intracellular trafficking of these Cu-ATPases (Lutsenko et al 2007).

**Figure 5.** Topology and organization of copper transporting ATPases (Lutsenko et al 2007)
2. **Intracellular trafficking of Cu-ATPases**

There is no functional complementation between ATP7A and ATP7B, probably due to their differences in regulation and trafficking properties (Niciu et al 2007). In the presence of copper, ATP7B translocates from Trans Golgi network (TGN) towards the apical membrane and accumulates in the cytoplasmic vesicles, and returns to TGN when copper is exported (Hung et al 1997). As for ATP7A, a similar copper-dependent trafficking event takes place, except the movement is toward the basolateral membrane and sub-basolateral vesicles, although most of the proteins remain intracellular (Petris et al 1996).

In addition to copper-dependent trafficking, ATP7A in hippocampal neurons also undergoes copper-independent trafficking through NMDA receptor activation (La fontaine and Mercer 2007). Ca\(^{2+}\) entry as a consequence of synaptic glutamate NMDA receptor excitation activates a calcium dependent biochemical cascade leading to synaptic modifications as well as to the rapid trafficking of ATP7A both to the somato-dendritic and axonal compartments of cultured hippocampal neurons, independent of the intracellular copper concentration. ATP7A in its turn accumulates copper into membrane bound compartments forming a pool of releasable copper. NMDA activation also results in the release of the stored copper which act endogenously as an antagonist for the NMDA receptors limiting thus Ca\(^{2+}\) entry into the cell preventing excitotoxic damage, in a manner similar to the exogenous copper (Schlief, Craig and Gitlin 2005). The modulation of the NMDA receptor takes place through copper acting as a redox active ion in processes that involve disulphide bond formation and S-nitrosylation of cysteine residues within NMDA subunits (Figure 6) (Schlief et al 2006).
When ATP7A is mutated, as in Menkes disease (discussed below), Cu cannot be stored in the compartments. The loss of the releasable copper leads to the decrease in the ability of the neuron to modulate NMDA receptor activation. Consequently, Ca\textsuperscript{2+} entry increases in these neurons and this results in excitotoxicity, explaining the seizures and neurodegeneration in these patients (Schlief and Gitlin 2006). As for Wilson disease (also discussed below), the copper level in the brain is so high that it affects the LTP and consequently the learning ability and development. This is consistent with the fact that the pathology is mainly found in the basal ganglia where NMDA receptors and LTP are prominent (Doreulee, Yanovsky, and Haas 1997).

Figure 6. Model of NMDA receptor-mediated copper homeostasis (Schlief and Gitlin 2006)
3. **Expression pattern of Cu-ATPases in the CNS**

ATP7A and ATP7B are vital for the CNS physiology, as their dysfunction results in copper deficiency (Menkes disease or Occipital horn syndrome) and overload (Wilson disease) respectively, manifesting neuronal degeneration, demyelination of neurons, impairment of synaptogenesis as well as severe neurological, developmental and psychiatric problems (Lutsenko et al 2007).

The two ATPases have a different expression pattern in the CNS: ATP7A is expressed all through the brain in both embryonic and postnatal stages. In early postnatal period, ATP7A is mainly expressed in neocortex and cerebellum, whereas in the developing and adult brain, its expression is highest in the choroid plexus/ependymal cells of the lateral and third ventricles. The expression level of this ATPase decreases in the neuronal subpopulation from birth to adulthood, except for the CA2 hippocampal pyramidal layer where the level increases (Lutsenko, Bhattacharjee and Hubbard 2010).

As for ATP7B, the expression is highest in the postnatal cerebellum, but also remarkable in the neuronal cells of CA1-CA4 layers of the hippocampus, in the glomerular cell layer of the olfactory bulbs, in the cerebral cortex, and nuclei of the brainstem (Lutsenko et al 2007).

4. **ATP7A**

ATP7A protein is coded by a gene located on chromosome Xq13.2-13.3 (Telianidis et al 2013). This 175 KDa Cu-ATPase is responsible for copper absorption and transport: Highly expressed in the duodenum, it exports copper from the enterocytes into circulation for delivery to the other organs of the body (Nyasae et al 2007). It is also expressed in many other tissues such as the brain, muscle, kidney, lung, placenta.
and pancreas, and thus participates in the transport of copper across these organs. Unlike ATP7B, this ATPase is not expressed in the liver (Harris et al 1999).

ATP7A is also crucial for copper incorporation in many copper-dependent proteins, specifically lysyl oxidase. The latter is a cuproenzyme that plays a crucial role in the formation, maturation, and stabilization of connective tissue as it catalyzes the oxidation of the side chain of lysine, the first step of cross-linking of important components of connective tissue, collagen and elastin (Tapiero, Townsend and Tew 2003). In addition to this peripheral function, it also plays a role in the CNS. Present in the choroid plexus, blood vessel walls, brain matrix and neurons, it plays an essential role in the formation and remodeling of the extracellular matrix during development, as well as in the maintenance of normal structural organization of tissue (Li et al 2004; Bronson et al 2005).

Consequently, defects in ATP7A gene leads to an increase in copper concentration in the intestine and poor copper supply to other tissues and incorporation in cuproenzymes due to diminished copper export, with brain and connective tissues being mostly affected. The two Cu deficiency disorders caused by ATP7A inactivation are the Menkes disease (MD) and the less deadly Occipital Horn Syndrome (OHS).

**Menkes disease** is an X-linked fatal childhood disease, characterized by failure of Cu transport from intestinal enterocytes to blood, after absorption from diet. This leads to Cu deficiency in peripheral organs and tissues and a severe impairment of physiological functions dependent on cuproenzymes. Patients with this metabolic disorder die within 3 years of birth (Wadwa et al 2014). The phenotypic manifestations of MD include growth failure, brittle hair, hypopigmentation, arterial tortuosity, and neuronal degeneration. The latter is marked by deficient myelination with cerebellar and
cerebral atrophy, focal degeneration of the gray matter and neuronal loss in the hippocampus and cerebellum, and appears in early childhood, revealing the critical role of ATP7A and copper in neuronal development (Madsen and Gitlin 2007). Copper replacement therapy is helpful for individuals affected with this disorder, if as little as 5-10% of ATP7A activity is intact, since this Cu-transporting ATPase is crucial for the copper entry to the brain by crossing intracellular barriers. Current studies are oriented to gene therapy restoring ATP7A function in these patients (Kaler 2011).

**Occipital horn syndrome** is a neurologically milder allelic variant of Menkes disease, because of its splice junction mutations that allow 20-30% of ATP7A transcription to be correctly processed. It is characterized by calcifications within the attachment point of muscle tendons and occiput in affected individuals by the second decade of life (Tang et al 2006). OHS, like MD, present with hair and connective tissue abnormalities as a result of lysyl oxidase deficiency (Gacheru et al 1993). However, the neurological manifestations in patients with OHS are milder than MD with symptoms such as generalized muscle weakness and dysautonomia. The biochemical features are also similar between the MD and OHS: Low serum copper and ceruloplasmin, and abnormal plasma and CSF neurochemical levels due to DBH deficiency are detected in individuals affected with both ATP7A disorders. OHS patients can also benefit from copper replacement therapy, if this treatment enhances the metallation of copper-requiring lysyl oxidase (Kaler 2011). Currently, treatment with L-threo-3,4-dihydroxyphenylserine, a compound that is converted to norepinephrine in a copper-independent manner, seems promising to manage the dysautonomic symptoms of these patients (Goldstein 2006).
5. **ATP7B**

The gene coding for ATP7B is located on chromosome 13q14.3. This membrane protein is crucial for copper transport and excretion: In the liver, it incorporates copper into apoceruloplasmin following which it is secreted from the hepatocytes into the plasma to deliver copper to other organs. It also facilitates the excretion of excess copper through its transport to the bile (Bull et al 1993). ATP7B is highly expressed in the liver, kidney, and in lower extent in the placenta, brain, heart, lung, muscle, and pancreas (Harada 2002).

In the human hepatic cells, ATP7B is present in 2 isoforms: a full length 160 KDa form that is targeted to the Trans Golgi network; and a modified 140 KDa form that is localized in the mitochondria. The modified ATP7B is likely to be formed after proteolytic cleavage of the ATP7B at the N-terminus. The unique subcellular localization of this Cu-ATPase points at the importance of copper-dependent processes taking place in this organelle. Indeed, a number of key enzymes of the mitochondria require copper as cofactor in order to function (Lutsenko and Cooper 1998).

Mutations in ATP7B gene leads to impaired function of the copper transporter Cu-ATPase, resulting in reduction of Cu excretion in the bile and impaired Cu incorporation into the serum copper protein ceruloplasmin (Figure 7). Because ATP7B is highly expressed in the liver, the outcome of this Cu transport defect would be the deposition of copper primarily in this organ. Copper leakage in the blood also leads to Cu accumulation in the brain and other organs (Cox and Moore 2002). This copper metabolism disorder is known as Wilson disease (WD).
E. Wilson disease

Wilson disease is an inherited autosomal recessive disorder resulting in hepatic cirrhosis and/or progressive basal ganglia degeneration (Madsen and Gitlin 2007) and its incidence rate is estimated to be 1 in 30,000 (Terada et al 1998). The age of onset as well as the clinical presentations in WD patients is highly variable (Ferenci et al 2007): Even monozygotic twins can manifest completely different symptoms and ages of onset (Czlonkowska, Gromadzka and Chabik 2009). The first type of manifestation of the disease occurs in the liver at an early age (10 to 13 years old) with symptoms ranging from asymptomatic transaminasemia, recurrent jaundice, acute or chronic hepatitis, fulminant hepatic failure, and cirrhosis (Ferenci et al 2003). Contour irregularity, multiple nodular lesions and presence of peri-hepatic fat layer and normal caudate lobe, contrary to other types of cirrhosis, are observed (Akhan et al 2007). The second is the neurological manifestation commonly occurring at a later age than cases of purely hepatic diseases (20 to 30 years old) with features such as rigidity, clumsiness, mild
tremors, speaking and writing problems as well as dystonia, dysarthria, Parkinson-like symptoms and psychological abnormalities (Oder et al 1991). Occasionally, a patient may have a mixed manifestation and present both hepatic and neurological symptoms.

