EVALUATION OF NEUROPROTECTIVE PROPERTIES OF *ECHIUM AMOENUM L.* ETHANOLIC EXTRACT

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EVALUATION OF NEUROPROTECTIVE PROPERTIES OF *ECHIUM AMOENUM L.* ETHANOLIC EXTRACT

by

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This thesis is dedicated to …

My Parents, Farah and Majid

whose love is the biggest treasure in my life and never can be repaid

My Husband, Roozbeh

who loves me, encourages me, supports me,

accepts my flaws and is never hard on me

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LIST OF ABBREVIATIONS

ABTS	2,2’-Azino-bis-(3-ethylbenthiazoline-sulphonic acid)
ACS14	Hydrogen sulfide releasing derivative of aspirin
ACS67	Latanoprost acid derivative
ALA	α-lipoic acid
ATCC	American type culture collections
ATP	Adenosine triphosphate
Bcl-2	β-cell CLL/lympha 2
BHT	Butylated Hydroxytoluene
CO_2	Carbon dioxide
CNS	Central nervous system
cm	Centimeter
DEVD-,	Amino acid sequence substrate for caspase 3/7
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELISA	Enzyme-linked immnuosorbent assay
ECA-EE	*Echium amoenum* ethanol extract

ECA-WE	*Echium amoenum* water extract
E. amoenum	*Echium amoenum L.*
eV	Electron volt
FBS	Fetal bovine serum
g	Gram
HIF-1α	Hypoxia inducible factor 1- alpha
HIFCS	Heat inactivated fetal calf serum
GC-MS	Gas chromatography-mass spectrometry
GSH	Glutathione
GST	Glutathione S-Transferase
h	Hour
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HUVEC	Human umbilical vein endothelial cells
IC_{50}	Inhibitory concentration of 50%
IOP	Intraocular Pressure
km	Kilometer
LEHD-	Amino acid sequence substrate for caspase 8
LETD-	Amino acid sequence substrate for caspase 9

m/z	Mass-charge ratio
mCB	Monochlorobimane
mg/mL	Miligram per millilitre
mg	miligram
min	Minute
mL	Millilitre
mm	Millimeter
mM	Millimolar
nm	nanometer
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide
NAION	Nonarteric Anterior Ischemic Optic Neuropathy
NMDA	N-methyl-D- aspartate
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
$NaNO_2$	Sodium nitrate
NIST	National institute of standards
NFk-B	Nuclear factor kappa B

P53	tumor suppressor protein 53
PBS	Phosphate buffered saline
pg/mL	picogram per millilitre
RGC	Retinal ganglion cells
ROS	Reactive oxygen species
rpm	Revolutions per minute
R^2	Regression coefficient
RT	Retention time
s	Second
S.E.M	Standard error of the mean
TMB	Tetramethylbenzidine
U/mL	Units per millilitre
UV	Ultraviolet
V/V	Volume per volume
VEGF	Vascular endothelial growth factor
WHO	World health organization
µg/mL	Microgram per millilitre
µM	Micromolar
µm	Micrometer

LIST OF SYMBOLS

α	Alpha
β	Beta
ºC	Degree Celsius
%	Percent

LIST OF PUBLICATIONS

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PENILAIAN SIFAT-SIFAT PERLINDUNGAN NEURO BAGI EKSTRAK ETANOL *ECHIUM AMOENUM L.*