The main areas of the brain affected in WD patients are the lenticular nuclei which appear brown under the microscope due to Cu deposition. As the disease progresses, the brain degenerates with necrosis, gliosis and cystic changes, as well as lesions at the level of brainstem, thalamus, cerebellum, and cerebral cortex. Proliferation of large protoplasmic astrocytes is also remarkable (Das and Ray 2006). Copper overload in the brain of WD patients is due to its accumulation in the plasma following liver injury and not defective ATP7B of the brain, because the neurological manifestations are entirely reversed following liver transplantation (Emre et al 2001, Schumacher et al 2001). The reversible nature of these symptoms is consistent with the concept that copper modulates synaptic function (Schlief and Gitlin 2006).

1. Diagnosis

Diagnosis of Wilson disease is based on a combination of clinical symptoms (described above), laboratory tests (table 3), and genetic screening of ATP7B gene.

The decrease in serum ceruloplasmin concentration (<20 mg/dl) is a diagnostic hallmark of this disorder since lack of functional ATP7B limits the copper incorporation into ceruloplasmin, resulting in the secretion of rapidly degradable enzymatically inactive apoceruloplasmin. However, since ceruloplasmin is an acute-phase protein, its serum concentration increases during inflammation, infection and trauma. Thus, the concentration of this protein may appear normal in WD patients manifesting active liver disease, leading to diagnostic difficulty (Gitlin 2003). In fact, a normal serum
ceruloplasmnin concentration is found in at least 5% of WD patients with neurological manifestations and in up to 40% if WD patients with hepatic symptoms, making the serum ceruloplasmnin concentration an unreliable screening test for WD (Steidl et al. 1997).

Investigation of tissue copper levels is another diagnostic tool given that copper content of cornea, brain, and liver are significantly increased in WD patients.

Kayser-Fleisher (KF) corneal ring, secondary to the deposit of copper in Descemet’s membrane of the cornea, is an important physical sign detected by slit-lamp examination. It is found in about 50% of WD patients with hepatic manifestation but almost always in patients with neurological or psychiatric symptoms (Steindl et al 1997).

Changes in the CNS due to copper increase in the brain of patients with neurological manifestations can be detected by neuroimaging, preferably magnetic resonance imaging (Machado et al 2006).

Measurement of hepatic copper content on a liver biopsy may be performed by mass spectroscopy or atomic absorption spectroscopy to check for an increase in hepatic copper (Ferenci et al 2003). Normal adults typically have less than 50 µg of copper per gram of dry liver, whereas in WD patients, it exceeds the 250 µg/g dry weight (Forbes and Cox 2000).

Urinary copper excretion greater than 100 µg per day also reflects excess of copper in the body stores. However, hypercupriuria is not specific for Wilson disease and can also be observed due to other causes (Packman 2003).
The best diagnostic tool remains the molecular genetic screening of the ATP7B. More than 500 mutations have been so far reported, the majority of which are compound heterozygous. The most common mutation in European populations is an amino acid substitution in a highly conserved motif close to the ATP-binding region (His1069Gln) with a frequency of 26-70% in various populations. As for the Asian population, the most common mutation is an amino acid substitution in exon 8 (Arg778Leu) (Weiss 2013).

<table>
<thead>
<tr>
<th>Test</th>
<th>Typical finding</th>
<th>False “negative”</th>
<th>False “positive”</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum ceruloplasmin</td>
<td>Decreased</td>
<td>Normal levels in patients with marked hepatic inflammation</td>
<td>Low levels in hepatic inflammation, malabsorption, aceruloplasminemia, liver insufficiency, heterozygotes</td>
</tr>
<tr>
<td>24-h urinary copper</td>
<td>&gt;100 µg/d</td>
<td>Normal; incorrect collection of urine</td>
<td>Increased; hepatocellular necrosis, contamination</td>
</tr>
<tr>
<td>Serum “free” copper</td>
<td>&gt;10 µg/dL</td>
<td>Normal if ceruloplasmin overestimated by immunologic assay</td>
<td>N/A</td>
</tr>
<tr>
<td>Hepatic copper</td>
<td>&gt;250 µg/g dry weight</td>
<td>Due to regional variation in patients with active liver disease, regenerative nodules</td>
<td>Cholestatic syndromes</td>
</tr>
<tr>
<td>Kayser-Fleischer rings</td>
<td>Present</td>
<td>In up to 40% of patients with hepatic Wilson disease, in most asymptomatic siblings</td>
<td>Primary biliary cirrhosis</td>
</tr>
</tbody>
</table>

Table 3. Routine tests for Diagnosis of Wilson disease (Ferenci et al 2003)

2. Treatment

The therapeutic objective in WD patients is to restore the normal copper homeostasis. This is performed on two levels: reduction of dietary copper intake and its absorption, and promotion of copper excretion (Gilroy et al 2014).

D-Penicillamine (PA), marketed as Cuprimine (250 mg capsules) or Depen (250 mg tablets), is the drug of choice to treat Wilson Disease patients. Its enantiomeric isoform, L-Penicillamine is highly toxic. D-PA (Figure 8) is also used to treat heavy metal poisoning, cysteinuria, rheumatoid arthritis, primary biliary cirrhosis, and progressive systemic sclerosis (Naik et al 2013). It was 1st identified by Abraham et al
as a product of penicillin hydrolysis (Stephenson and Roberson 1960). This copper chelating agent is well absorbed from the gastrointestinal tract (Caillie-Bertrand et al 1985) and binds to the excess copper with its sulphydryl and amino groups forming a non-toxic ring complex (Walshe 2009). In this manner, it mobilizes the intracellular copper into circulation to be later excreted in urine (McArdle et al 1990). This drug, like any other treatment, has mild side effects such as loss of taste, headache, or abdominal pain (Czlonkowska, Gajda, and Rodo 1996), but can also cause more serious problems like hypersensitivity, pyridoxine deficiency, suppression of bone marrow, skin toxicity, and autoimmune diseases (Scheinberg et al 1987).

Penicillamine has been reported to result in deterioration in up to 50% of WD patients with neurologic symptoms at the early stage of administration and half of these patients do not recover even after discontinuation of the drug (Kalita et al 2014). The reasons for the worsening of Wilson disease patients with neurological manifestations following Penicillamine treatment are still not fully understood.

Depletion of pyridoxine (Vitamin B) may be one of them, since PA acts as a pyridoxine antagonist (Packman 2003), by forming a thiazolidine with this vitamin (Walshe 2011). For this reason, pyridoxine is routinely added to the course of therapy in a daily dosage of 20-50 mg (Gibbs and Walshe 1966).

Another reason might be that PA mobilizes copper stores in the brain or liver and further elevates free copper (Brewer et al 2009). In fact, in untreated WD patients, the blood brain barrier was shown to be abnormally leaky, because the ratio of CSF albumin to serum albumin was abnormally high. However, when patients with neurological manifestations were administered with PA, a further increase in BBB leakiness and worsening of the neurological manifestations was observed. The leakiness
of the BBB might be from the elevation of the serum free copper due to the mobilization by PA (Stuerenburg 2000). Furthermore, in a recent study, increased free copper concentrations in the serum and brain, resulting in oxidative stress, as well as decreased protein-bound copper concentrations in the cortex and basal ganglia were found during D-PA administration in WD animal model toxic milk mice. Following PA administration, ATP7A expression was also reported to be increased in choroid plexus and decreased in the BBB, suggesting that the increased free copper in the brain is mobilized from the brain parenchyma but not from serum (Chen et al 2012).

![Figure 8. Structure of D-Penicillamine](image)

F. Ceruloplasmin

Ceruloplasmin is the main copper transporting protein in the blood as it carries 95% of plasma copper (Meyer et al 2001). It is initially synthesized in the liver as apoceruloplasmin. Copper loading into apoceruloplasmin occurs in the Trans Golgi network, giving rise to the active holoceruloplasmin. Cp in its active form is then secreted in the plasma to deliver copper to other organs (Terada et al 1998).

Cp contains three type I, a single type II, and two type III copper binding sites. Type I copper ions are arranged in 3 mononuclear sites in domains 2, 4 and 6 and are coordinated to a cysteine, and two histidine residues. A charge transfer between the sulfur of cysteine and copper at these sites takes place giving rise to the intense blue
color of the protein. Type II copper is coordinated by four imidazole nitrogens and is in close proximity to the two type III copper ions. The latter are in their turn coordinated to the single type II copper at the interface of domain 1 and 6 to form a trinuclear copper cluster to which oxygen binds during the catalytic cycle (Figure 9) (Hellman and Gitlin 2002; Bento et al 2006).

In addition to its role as a copper transport protein, ceruloplasmin plays a crucial role in iron homeostasis. Iron, like copper, is an essential trace element that plays an important role in many biological processes. However, free ferrous iron can also generate highly toxic hydroxyl and superoxide free radicals in the presence of hydrogen peroxide or molecular oxygen, leading to oxidative damage to proteins, lipids, and DNA (Patel et al 2002).

Cp belongs to the multicopper oxidase family of proteins which utilize copper to couple substrate oxidation with the reduction of oxygen to water (Bielli and Calabrese 2002). Thus, Cp acts as an antioxidant through its potent ferroxidase activity.
as it converts the toxic ferrous iron into relatively non-toxic ferric iron, and so protects the cells from oxidative stress (Attieh et al 1999). This facilitates the cellular iron homeostasis because the dietary and recycled iron are in the Fe\(^{2+}\) state but the iron transporter, transferrin (Tf), carries iron in serum only in the Fe\(^{3+}\) state (Eid et al 2014). Iron-bound transferrin interacts rapidly with transferrin receptor present on the plasma membrane and gets internalized by receptor-mediated endocytosis (Moos et al 2006).

Since ceruloplasmin does not cross the blood brain barrier, it is also expressed in the astrocytes surrounding the brain microvasculature and in the dopaminergic neurons of the substantia nigra (Hellman and Gitlin 2002). It is important to note that ceruloplasmin in astrocytes is synthesized as a glycosylphosphatidylinositol (GPI)-linked isoform in addition to the soluble form, suggesting that this membrane bound ferroxidase facilitates the rate of iron release from the astrocytes within the CNS (Madsen and Gitlin 2007). The GPI-Cp, resulting from a splice variation in the last two exons (exons 19-20), is 98\% identical to the soluble ceruloplasmin having only a different N-terminal peptapeptide and additional 25 amino acids (Patel, Dunn, and David 2000).

1. **Iron homeostasis in the brain**

   To enter the brain, iron in Fe\(^{3+}\) state binds to transferrin and interacts with the transferrin receptors of the blood brain barrier. After receptor internalization into the endothelial cells of the brain capillary, iron in Fe\(^{2+}\) state is dissociated from the complex with the help of the acidic and reductive environment in the endosome, and is maintained in the storage protein of these cells, ferritin, in the form of ferrihydrite crystals (5Fe\(_2\)·9H\(_2\)O). As for the apotransferrin-transferrin receptor complex, it is
brought back to the surface where the two components dissociate from each other and are recycled. In response to iron deprivation, iron is released from ferritin following lysosomal or proteosomal degradation, and exits the endothelial cells via the iron exporter ferroportin, to be oxidized to Fe$^{3+}$ by the GPI-ceruloplasmin of astrocytes. Afterward, Fe$^{3+}$ binds to transferrin synthesized by the oligodendrocytes, and gets internalized in the neurons through the transferrin receptors present on these cells (Figure 10) (Texel, Xu, and Harris 2008; Linder 2013).

![Diagram of iron metabolism in the CNS](image)

**Figure 10.** Iron metabolism in the CNS (Texel, Xu, and Harris 2008)

The rate of iron uptake into the brain is much lower than the iron requirements of this organ, indicating that the brain iron is recycled behind the blood brain barrier between the glial and neuronal cells, similar to the periphery (Figure 11). Iron is distributed in all cell types within the CNS but is present most abundantly in the glial cells for storage, because they contain abundant ferritin (Bradbury 1997).

Cp is essential for stabilization of ferroportin on the basolateral membrane surface of neurons, oligodendrocytes, astrocytes, choroid plexus and ependymal cells. Thus under conditions of Cp deficiency, as in aceruloplasminemia or Wilson disease,
ferroportin is not expressed on the basolateral membrane leading to decreased iron efflux and cellular iron overload (De Domenico et al 2007).

2. Aceruloplasminemia

Aceruloplasminemia is an autosomal recessive disease characterized by iron overload, particularly in the retina and the brain (Klomp et al 1996). It is caused by a loss of function in the gene coding for ceruloplasmin (chromosome 3q23-q24). The most common clinical presentations include retinal degeneration, diabetes mellitus, and neurological signs and symptoms such as dementia, dystonia and dysarthria due to neuronal loss and cavitary degeneration of the basal ganglia (Nittis and Gitlin 2002). The age of onset of this disease is at the age of 45 to 55 years, indicating that severe iron overload in the CNS takes several decades to show (Patel et al 2002).
G. Preliminary data and objectives of the Study

D-Penicillamine, is the drug of choice for treating copper overload in Wilson’s disease patients. However, this copper chelating agent has been reported to cause worsening of the neurological syndrome in 50% of Wilson disease patients; about half of these patients do not recover even after they discontinue the treatment (Kalita et al 2014).

We have previously investigated the *in vitro* effect of the copper chelator PA on HepG2 cells. We have demonstrated a 50% and 70% decrease in viability in HepG2 cells treated with Cu (50µM) and Cu-PA (50-250µM) respectively. PA alone exerted no effect on HepG2 viability. Concomitant with the obtained cell death, there was an increase in ROS generation, but no LDH release, with Cu-PA treated HepG2 cells. Moreover, this co-treatment has caused significant morphological changes in HepG2 cells, with a sharp increase in G2M phase and complete mitochondrial membrane depolarization. The expression of Fe and Cu binding proteins was also examined. PA decreased the expression levels of ceruloplasmin and transferrin, and Cu-PA decreased the expression level of transferring and ferritin. Moreover *in vitro* and *in vivo* ceruloplasmin ferroxidase activity was significantly reduced in PA and PA-Cu of HepG2 treated cells and serum of WD patients treated by PA.

These findings pose important questions regarding the consequences of the lifetime treatment of Wilson disease patients with PA. The copper chelator PA will decrease copper incorporation into apoceruloplasmin; reduce ceruloplasmin ferroxidase activity; and consequently may disturb iron homeostasis.
A general consensus regarding management of WD patients advised those with hepatic manifestations may be treated with PA, while those presenting neurologically avoiding PA is highly recommended.

In the current study we have investigated the *in vitro* effects of PA on brain cells comparing them to those of HepG2 cells. More specifically, cultured neuronal (PC12) and glial (U251) cells were treated with Cu/PA or Cu-PA following which we assessed:

1. **Cytotoxicity of the treatment by determining:**
   a. Viability of treated cells using MTT and Trypan blue exclusion assays
   b. Level of ROS generated using NBT reduction assay
   c. Intracellular GSH level using fluorometric glutathione detection kit
   d. Membrane integrity by measuring level of LDH released

2. **Apoptotic events by examining:**
   a. Morphological changes using a light microscope
   b. Gradient potential across mitochondrial membrane using fluorescent MitoPT-JC
   c. Cell cycle phases using flow cytometry analysis

3. **Expression levels of proteins including:**
   a. Cleavage of α-fodrin as apoptotic marker
   b. Copper binding protein: ceruloplasmin
   c. Iron binding proteins: ferritin and transferrin.
CHAPTER II
MATERIALS AND METHODS

A. Materials:

1. Cell Lines

Two different cell lines, used in this study, were originally purchased from the American Type Culture Collection, Manassas, VA, USA.

PC12: Rat pheochromocytoma (cat# CRL-1721)
U251 (formerly known as U373): Human Glioblastoma astrocytoma (cat# HTB-17)

2. Disposable Lab Ware

- **Corning**–Cell culture plastic wares.

- **GAIGGER brand**–Microscope cover glasses for Mito-PT (JC-1) immunofluorescent assay (cat # G15973C).

3. Cell Culture Reagents

- **Lonza**–Penicillin-Streptomycin mixture (PS) (cat# P4333-110M0790); 10X Trypsin (cat# BE 17-160E); 10X Phosphate buffered saline (PBS) without calcium and magnesium (cat# BE17-517Q).

- **Sigma**–Heat inactivated Fetal Bovine Serum (FBS), (cat#SF9665); Dulbecco's Modified Eagle's medium (DMEM), (cat#D5796); Heat Inactivated Horse Serum Donor Herd, (cat# H1138).
• **Calbiochem** – 7S NGF (Neural Growth Factor), mouse submaxillary glands (cat# 480354)

4. *In Vitro Kits*

• **Roche**–Cell Proliferation Kit I (MTT, cat # 11699709001); Cytotoxicity Detection Kit^{PLUS} (LDH, cat# 04744934001).

• **ImmunoChemistry TECHNOLOGIES**–MitoPTTM Kit (JC-1) (cat# 911).

• **Abcam**– Glutathione Detection Assay Kit (Fluorometric, cat# ab65322).

5. *Chemicals and Reagents*

• **Amresco**–Polyxyethylene-20-Sorbitan Monolaurate (Tween; cat# 0777-1L); Sodium Dodecyl Sulfate (SDS); Dimethyl sulfoxide (DMSO).

• **BIO-Rad**–N, N, N’, N’ tetra-methylethylenediamine (Temed); Glycine; Acrylamide/bisacrylamide; Nitrocellulose membrane (2μm); PVDF membrane; Ammonium Persulfate (APS); Bio-rad protein assay (cat# 500-0006).

• **BDH laboratory reagent**– Sucrose (cat# 302997J); Trichloroacetic acid (TCA).

• **GE Healthcare**– Rainbow molecular weight ladder (RPN800E).

• **ICN**–4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid(Hepes, cat# 194550).

• **MERCK**–Potassium Hydroxide pellets (KOH); Sodium Chloride (NaCl).

• **Roche**–Enhanced Chemi Luminescence’s Reagent (ECL) Kit.

• **SIGMA**–Tris-Acid; Tris-Base; Methanol; Triton 100x; β-Mercaptoethanol; Isobutanol; Ribonuclease A, (cat # R6513); Propidium Iodide, (cat# P4170); Nitro Blue Tetrazolium Chloride (NBT) (cat#N-6876); D-Penicillamine (cat#P-4875); Trypan Blue; Bovine Serum Albumin (BSA); Ponceau Xylinde.
6. Antibodies

- **Abcam** – Ceruloplasmin, rabbit monoclonal (ab131220); Ferritin, rabbit polyclonal (ab7332); Transferrin, rabbit polyclonal (ab1223)
- **Santa Cruz** – GAPDH, mouse monoclonal IgG antibody (Sc-32233)
- **Enzo Life Sciences** – α-Fodrin, mouse monoclonal IgG antibody (BML-FG6090)
- **Jackson ImmunoResearch** – Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (cat# 115-035-166); Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (cat# 111-035-003).