ABSTRAK

Dalam penyakit degeneratif neuro iskemia dan hipoksia, tekanan oksidatif, eksitotoksisiti dan keradangan semuanya boleh membawa kepada kehilangan neuron. Kematian sel ganglion akibat degenerasi saraf optik boleh disebabkan oleh kesakitan iskemia berselang-selang sepanjang tempoh yang ditetapkan. Perlindungan neuro bertujuan untuk mencegah, melambatkan atau menterbalikkan kerosakan neuron akibat daripada keadaan asas patofisiologi. Matlamat kerja ini adalah untuk menilai sifat-sifat perlindungan neuro daripada tumbuhan asli Iran *Echium amoenum L.*. Kajian telah dijalankan untuk menyimpulkan sama ada *E. amoenum* boleh menumpulkan pengaruh negatif kekurangan serum terhadap sel-sel ganglion retina (RGC-5) dalam kultur dan juga terhadap kesakitan iskemia tertakrif dalam saraf optik tikus. Anti-apoptotik, anti-oksidan, gerak balas angiogenik dan aktiviti-aktiviti anti-radang semuanya disiasat. Kajian lanjut seterusnya dijalankan untuk mencirikan entiti perubatan aktif yang hadir dengan menggunakan Jisim Spektrometri-Kromatografi Gas (GC/MS). Ekstrak kloroform, etanol, n-heksana, dan akueus daripada kelopak *E. amoenum* telah disediakan melalui teknik maserasi/pemaseratan dan disaring dengan titisan sel RGC-5 untuk sifat-sifat neurotoksiknya. Ekstrak etanol dan air adalah tidak toksik. Walau bagaimanapun kajian *in vitro* ischemia mendedahkan bahawa ekstrak etanol *E. amoenum* pada 5 μg/mL adalah berkesan dalam menyelamatkan RGC-5 sel-sel daripada kesan buruk kekurangan serum dengan ketara mengekalkan daya maju (cerakin MTT), mengurangkan sel-sel apoptotik (pewarnaan Hoescht) dan penanda caspases 3/7, 8 dan 9 (cerakin Caspase)

mereka. Sifat-sifat perlindungan neuro *E. amoenum* dalam melemahkan keadaan tekanan oksidatif telah disimulasi dengan menguji kapasiti antioksidan menggunakan DPPH, ABTS dan ujian pelunturan β-karotena serta kemampuannya untuk meningkatkan tahap *glutation* (GSH) dan *glutation-S-transferase* (GST). Aktiviti IC_{50} yang radikal masing-masing ialah 10.89 ± 0.082 μg/mL, 15.91 ± 0.10 μg/mL dan 9.49 ± 0.12 μg/mL. *E. amoenum* ekstrak etanol pada 5 μg/mL dengan ketara meningkatkan tahap GSH dan GST. Saringan dengan GC/MS mendedahkan jumlah fenolik dan flavanoids yang tinggi menyokong sifat mujarab antioksidan. Kesannya terhadap angiogenesis telah dinilai melalui cerakin cincin aorta tikus (RARA), ekstrak etanol *E. amoenum* pada 100 μg/mL menghalang daripada 93.22 ± 0.127% pembentukan saluran darah dan mengurangkan dengan ketara aktiviti hipoksia yang disebabkan faktor kepekatan VEGF dalam Lisat HUVECs daripada 240.89 ± 0.00 pg/mL hingga 68.15 ± 9.56 pg/mL. Tambahan pula *E. amoenum* adalah sitotoksik terhadap HUVECs dalam cerakin percambahan. Dalam model tikus albino BALB/c iskemia neuropati optik, ProSense 750 telah digunakan untuk menyiasat pengaktifan katepsin pengantara keradangan dan aktivitinya dipantau menggunakan sistem pengimejan *in vivo* Tomografi Molekul Pendarfluor (FMT). Tikus dirawat *E. amoenum* (200 and 400mg/kg) mempunyai pengurangan 61.43% dan 75.63% daripada iskemia disebabkan gerak balas keradangan masing-masing. *E. amoenum* menunjukkan kesan mujarab perlindungan neuro dalam model *in vitro* dan *in vivo* degenerasi neuro retina. Ini mungkin disebabkan oleh anti-apoptotik, aktiviti antioksidan, keupayaan untuk meningkatkan spesis sel dalaman antioksidan, modulasi angiogenesis, dan ciri-ciri anti-radang.

Keywords: Perlindungan neuro; RGC; Model Tikus Iskemik Neuropati Optik; FMT; *Echium amoenum L.*; Perubatan tradisional.