7. Buffer Preparations

- Sucrose Hepes Tris-Base buffer, SHT (250mM: 10mM: 50mM): 85.6g of sucrose, 2.83g of Hepes and 60g of Tris-Base dissolved in 1L of ddH2O with pH adjusted to 7.4.
- Lysis buffer, SHT-Triton (1%): 10μl of triton (100%) added to 1mL of SHT.
- Laemmli (4X): 8mL of 10% SDS, 1mL of 100% glycerol, 100μl of 0.01% bromophenol blue and 2mL of 5% β-Mercaptoethanol.
- 10% Sodium Dodecyl Sulfate (SDS): 10g SDS dissolved in 100mL ddH2O.
- Acrylamide (30%): 29.2g acrylamide and 0.8g N,N’-methylene-bisacrylamide dissolved in 100mL ddH2O and stored at 4°C.
- Ammonium PerSulfate (APS, 10%): 0.1g APS dissolved in 1mL ddH2O.
- Ponceau Red (0.4%): 0.4g ponceau red dissolved in 100mL of 0.3% TCA.
- Resolving gel (6%): 2.6 mL ddH₂O, 1 mL acrylamide (30%), 1.3 mL Tris-HCl (1.5M, pH 8.8), 50 μl SDS (10%), 50 μl APS (10%) and 4 μl Temed.
- Resolving gel (8%): 2.3 mL ddH₂O, 1.3 mL acrylamide (30%), 1.3 mL Tris-HCl (1.5M, pH 8.8), 50 μl SDS (10%), 50 μl APS (10%) and 3 μl Temed.
- Resolving gel (12%): 1.6 mL H₂O, 2.0 mL acrylamide (30%), 1.3 mL Tris-HCl (1.5M, pH 8.8), 50 μl SDS (10%), 50 μl APS (10%) and 2 μl Temed.
- Stacking gel (4%): 1.4 mL ddH₂O, 330 μl acrylamide (30%), 250 μl Tris-HCl (0.5M, pH=6.8), 20 μl SDS (10%), 20 μl APS (10%) and 2 μl TEMED.
- Running buffer (5X, pH 8.3): 15.1 g of Tris-Base, 72 g of Glycine and 5 g of SDS dissolved in 1 L ddH₂O.
- Transfer buffer (1X): 3.03 g of Tris-Base, 14.4 g of Glycine and 10 mL of 10% SDS dissolved in 200 mL methanol and completed to 1 L by ddH₂O.
- Blocking buffer (10%): 10 g of fat free milk dissolved in 100 mL TBS and 50 μl tween.
- Tris-HCl (1M, pH 8): 121.14 g of Tris-HCl dissolved in 1 L ddH₂O, and pH adjusted to 8.
- TBS (1X): 10 mL Tris-HCl (1M, pH 8) and 30 mL NaCl (5M) completed to 1 L by ddH₂O.
- Washing buffer TBS-Tween: 50 μL of Tween20 added for each 100 mL TBS.
- Trichloroacetic acid (0.3%): 0.3 g TCA dissolved in 100 mL ddH₂O.
- Stripping buffer: 6.25 mL of 1 M Tris-HCl (pH = 6.7) and 20 mL of 10% SDS completed to 100 mL by ddH₂O.
- Potassium Hydroxide (KOH, 2M): 11.22 g of KOH up to 100 mL of ddH₂O.
- Sodium Chloride (NaCl, 5M): 146.1 g of NaCl up to 500 mL ddH₂O.
8. **Equipments**

- Centrifuge 5416 (Eppendorf)
- Centrifuge 5810 (Eppendorf)
- Epson Expression 1680 Pro (Epson)
- Flow cytometer (Guava EasyCyte8)
- Fluroskan Ascent FL (Thermo).
- Fluorescent Microscope (OLYMPUS; BH2-RFCA)
- Hotplate stirrer (Corning)
- Hematocytometer (Fisher Scientific) (cat # 0267110)
- Inverted microscope (Axiovert 25 – Zeiss)
- Mini spin centrifuge (Thermo)
- Multiskan EX (ELISA reader) (Thermo).
- RP X-OMAT processor (Model M6B) (KODAK)
- UV-VIS scanning Spectrophotometer (UV-2101 PC) (SHIMADZU).
- Vortex (Fischer Scientific)
B. Methods

1. Cell Culture

   PC12 and U251 cells were incubated in a humidified 5% CO2 incubator at 37°C and cultured in their respective media as such: PC12 cell line was cultured in Dulbecco’s Modified Eagle’s medium DMEM (500 mL) supplemented with 10% FBS, 0.5% PS and 2.5% Horse Serum; whereas U251 cell line in DMEM (500 mL) supplemented with 10% FBS and 0.5% PS. PC12 and U251 cells were seeded in cell culture plates at a density of 1 x 10^5 cells/mL and 0.8 x 10^5 cells/mL respectively and then treated for 24 hours with the appropriate concentration of CuSO₄ and/or PA.

2. Viability Assays

   a. MTT Cell Proliferation Assay

      PC12 and U251 cells were seeded in triplicates in 96-well plates at an average density of 1 x 10^4 and 0.8 x 10^4/100μl media respectively. Effect of Cu and/or PA was determined at final concentrations varying between 0-200 μM and 250 μM respectively compared to a control. Viability was assessed using MTT proliferation kit after 24 hours of treatment.

      Briefly, 10 μl of yellow tetrazolium salt was added to control and treated cells and incubated for 4 hours. Salts reduced into purple formazan crystals by NADH/NADPH dehydrogenases of metabolically active cells were solubilized by adding 100 μl of solubilizing reagent for an overnight. The intensity of the purple color was measured using an ELISA reader at 595 nm.
Viability was calculated as follows:

\[
\% \text{ Viability} = \frac{\text{Abs (treated cells)} - \text{Abs (media + treatment)}}{\text{Abs (untreated cells)} - \text{Abs (media)}} \times 100
\]

b. **Trypan Blue Exclusion Test**

To confirm the results of MTT proliferation assay, PC12 and U251 cells were seeded in 100 mm petri dishes at an average density of 1 x 10^6 cells/10 mL and 8 x 10^5 cells/10 mL respectively and treated with Cu (50 and 200 μM), PA (250 μM) and Cu-PA (250 μM) + Cu (50 μM) for 24 hours. Cells were then washed with 1X PBS, trypsinized with 1X trypsin, centrifuged and suspended in 1 ml of medium. Cells mixed with Trypan blue with a ratio of 1v/1v were then introduced to a hemocytometer and counted under a light microscope. Dead cells are stained blue due to the diffusion of the dye, whereas viable cells remain unstained and appear opaque.

Viability was calculated as follows:

\[
\% \text{ Viability} = \frac{\text{number of unstained cells}}{\text{total number of cells}} \times 100
\]

3. **Intracellular ROS Level Determination**

PC12 (1 x 10^4/100 μl) and U251 cells (0.8 x 10^4/100 μl) were seeded in 96-well plates and treated for 24 hours with Cu (0-200 μM) and PA (250 μM).

Intracellular ROS level was determined using P-Nitro-Blue-tetrazolium salt (NBT) assay. Briefly, media was aspirated and 100 μl of NBT (1 mg/ml) was added and incubated for 1 hour in a humidified 5% CO2 incubator at 37°C. The wells were then washed with 100 μl of methanol and left to dry at room temperature. The NBT salts reduced into formazan crystals were solubilized by the addition of 120 μl of KOH (2M) then 140 μl
of DMSO. Control cells display an intense blue-turquoise color compared to the ROS generating cells which show a fainter color. Hence the reduction of NBT is inversely proportional to the generation of ROS. The intensity of blue-turquoise color was quantified at 630 nm using an ELISA reader.

ROS level was calculated as follows:

\[
\% \text{ NBT reduction} = \frac{\text{Abs (treated cells)}}{\text{Abs (control)}} \times 100
\]

\[
\% \text{ ROS production} = 100\% - \% \text{NBT reduction}
\]

4. **LDH release assay**

Cytotoxicity of Cu and/or PA was evaluated by measuring lactate dehydrogenase (LDH) release from the cells, using the Cytotoxicity Detection kit\(^\text{plus}\) (LDH). PC12 (1 x 10\(^4\)/100 μl) and U251 (0.8 x 10\(^4\)/100 μl) cells were seeded in 96-well plates and treated for 24 hours with Cu (0-200 μM) and PA (250 μM). Two sets of triplicates were used for control: “low control” (LC) with minimal LDH release and “high control” (HC) with maximal LDH release. 5 μl of lysis reagent of the kit was added to the HC triplicates and incubated for 15 minutes in the incubator at 37ºC to induce maximum LDH release. Then, 100 μl of reaction mixture was added to all wells, and incubated for 20 minutes at room temperature in dark. Finally, 50 μl of stop solution was added to terminate the reaction. The intensity of the orange-red color formed was read at 492 nm on ELISA reader. LDH release was calculated as follows:

\[
\text{Cytotoxicity (\%)} = \frac{\text{Abs (treated cells)} - \text{Abs (LC)}}{\text{Abs (HC)} - \text{Abs (HC)}} \times 100
\]
5. **Immunofluorescent Detection Of Mitochondrial Depolarization**

In order to examine the effect of Cu and/or PA on the mitochondrial membrane potential, the cell permeable lipophilic fluorescent dye known as JC-1 (5, 5’, 6, 6’ – tetrachloro – 1, 1’, 3, 3’ – tetraethyl – benzamidazolocarbocyanin iodide) was used. The delocalized positive charge on JC-1 favors the entry of the dye into the negatively charged non-apoptotic mitochondria, forming red-orange fluorescent aggregates. However, in compromised cells, JC-1 disperses throughout the cell forming green fluorescent monomers.

PC12 and U251 cells were seeded on sterile glass cover slips in 12-well plates at a density of 1 x 10^5 and 8 x 10^4/1ml of media respectively and treated with with Cu (50 and 200 μM), PA (250 μM) and PA (250 μM) + Cu (50 μM) for 24 hours. Following media aspiration, 300 μl of stain/well was added to the cells and incubated for 15 minutes at 37ºC. Cells were then washed twice for 10 minutes with washing buffer provided by the kit. Finally, the cover slips were mounted on microscope slides, left to dry and visualized using a fluorescent microscope. Images were taken by OLYMPUS Micro Software.

6. **Cell Cycle Analysis**

Cell cycle analysis was performed in order to assess the effect of the different treatments on cell cycle phases. Propidium Iodide (PI) stains the DNA material thus its fluorescence intensity quantified using a flow cytometer is directly proportional to the DNA content in a cell. In turn, DNA content is used to assess the proportion of cells in the different cell cycle phases.
a. **Seeding and Fixation**

PC12 (1 x 10^6 cells/10 ml) and U251 cells (8 x 10^5 cells/10 ml) were seeded in 100mm petri dishes and treated with Cu (50 and 200 μM), PA (250 μM) and PA/Cu (250 μM/50 μM). After 24 hours, cells were washed with 1X PBS, trypsinized by 1X trypsin-EDTA, collected and centrifuged at 1500 rpm for 5 minutes at 4 ºC. The pellet was washed twice by 1X ice-cold PBS. The fixation was then performed by the resuspension of the pellet with 1 ml of 1X ice-cold PBS and the dropwise addition of 4 ml of 70% ice-cold ethanol, while vortexing. Cells were then stored at -20 ºC for at least an hour before staining (they are stable up to 10 days).

b. **Staining**

Fixed cells were warmed to room temperature, and centrifuged at 1500 rpm for 5 min at room temperature. The pellet was washed with 1 ml of PBS, centrifuged, then resuspended in 100 μl of 200 μg/mL RNase A and incubated for 1 hour in a 37 ºC waterbath. After 5 minute centrifugation at 2000 rpm, the obtained pellet was resuspended in 350 μl of PBS and transferred to a 1.5 ml eppendorf tube. 20 μl of 1mg/mL Propidium iodide stain was then added to the cells, vortexed and incubated for 10 minutes in the dark.

c. **Cell Cycle Analysis**

Cell cycle analysis was performed using Guava EasyCyte8 Flow Cytometer. Each sample was collected as 5,000 ungated events and the corresponding cell cycle distribution, according to the DNA content, was then determined.
7. **Intracellular GSH Level Determination**

Glutathione is the principal intracellular low-molecular-weight thiol that plays a crucial role in the cellular defense against oxidative and nitrosative stress in mammalian cells. Diminished glutathione levels have been observed in the early stages of apoptosis. To check for changes in the intracellular total GSH level, fluorometric glutathione detection assay kit was used. PC12 (1 x 10^6 cells/10 ml) and U251 cells (0.8 x 10^6 cells/10 ml) treated with Cu (50 and 200 μM), PA (250 μM) and PA (250 μM) + Cu (50 μM) for 24 hours were collected in 1.5 ml eppendorf tubes and centrifuged at 700xg for 5 minutes. After discarding the supernatant, cells were lysed by kit cell lysis buffer (100 μl) and were incubated on ice for 10 minutes. The eppendorfs were then centrifuged at top speed (13400 rpm) for 10 minutes and the supernatant of the samples were transferred into new tubes for glutathione assay.

Different volumes of the samples were added into black clear-bottom 96 well plates and the total volume was then brought to 100μl with lysis buffer followed by addition of GST reagent (2 μl) and MCB (2 μl) to each well. The plate was incubated at 37 ºC for 1 hour. The fluorescence was measured by fluoroscan at Ex/Em.= 360±20nm/ 460±20nm. The total GSH level was determined using the standard curve plot obtained using the 0.1μg/μl standard GSH stock solution of the kit.
8. **Western Blot Analysis**

a. **Cell Lysate Preparation**

   As follows, cells were scraped, collected and centrifuged at 1500 rpm for 5 minutes. The pellet was washed twice by ice-cold 1X PBS, resuspended in SHT-Triton lysis buffer and lysed. Protein quantification was performed according to the instructions of Bradford assay Bio-Rad kit and compared to BSA standard (2-10μg). Then, 30 μl of samples were mixed with 10 μl of 4X laemmli buffer and denatured by boiling for 10 minutes.

b. **Western Blot**

   The expression of Ceruloplasmin, Transferrin, Ferritin and α-Fodrin in treated and untreated samples was examined. Samples were loaded on SDS-PAGE and allowed to migrate in 1X running buffer, following which proteins were transferred from the gel onto a Nitocellulose or PVDF membrane using BIORAD electro-transfer set up. The membrane was then blocked with 10% fat free milk for 1 hour, and incubated with the appropriate primary antibody overnight at 4 °C. After three washes with TBS-Tween (each 20 minutes), it was incubated with the suitable secondary antibody for 1 hour at room temperature. The membrane was washed again three times and the protein expression was visualized using the enhanced chemiluminescence ECL reagent. The protein bands were scanned and quantified using Image J software. They were first normalized to their respective GAPDH band and then fold expression was determined relative to the control.

   The table below represents the concentration of protein loaded in each well, the gel percentage, the type of membrane and the dilution of the antibody used for each protein of interest.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein loaded (µg)/well</th>
<th>Gel percentage</th>
<th>Type of membrane</th>
<th>Dilution of the antibody (µl/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceruloplasmin</td>
<td>100</td>
<td>8%</td>
<td>Nitrocellulose</td>
<td>1:300</td>
</tr>
<tr>
<td>Transferrin</td>
<td>100</td>
<td>12%</td>
<td>PVDF</td>
<td>1:1000</td>
</tr>
<tr>
<td>Ferritin</td>
<td>100</td>
<td>12%</td>
<td>PVDF</td>
<td>4:3000</td>
</tr>
<tr>
<td>α-Fodrin</td>
<td>75</td>
<td>6%</td>
<td>Nitrocellulose</td>
<td>1:500</td>
</tr>
</tbody>
</table>

**Table 1.** Protein concentration, gel percentage, type of membrane and dilution of Ab

### 9. Statistical Analysis

SPSS software was used to analyze data and determine significance. Both One-Way Anova and Independent sample t-test were applied. For One-Way Anova analysis, Post Hoc tests, Bonferroni and Scheffe were used for multiple comparisons. p-value<0.05 is considered significant. For each parameter tested, a set of at least three experiments were done where a triplicate of three determinants was applied in each experiment. Moreover, for each parameter, both, inter-categorical statistical significance and significance relative to control were analyzed.
CHAPTER III

RESULTS

A. Effect of CuSO$_4$ and/or Penicillamine on viability of PC12 and U251 cells

1. MTT Cell Proliferation Assay

PC12 and U251 cells were treated with different concentrations of CuSO$_4$ (10 μM – 200 μM) in order to determine the Cu concentration at which 50% of cell death occur (IC$_{50}$).

In both cell lines, CuSO$_4$ induced a significant (p<0.001) dose-dependent decrease in viability reaching a 50% cell death (p<0.001) at 50 μM CuSO$_4$ and a maximum of 90% death (p<0.001) at 200 μM (Figure 1).

![Figure 1](image)

*Figure 1:* Effect of different CuSO$_4$ concentrations on viability of PC12 and U251 cells. Cells were treated with increasing concentrations of CuSO$_4$ (10-200 μl) for 24 hours following which viability was determined using MTT assay. Data presented is the mean ± SEM of nine determinations from three different experiments. Asterisks on bars represent inter-categorical statistical significance (each category with the preceding one), and those drawn upwards represent significance relative to the control. (*), (**), and (***) correspond to P<0.05, 0.01, and 0.001 respectively.
PA, at a concentration of 250 μM, exerted no effect on the viability of PC12 cells; however, it decreased the viability of U251 cells by 11 % (p<0.001).

To investigate the protective effect of PA as chelator, the two cell lines were also co-treated with PA and Cu (IC50) (Figure 2). Surprisingly co-treatment with PA-Cu exhibited no protective effect; instead a further decrease in the viability to 65% (p<0.001) and 85 % (p<0.001) was obtained with PC12 and U251 cells respectively.

![Figure 2](image)

**Figure 2**: Effect of copper chelator Penicillamine on viability of PC12 and U251 cells. Cells were treated with 250 μM PA and/or 50 μM CuSO₄ for 24 hr. Data presented is the mean ± SEM of nine determinations from three different experiments. Asterisks on bars represent inter-categorical statistical significance (each category with the preceding one), and those drawn upwards represent significance relative to the control. (*), (**), and (***) correspond to P<0.05, 0.01, and 0.001 respectively.
2. **Trypan Blue Exclusion Test**

To confirm the results of MTT proliferation assay, viability of PC12 and U251 cells treated with 250 µM PA and/or 50 µM CuSO₄ was assessed by trypan blue exclusion test (Figure 3).

Treatment with CuSO₄ (50 µM) and/or PA (250 µM) had no significant effect on the viability and membrane integrity of the PC12 cells. CuSO₄ at 200 µM decreased the viability insignificantly by 10% only (p>0.05).

As for the U251 cells, CuSO₄ at 50 µM or 200 µM, and PA at 250 µM decreased the viability by 5%, 22% (p<0.001), and 17% (p<0.05), respectively. However, co-treatment of U251 with CuSO₄·PA (50-250 µM) further decreased the viability by 92% (p<0.001).

**Figure 3:** Effect of copper and/or PA on viability and membrane integrity of PC12 and U251 cells. Cells were treated with 250 µM PA and/or 50 µM CuSO₄ for 24 hr. Data presented is the mean ± SEM of three determinations from three different experiments. Asterisks on bars represent inter-categorical statistical significance (each category with the preceding one), and those drawn upwards represent significance relative to the control. (*), (**), and (***) correspond to P<0.05, 0.01, and 0.001, respectively.
B. Effect of CuSO₄ and/or Penicillamine on ROS generation in PC12 and U251

The level of ROS generation was determined in PC12 and U251 cells treated with CuSO₄, PA, and PA+ Cu (EC50).

Following 24 hour treatment of PC12 and U251 cells, Cu (10 μM – 200 μM) showed no significant effect on ROS generation (data not shown).

Similar results were obtained with PA (250 μM) treatment.

However co-treatment of PC12 and U251 cells with Cu-PA (50μM, 250 μM) decreased NBT reduction by 84% and 49% indicating an increase of 16% and 51% in ROS levels respectively.

![Figure 4: Effect of copper and/or PA on ROS generation in PC12 and U251 cells.](image)

Figure 4: Effect of copper and/or PA on ROS generation in PC12 and U251 cells. Cells were treated with 250 μM PA and/or 50 μM CuSO₄ for 24 hr. Data presented is the mean ± SEM of nine determinations from three different experiments. Asterisks on bars represent inter-categorical statistical significance (each category with the preceding one), and those drawn upwards represent significance relative to the control. (*), (**) and (***) correspond to P<0.05, 0.01, and 0.001 respectively.
C. Effect of CuSO$_4$ and/or PA on membrane integrity in PC12 and U251

Cytotoxicity of Cu and/or PA was conducted by measuring LDH release in PC12 and U251 cells. No significant LDH release was observed in PC12 and U251 cells treated with PA or Cu; however, with cells co-treated with PA and Cu (EC50), a significant release in LDH (16%) (p<0.001) was obtained with PC12 (Figure 5) but not with U251 cells (data not shown).

Further examination of the morphological changes in PC12 and U251 cells following treatment with CuSO$_4$ or PA using a light microscope showed no variation in the morphology of the cells. However, unlike the co-treatment of PC12 with Cu-PA, U251 lost their elongated shape assuming a rounded morphology (Figure 6).

Figure 5: Effect of copper and/or PA on LDH release in PC12 cells. Cells were treated with 250 µM PA and/or 50 µM CuSO$_4$ for 24 hr. Data presented is the mean ± SEM of nine determinations from three different experiments. Asterisks on bars represent intercategorical statistical significance (each category with the preceding one), and those drawn upwards represent significance relative to the control. (*), (**), and (***)) correspond to P<0.05, 0.01, and 0.001 respectively. LC: low control, HC: high control.
Figure 6: Effect of CuSO₄ (6a and 6c), PA and PA+Cu (6b and 6d) on morphology of PC12 and U251 cells respectively. Cells were seeded in 6 well plates and treated for 24h
D. Depolarization of Mitochondrial membrane by Cu and/or PA

The effect of Cu (50 μM, 200 μM), PA (250 μM), and PA+Cu on mitochondrial membrane potential was investigated using MitoPT-JC assay (Figure 7). In PC12 cells, 50 μM (Figure 7b) and 200 μM (Figure 7c) CuSO₄ did not induce any depolarization of the mitochondrial membrane. However, partial depolarization was detected in cells treated with PA (Figure 7d) and PA+Cu (Figure 7e). As for U251, a partial depolarization was observed in some of the Cu-treated cells (Figure 7g and 7h) and all PA-treated cells (Figure 7i). Complete depolarization of the mitochondrial membrane was only detected in PA+Cu treated cells (Figure 7j).

![Images of PC12 cells treated with different conditions](7a) PC12 – Control  
(7b) PC12 – Cu50  
(7c) PC12 – Cu200  
(7d) PC12 – PA  
(7e) PC12 – PA+Cu
Figure 7: Effect of CuSO₄ and/or PA on mitochondrial membrane potential of PC12 and U251 cells. Mitochondrial depolarization was detected by MitoPT JC-1 dye that fluoresces orange in healthy mitochondria and green in compromised ones. Representative figure of cells treated with 50 µM CuSO₄ (7b and 7g), 200 µM CuSO₄ (7c and 7h), 250 µM PA (7d and 7i) and PA+Cu (7e and 7j) in PC12 and U251 cells respectively.
E. Cell cycle analysis of Cu and/or PA treated PC12 and U251 cells

Cell cycle distribution of PC12 and U251 cells treated with Cu and/or PA was examined by flow cytometry. In PC12, the average distribution of PA, Cu50 or Cu200 treated cells in the cell cycle phases subG0, G0/G1, S, and G2/M was found to be similar to PC12 untreated cells. A minimal increase was detected in subG0 phase (12%) of PC12 cells co-treated with PA and Cu50 (Figure 8a and 8c).
In U251, the cell cycle distribution of PA or Cu50 treated cells was similar to the control cells. A minimal increase was identified in subG0 (7%) and G2M (29%) phases of Cu200 treated U251 cells. However, a sharp increase in subG0 (99%), signifying cell death, was distinguished in the PA+Cu50 co-treated cells (Figure 8b and d).

*Figure 8*: Cell cycle analysis of PC12 (8a and 8b) and U251 (8c and 8d) cells treated with Cu and/or PA. Cell cycle analysis was performed using Guava EasyCyte8 Flow Cytometer. The results are average of three representative experiments.
F. Effect of PA and/or Cu on intracellular GSH level of PC12 and U251 cells

Intracellular GSH level of cells treated with Cu and/or PA was investigated using the fluorometric glutathione detection assay kit. None of the Cu or PA treatments exerted any effect on the GSH level of the 2 cell lines, whereas a remarkable decrease of 49% was identified in U251 cells co-treated with PA and Cu50, characterizing early stage apoptosis. A non-significant decrease of 5% was detected in PA+Cu treated PC12 cells.

![Figure 9](image)

**Figure 9:** Effect of copper and/or PA on intracellular GSH level of PC12 and U251 cells. Cells were treated with 250 µM PA and/or 50 µM CuSO₄ for 24 hr. Data presented is the mean ± SEM of three determinations from three different experiments. Asterisks on bars represent inter-categorical statistical significance (each category with the preceding one), and those drawn upwards represent significance relative to the control. (*), (**), and (***) correspond to P<0.05, 0.01, and 0.001 respectively.
G. Effect of Cu and/or PA on α-Fodrin in U251 cells

To confirm that cell death observed in PA+Cu treated U251 cells was due to apoptosis and not necrosis, western blot analysis was used to observe the cleavage of α-fodrin, an apoptotic marker cleaved by calpain and caspase-3. No fragmentation was detected in U251 cells treated with PA or Cu alone. Cleavage of α-Fodrin was observed in PA+Cu treated U251 cells showing that apoptosis is the cause of cell death in these co-treated U251 cells.

Figure 10: U251 cells were treated with PA and Cu for 24 hours and α-Fodrin cleavage was observed using western blot analysis.

H. Effect of Cu and/or PA on the expression of Transferrin, Ferritin & Ceruloplasmin

Expression levels of iron and copper binding proteins (transferrin, ferritin and ceruloplasmin) in PC12 and U251 cells treated with Cu and/or PA were determined by western blotting.

Compared to untreated PC12 cells, transferrin expression was significantly decreased by 0.33 and 0.54 fold (p<0.05) in PA and PA+Cu treated PC12 cells, respectively. In U251, there were no significant differences in Tf expression between the control and the treated cells. However, compared to PA+Cu treated U251 cells, the expression of transferrin in PA treated U251 cells significantly decreased by 0.23 fold (p<0.05) whereas upon treatment with Cu50, it increased by 0.21 fold (p<0.01) (Figure 11a).
Figure 11a. Effect of Cu and/or PA on the expression level of Transferrin in PC12 and U251 cells. Protein expression was assayed using western blot analysis. Quantitative assessment of bands was done using Image J. Expression level of treated samples and controls were normalized to their respective GAPDH, and then fold expression was determined relative to the control. All bars represent mean ± SEM. Asterisks on bars represent inter-categorical statistical significance (each category with the preceding one), and those drawn upwards represent significance relative to the control. (*), (**) and (***) correspond to P<0.05, 0.01, and 0.001 respectively. Image represents one of 3 experiments.
As for ferritin, the only significant variation observed was that between PA+Cu and Cu50 treated U251 cells: Cu treated cells showed a significant increase (0.65 fold, p<0.001) in the expression level of ferritin compared to cells treated with PA+Cu (Figure 11b).

**Figure 11b.** Effect of Cu and/or PA on the expression level of Ferritin in PC12 and U251 cells. Protein expression was assayed using western blot analysis. Quantitative assessment of bands was done using Image J. Expression level of treated samples and controls were normalized to their respective GAPDH, and then fold expression was determined relative to the control. All bars represent mean ± SEM. Asterisks on bars represent inter-categorical statistical significance (each category with the preceding one), and those drawn upwards represent significance relative to the control. (*), (**) and (***) correspond to P<0.05, 0.01, and 0.001 respectively. Image represents one of 3 experiments.
Finally, the expression level of ceruloplasmin varied significantly between most conditions in both PC12 and U251 cells.

In PC12 cells, a significant increase of 0.54 fold (p<0.001) in the expression level of Cp was observed in PA+Cu treated cells, compared to control. In addition, a significant decrease in Cp expression was obtained in PC12 cells treated with PA (0.66 fold, p<0.001) and Cu50 (0.39 fold, p<0.001), when compared to PA-Cu treated cells. Regarding U251, compared to control, cells treated with PA+Cu showed a significant decrease of 0.6 fold in Cp expression level (p<0.001). Moreover, Cu50 and PA significantly increased the expression of Cp by 0.53 fold (p<0.001) and 0.38 fold (p<0.05) respectively, compared to PA+Cu. An increase in the Cp expression by 0.89 fold (p<0.001) was also observed in Cu200 treated U251 cells when compared to Cu50 (Figure 11c).

**Figure 11c.** Effect of Cu and/or PA on the expression level of ceruloplasmin in PC12 and U251 cells. Protein expression was assayed using western blot analysis. Quantitative assessment of bands was done using Image J. Expression level of treated samples and controls were normalized to their respective GAPDH, and then fold expression was determined relative to the control. All bars represent mean ± SEM. Asterisks on bars represent inter-categorical statistical significance (each category with the preceding one), and those drawn upwards represent significance relative to the control. (*), (**) and (***) correspond to P<0.05, 0.01, and 0.001 respectively. Image is representative of 2 experiments.
I. Effect of PA-Cu on PC12 cells grown in the presence of NGF

To induce differentiation in PC12 cells, 50 ng/ml of nerve growth factor (NGF) was added to the seeded cells (100,000 cells/10 ml). The culture media was changed every two days until differentiation was observed under the light microscope. Then, cells were treated with PA+Cu, in order to study the effect of this treatment in differentiated PC12 cells as well. No significant difference in results was observed between cells grown in the absence or presence of NGF when treated with PA+Cu using trypan blue exclusion assay, MTT assay, and cell cycle analysis. Also, no morphological change was observed between control and PA+Cu treated PC12 cells grown with NGF supplemented media (Figure 12).

![Image of PC12 cells grown with or without NGF](image1)

**Figure 12.** Effect of PA and Cu on morphology of PC12 cells grown with or without NGF
CHAPTER IV
DISCUSSION

Copper is an important transition element that plays a key role in several biochemical processes. Being a cofactor in many cuproenzymes, copper is required in cellular respiration, neurotransmitter biosynthesis, connective tissue, pigment formation, antioxidant defense enzymes, and iron homeostasis (Puig et al 2002). Cu as well, is crucial for the brain function and development (Lutsenko, Bhattacharjee and Hubbard 2010). Disturbances in copper homeostasis have been associated with many neurodegenerative disorders such as Alzheimer, Parkinson, Menkes, and Wilson diseases. Demyelination and neurodegeneration evidenced in Menkes patients resulted from impaired copper transport, leading to copper deficiency in the brain (Kaler 1998). On the other hand, dystonia, dysarthria, depression, schizophrenia and cognitive deterioration were reported in Wilson disease patients resulting from copper accumulation in the brain (Ferenci 2004; Madsen and Gitlin 2007). In addition to brain, copper overload may also occur in the liver caused by defective ATP7B gene (Emre et al 2001; Schumacher et al 2001). WD Patients are managed by lifetime treatment with copper chelators such as penicillamine, trientine, and zinc. Penicillamine, commonly used in treating WD patients, is not recommended for patients with neurological manifestation, as it worsens their symptoms (Kalita et al 2014).

This study was undertaken to determine the effect of Cu and its chelator Penicillamine on brain cells, more specifically neuronal PC12 and gliomal U251. We hereby show that U251 cells were more sensitive to Cu-PA co-treatment, than PC12. On the other hand, neither of the examined cell lines was significantly affected by either Cu
or PA treatment. U251 cells co-treated with Cu-PA showed a significant increase in reactive oxygen species with a concomitant decrease in glutathione level. This has led to apoptotic cell death as evidenced by: the decrease in mitochondrial membrane potential; the increase in subG0 cell phase; and fragmentation of apoptotic marker α-fodrin. In addition, the expression levels of Fe and Cu binding proteins namely transferrin, ferritin and ceruloplasmin varied with treatment in both cell lines.

Two cell lines were used in this study: the neuronal PC12 and glioma U251 (also known as U373) cells. PC12 cells, neuron-like, derived from a pheochromocytoma of the rat adrenal medulla has been widely used in in vitro investigations of the neurotoxic/protective properties of specific compounds (Malagelada and Greene 2012). They synthesize and store acetylcholine, catecholamine, dopamine and norepinephrine (Greene and Tischler 1976) and contain Na+, K+, and Ca2+ channels (Shafer and Atchison 1991). In addition, they express ATP7A, a homologue of the Cu-transporting ATP7B, the Wilson gene product (Qian, Tiffany and Harris 1997).

U251 cell line, commonly used as a glioblastoma model, is derived from a male patient with malignant astrocytoma (Torsvik et al 2014) with adaptive plasticity and self-renewal capability (Zhang et al 2013). Astrocytes are the first parenchymal cells that get exposed to copper entering the brain. They are fully equipped with the machinery required to take up, store and distribute copper to other brain cells such as neurons (Scheiber, Mercer and Dringen 2014). They are among the pharmaceutical companies’ targets that aim to develop therapies to correct copper homeostasis dysregulation in brain (Brown 2004; Haywood et al 2008).

To mimic condition of WD patients, cells (PC12 and U251) were treated for 24 hours with varying concentrations of Cu (0-200 µM), PA (250 µM) or were co-treated
with Cu-PA (50-250 µM) mix. The chosen Cu concentration took into consideration the approximate physiological concentrations of Cu in body fluid (10 µM), and cerebral spinal fluid (78 µM) (Kardos et al 1989; Linder 1991). PA, at the concentration used, had no significant effect on either cell line. Surprisingly, the viability of either cell following treatment with Cu (50 µM) decreased by 50%, using MTT assay, but was minimal when assessed using trypan blue exclusion assay. Similar findings were also obtained with higher concentrations of 200 µM of Cu (90% cell death in MTT assay but insignificant effect in trypan blue). On the other hand, co–treatment with Cu-PA (50-250 µM) decreased the viability by 65% and 85% in PC12 and U251 cells respectively using MTT assay. A similar decrease (92%) in viability of U251 cells, but not PC12, was obtained with trypan blue exclusion assay. Our findings suggest that U251 cells are more sensitive than PC12 to Cu-PA treatment. Previous report from our lab with Cu (50 µM) treated HepG2 cells showed 50% decrease in viability (Majarian 2014). This discordance in the response between the 2 assays (MTT assay and Trypan blue exclusion test) may be attributed to interfering component with the MTT assay; that seems to be copper in this case. Trypan blue exclusion assay qualitatively determines the number of viable cells based on the cell membrane integrity (Strober 2001), while MTT assay measures mitochondrial function in live cells by monitoring the effect of agent, in this case Cu, on mitochondrial dehydrogenase activity (Meerloo, Kaspers, and Cloos 2011). Previous in vivo and in vitro studies have reported inhibition of the activity of the mitochondrial NADH-dependent dehydrogenases by copper (Sheline, and Choi 2004) that may not lead to cell death.

Consistent with the obtained results, no morphological changes in PA or Cu treated cells were observed. However, Cu-PA co-treatment induced loss of the
distinctive elongated morphology of U251 into rounded shape while no changes occurred with PC12 cells. Previous study with cultured rabbit articular chondrocytes treated with either CuSO₄ or D-PA resulted in a small decrease in cell proliferation; however, D-PA in association with heavy metals, including copper, was shown to markedly increase the extent of growth inhibition (Clain et al 1988).

For further assessment of the morphological changes, and membrane derangement or rupturing, we examined the extent of necrosis, if any, following the different treatments. Necrosis is characterized by cellular swelling, rupturing of plasma membrane, leakage of cellular components and cell lysis (Vairetti et al 2004). A marker of necrosis is the extracellular release of LDH (media in cultured cells) following loss of plasma membrane integrity (Chan, Moriwaki, De Rosa 2013). In our study, no significant release of LDH was detected in Cu or PA treated cells (PC12, U251). Our findings are in congruence with those reporting 10% LDH release from PC12 cells treated with 500µM Cu (10 fold the concentration used in the current study) for 48 hours (Belyaeva et al 2012). On the other hand, Cu-PA co-treatment caused a mild release of LDH from PC12 cells only (16%).

Copper is an important component of electron transport chain protein complexes such as cytochrome-c oxidase mediating electron transfer to oxygen (Horn and Barrientos 2008). Isolated liver mitochondria treated with copper chelators were reported to show structural and functional changes. Moreover, ATP7B -/- animals with apparent clinical manifestations had impaired mitochondrial enzymatic activities (Zischka et al 2011). Although intracellular level of copper was not determined in either of the 2 cell lines, mobilization of intracellular copper sites of mitochondria may perturb copper levels and derange mitochondrial proteins’ function (Bush 2000). Hence we
examined the effect of Cu, PA and Cu-PA on mitochondrial function. Mitochondrial membrane potential provides a sensitive indicator of the normal coupling between electron transfer and energy state of the cell, and consequently viability. Mito JC-1 assay qualitatively assesses the membrane potential across the mitochondrial membrane hence the coupling state of the mitochondria. Treatment with Cu alone in PC12 and U251 cells showed no significant effect on membrane potential whereas PA induced partial depolarization in both cell lines. As for the Cu-PA treatment, a partial and complete depolarization was observed in PC12 and U251 cells, respectively. Our findings suggest that dissipation of the mitochondrial membrane potential do not solely result from the possibility of Cu mobilization induced by PA, but from a toxic intermediate or component yielded from PA interaction with Cu.

Starkebaum et al reported that during chelation, PA reduces Cu$^{2+}$ to Cu$^+$ generating PA radicals. The reduction of O$_2$ first to superoxide (O$_2^-$) radical, and then to hydrogen peroxide (H$_2$O$_2$) occurs while Cu$^+$ is reoxidized to Cu$^{2+}$. The net reaction for PA oxidation results in H$_2$O$_2$ generation, occurring at a molar ratio of 2:1 (Figure 12) (Starkebaum and Root 1985). In our study, PC12 and U251 treated with either Cu or PA exhibited no significant effect on level of ROS, whereas co-treatment with Cu-PA increased ROS by 16% and 51% in PC12 and U251, respectively; and in line with previous results (Majarian 2014) obtained with HepG2 cells co-treated with Cu-PA (56% increase in ROS). Previous investigations (Gupte and Mumper 2007) using human leukemia and breast cancer cells reported no production of ROS with either PA (200 µM) or CuSO$_4$ (10 µM) treatments; whereas co-treatment with PA-Cu ($\leq$400 µM-10 µM) exhibited a concentration-dependent H$_2$O$_2$-mediated cytotoxicity with a concomitant decrease in intracellular GSH levels.
A recent study has established that the toxic effects of copper are mediated by an increased oxidative damage (Jazvinscak et al. 2014; Beyaeva et al. 2014). *In vitro* studies with neuronal P19 and PC12 cells reported that treatment with CuSO₄, at a concentration greater than 0.5 mM, increased ROS while decreasing GSH. Others have documented that apoptotic cell death of somatosensory neurons and PC12 cells treated with copper nanoparticles occur by increasing oxidative stress that disturbs intracellular redox homeostasis activating the apoptotic events (Prabhu et al. 2010; Xu et al. 2012). Mitochondrial dysfunction such as depolarization of membrane potential is an early marker or sign of cell injury that may result from ROS generation and/or depletion of GSH level (Gyulkhandanyan et al. 2003).

Glutathione is the most abundant intracellular non-protein thiol antioxidant that plays an essential role, such as scavenging free radicals generated during oxidative stress and maintaining the redox state (Jan, Ali and Hag 2011). Oxidative stress increases when generation of ROS level exceeds the antioxidant defense capacity of the cell causing lipids, proteins, and DNA damage (Thannickal and Fanburg 2000), leading to apoptosis and/or necrosis (Vairetti et al. 2004).

White et al have investigated the role of GSH in underlying resistance of primary neurons to toxicity. They showed that GSH depletion results in Cu, but not Fe,
toxicity in neurons (White et al 1999). In our current study, we obtained no significant variation in GSH level in either PC12 or U251 cells treated with Cu or PA. However in Cu-PA co-treated cells, depletion in GSH level was only obtained in U251 cells (49%), in line with ROS findings (51% increase) and depolarization of mitochondrial membrane potential. Our findings were as well in concordance with those reported by Gyulkhandanyan et al (2003) showing that CuSO$_4$ does not significantly induce changes in ROS and GSH levels as well as on mitochondrial membrane potential of astrocytes.

The role of GSH in ROS disposal has been verified and documented in brain cells. There is increasing evidence that oxidative stress is initiated in glial cells that alters its function and contributes to the pathogenesis of neurodegenerative diseases as well as the selective vulnerability of neurons (Dringen 2000). A decrease in GSH level triggers apoptotic events that may be assessed by cell cycle analysis. No variation in % distribution of cell cycle phases (subG0, G0/G1, S, and G2/M) distribution was obtained between control and PA or Cu treated PC12 cells and U251 cells. This supports our hypothesis that the decrease in cell viability of Cu-treated cells observed in MTT assay is due to a reduction in the dehydrogenase activity and not cell death. Surprisingly, co-treatment of PC12 and U251 cells with Cu-PA caused 12% and 99% increase in subG0 phase compared to 5% in control cells. In a previous study (Majarian 2014), we have investigated the co-treatment effect of Cu-PA on HepG2 cells. Similar to U251 cells, HepG2 cells’ morphology was changed; viability decreased (70%); ROS level increased (56%); and mitochondrial membrane potential depolarized. However, HepG2 cells showed a significant increase (63%) in G2M phase contrary to the 99% increase in subG0 in U251 cells indicating DNA fragmentation (Sedlak et al 1999) leading to cell
death by apoptosis or necrosis (Higuchi 2003). The absence of LDH release in Cu-PA treated U251 cells supports apoptosis rather than necrosis.

Apoptosis, an active and highly organized process of cell death, is triggered by many factors and characterized by morphological changes (cell shrinkage, chromatin condensation, nuclear fragmentation and membrane blebbing) with concurrent activation of caspases. One hallmark of apoptosis includes intracellular GSH depletion that has been linked to the activation of cysteine protease caspases that cleave specific target proteins and initiate a cascade of events leading to cell death (Circu and Yee 2012; Franco, Panayiotidis and Cidlowski 2007).

Fodrin is an abundant intracellular cytoskeleton protein in eukaryotic cells, a heterodimer composed of α and β subunits. During apoptosis, proteolytic cleavage of α-fodrin into fragments occurs, catalyzed by consecutive action of calpain and caspase-3 yielding unstable cellular structure promoting apoptosis (Kahaly et al 2005; Janicke et al 1998). Using western blotting analysis, α-Fodrin cleavage was only detected in PA-Cu but neither PA nor Cu treated U251 cells.

Defects in the ATP7B gene, encoding a copper transporting ATPase, underlie the pathophysiology of Wilson disease. Failure to incorporate copper into ceruloplasmin, and reduced biliary copper excretion cause copper accumulation in tissues including liver, brain, and cornea, leading to liver and/or neurologic clinical presentation. Most, but not all, patients with WD patients have low serum Cp level.

Ceruloplasmin is an abundant copper containing glycoprotein and a member of the multicopper oxidase family of enzymes. Cp, mainly synthesized in the liver as well as in various tissues, is expressed as a soluble form or anchored to cell membrane. The isoform of Cp, namely Glycosylphosphatidylinositol-anchored ceruloplasmin (GPI-Cp),
is highly expressed in the CNS by astrocytes and regulates neuronal iron levels through copper effluxing (Patel et al 2000; Jeong and David 2003). The role of Cp in brain iron metabolism is well established, and so is its role in neurodegeneration (Qian and Ke 2001). As an oxidase, Cp plays an important function in converting the Fe^{2+} into Fe^{3+} that gets incorporated into iron transporter transferrin, metabolized and stored bound to ferritin (Eid et al 2014).

Though iron is an essential ion involved in many physiological processes, iron overload is toxic (Patel et al 2002). Hence, similar to copper, its level must be balanced and strictly regulated. In its divalent state, Iron reacts with hydrogen peroxide and molecular oxygen to generate highly toxic hydroxyl and superoxide free radicals, promoting lipid peroxidation, and degradation of biomolecules including DNA, leading to cell death (Halliwell and Gutteridge 1984).

The lifelong management of WD patients using copper chelator, such as PA, poses a question on its consequences on iron homeostasis; more specifically would deprivation of copper by PA decrease copper incorporation in ceruloplasmin, reduce its ferroxidase activity and disturb iron homeostasis, that ultimately leads to iron deposition.

A preliminary study from our lab (Majarian 2014) has shown in a decrease in ferroxidase activity of Cp in PA treated HepG2 cells and in serum of WD patients. In the current study, we opted to estimate the expression of Cp, ferritin and transferrin in Cu, PA, and Cu-PA treated PC12 and U251 cells. Using monoclonal antibody, Cp expression in the 2 cell lines varied with treatment. Cp expression increased (67%) in Cu-treated U251 but did not change in PC12. This is consistent with the ability of U251 to recognize, transport and store nutrients. Treatment with PA exerted no effect in both
cell lines; whereas co-treatment with Cu-PA decreased Cp expression (60%) in U251 while increased it (54%) in PC12. On the other hand, the expression levels of Fe binding proteins transferrin and ferritin in U251 did not vary with treatments; while in PC12 cells, the expression of transferrin decreased with PA (33%) and Cu-PA (54%) treatments respectively with no change in ferritin levels.

Cp, a crucial antioxidant, is effective during CNS injury when levels of free iron or ROS including H$_2$O$_2$ increases (Patel et al 2002). Tapryal et al have previously reported a decrease in Cp level in response to extracellular H$_2$O$_2$ resulting from mRNA decay mechanism. Intracellular oxidative stress was also noted to decrease Cp expression in hepatic and astroglial cells. Furthermore, synthesis of Cp in hepatic cells and membrane bound GPI-Cp in astroglial cells were found to decrease with ROS treatment such as H$_2$O$_2$ or inhibitors of mitochondrial electron transport chain (Tapryal et al 2009). We postulate that the extracellular H$_2$O$_2$ generated from Cu-catalyzed PA oxidation in U251 cells decreases the expression level of ceruloplasmin which leads to further increase in H$_2$O$_2$, consequently leading to cell death through apoptosis.

Maintaining homeostasis of metal ions in the brain is under strict mechanisms that regulate transporters or prooxidant potentials of these metals. Existence of energy dependent regulatory transport systems prevents excessive loading and abnormal compartmentalization of metal ions. Any damage to BBB or comprised energy production would perturb levels of metal ions which characterizes most of neurodegenerative disorders, including Wilson disease (Bush 2000).

We hereby show sensitivity of U251 but not PC12 to Cu-PA treatment at a Cu concentration (50 µM) that is way below the toxic levels reported for Cu (500 µM). Likewise, PA at 250 µM is not toxic; as reported in previous reports including current
study. Our findings identify U251 cells with both positive (preventive) and negative (toxic) roles. U251 cells are first target and site of exposure to external factors or drugs such as PA. The co-treatment with Cu-PA caused U251 cell death by apoptosis, providing a mechanism that might protect the underlying neuronal cells. On the other hand, the obtained toxicity in U251 cells co-treated with Cu-PA may shed some light about one of the underlying mechanisms that worsen manifestations of WD patients with neurologic symptoms. Figure 13 summarizes an overview of the Cu-PA induced toxicity on U251 cells.

We propose that following Cu-PA interaction, there is an increase in extracellular H₂O₂ which permeates the plasma membrane of U251 cells causing:

a) Decrease in Cp expression by inducing mRNA degradation. This in turn would decrease the Cp ferroxidase activity, consequently preventing oxidation of Fe²⁺ to Fe³⁺. Through the fenton reaction, the piled Fe²⁺ in the presence of H₂O₂ will produce hydroxyl and superoxide radicals leading ultimately to cell death.

b) Oxidative stress inside cell affecting mitochondrial function leading to complete depolarization and uncoupling, the consequences of which is a decrease in ATP level that would further increase ROS leading to cell death.

Should the co-treatment effects result from mobilization of intracellular Cu stores, one would expect similar effects with PA treatment alone. However, the generation of H₂O₂ in Cu-PA treated cells seems to initiate a cascade of events that eventually lead to apoptosis. In addition, H₂O₂ and ROS derived intermediates might influence Cu-loading on Cp, favoring unstable apo-Cp that is rapidly degraded explaining thus the decrease in Cp level in co-treated cells. Bearing in mind the role and function of these cells in storing and providing nutrients to underlying neurons, one
would expect both cells U251 and PC12 to respond similarly. Surprisingly PC12 were less sensitive.

The resistance of PC12 to the co-treatment is unexplainable and requires further exploration. Species variability may be one possible reason. The used PC12 cells are derived from rats, the sensitivity of which may possibly be different compared to that of humans. Another plausible explanation may be attributed to the undifferentiated state of the used PC12 cells. Cultured under normal conditions, PC12 are adrenal chromaffin-like cells in morphology, physiology, and biochemistry, sharing some fundamental mechanisms with neurons (Bornstein et al 2012). Adding NGF (50 ng/ml) triggers and allows PC12 differentiation into cells with morphological and biochemical characteristics similar to those of sympathetic neurons (Das, Freudenrich and Mundy 2004). Examining differentiated cells, our preliminary findings have shown no difference between un/differentiated PC12 response to Cu-PA treatment. However release of factors, by the U251 cells that acts on PC12 triggering or inducing an effect, may not be ruled out and needs further investigation.

To sum up, deterioration of neurological WD patients following PA treatment may be partially explained by findings of the current study. In a copper rich environment, such as the one characterizing Wilson disease, the expected response would be exaggerated.

In addition, our findings pose an important question on the consequence of PA treatment on Fe homeostasis, possibility of Fe deposition as well, requiring new modalities in managing side effects of the life treatment of WD patients.
Future studies

- Investigate the role of apoptotic markers in the Cu-PA observed toxic effect.
- Monitor the variation in GSH/ROS levels, and depolarization of membrane potential with time. This will allow us to determine the proper sequence of events.
- Study the protective role of NAC/trolox or GSH in reversing Cu-PA effect at mitochondrial and cell cycle analysis levels.
- Perform activity assay of the different antioxidant enzymes and determine their level of expression following Cu-PA treatment. These include Cu,Zn-SOD, Mn-SOD, catalase and Glutathione S-transferase.
• Co-culture U251 and human neurons or culture PC12 with media of U251 cells treated with Cu-PA to check if effect is due to factors released by U251 in response to treatment.

• Determine effect of Cu-PA on Cu-dependent enzymes for brain function (dopamine-β-hydroxylase and peptidyl-α-amidating mono-oxygenase enzymes).

• Examine the effect of PA both in vitro and in vivo on WD animal model, LEC rats where copper level is high.

• Assessment of intracellular Cu/Fe levels in PC12 and U251 before and after treatment.

Limitations

• Both cell lines presumably have normal ATP7B, with no copper overload as encountered in WD patients.

• Astrocytes and neurons are cultured separately and each alone may function differently than when they are co-cultured, because astrocyte-neuron coupling has been reported in ion (Cu, Fe) homeostasis.

• In this study, neurons and astrocytes were directly exposed to PA. Whether in real life a metabolite of D-PA rather than PA itself is reaching the brain is not established yet.

• PC12 are isolated from rat. Using human neuronal cells would eliminate species differences that may interfere in communication between astrocytes and neurons.


Chan, F., Moriwaki, K., and De Rosa, M. (2013). Detection of necrosis by release of lactate dehydrogenase