EVALUATION OF NEUROPROTECTIVE PROPERTIES

OF *ECHIUM AMOENUM L.* ETHANOLIC EXTRACT

ABSTRACT

In neurodegenerative diseases, ischemia and hypoxia, oxidative stress, excitotoxicity, and inflammation can all lead to neuronal loss. Ganglion cell death as a result of optic nerve degeneration can be caused by intermittent ischemic insults over defined periods. Neuroprotection aims to prevent, retard or reverse neuronal damage as a result of the underlying pathophysiological state. The goal of the present work is to evaluate the neuroprotective properties of *Echium amoenum L.* an Iranian native plant. Studies were carried out to deduce whether *E. amoenum* ethanol extract can blunt the negative influences of serum deprivation to retinal ganglion cells (RGC-5) in culture and a defined ischemic insult to the optic nerve in mice. Its anti-apoptotic, antioxidant, angiogenic response and anti-inflammatory activities were all investigated. Further studies were then undertaken to screen the active medicinal entities present by the use of Gas Chromatography-Mass Spectrometry (GC/MS). Petals of *E.amoenum* chloroform, ethanol, n-hexane, and aqueous extract were prepared via maceration technique and screened on RGC-5 for its neurotoxic properties. Ethanol and water extracts were non-toxic. However *in vitro* ischaemia studies revealed only the ethanol extract *of E. amoenum* at 5 µg/mL was effective in rescuing RGC-5 cells from the detrimental effect of serum deprivation by significantly maintaining viability (MTT assay), decreasing apoptotic cells (Hoescht staining) and their markers caspases 3/7, 8 and 9 (Caspase assay). *E. amoenum* ethanol extract neuroprotective properties in attenuating oxidative stress conditions was simulated by testing its antioxidant capacities using DPPH, ABTS,

and β-carotene bleaching tests and its ability to increase the levels of glutathione (GSH) and glutathione-S-transferase (GST). The IC_{50} of radical scavenging activities were 10.89 ± 0.082 µg/mL, 15.91 ± 0.10 µg/mL and 9.49 ± 0.12 µg/mL respectively. *E. amoenum* ethanol extract at 5 µg/mL significantly increased the levels of GSH and GST. Screening with GC/MS revealed high amounts of phenolics and flavonoids supporting its potent antioxidant property. Its effect on angiogenesis was evaluated in SD-Rat aortic ring assay (RARA), *E. amoenum* ethanol extract at 100 µg/mL inhibited of 93.22 ± 0.127% blood vessel formation and significantly decreased the activity of the hypoxia induced factor, VEGF concentration in HUVECs lysates from 240.89 ± 0.00 to 68.15 ± 9.56 pg/mL. Furthermore, *E. amoenum* ethanol extract was cytotoxic to HUVECs in proliferation assay. In albino Balb/c mice ischaemic optic neuropathy model, ProSense 750 was used to probe the activation of the inflammatory mediator cathepsin and its activity monitored using the Fluorescence Molecular Tomography (FMT) *in vivo* imaging system. *E. amoenum* ethanol extract treated mice (200 and 400 mg/kg) had 61.43% and 75.63% reduction of ischaemia induced inflammatory response respectively. *E. amoenum* ethanol extract showed potent neuroprotective effect in *in vitro* and *in vivo* models of retinal neurodegeneration. This is likely due to its anti-apoptotic, antioxidant activity, ability to enhance cellular endogenous antioxidant species, modulation of angiogenesis, and anti-inflammatory properties.

Keywords: Neuroprotection; RGC; Mice Ischaemic Optic Neuropathy Model; FMT; *Echium amoenum L.*; Traditional medicine

CHAPTER ONE- LITERATURE REVIEW

1. Neurodegeneration and Neurodegenerative Diseases- General Definition

Neurodegeneration is a collective term for the progressive loss of structure, function, or even death of neurons. It is a characteristic feature in the pathogenesis of diseases as for Alzheimer's disease, frontotemporal dementia, Parkinson's disease, amyotrophic lateral sclerosis, progressive supranuclear palsy, corticobasal degeneration, Huntington's disease, Pick's Disease, prion diseases, motor neuron diseases, glaucoma, and also optic neruropathies, etc. (G Kalesnykas, Tuulos, Uusitalo, & Jolkkonen, 2008; Stokin & Goldstein, 2006).

Identified risk factors for neurodegenerative diseases regard in disputable genetic polymorphisms and increasing age. Gender, poor education, endocrine conditions, oxidative stress, inflammation, stroke, hypertension, diabetes, smoking, head-trauma, depression, infection, tumors, vitamin deficiencies, immune and metabolic conditions, and chemical exposure are to be other possible causes included (Brown, Lockwood, & Sonawane, 2005; Hitzl, Mossler, & Schnait, 2011). The mechanistic features of neurodegeneration include aberrant cellular processing, protein misfolding and subsequent aggregation of normal proteins and their intracellular or extracellular depositions (Doyle et al., 2011; Jucker & Walker, 2013). At the molecular level, mechanisms that have been observed include defective neuronal plasticity through aberrant regenerative responses, reduced neurotrophin levels, or increased neuronal vulnerability to stress (Imitola, Snyder, & Khoury, 2003; Mattson et al., 2000).

Concerning the population of our world which is ageing, also an ever-increasing number of elderly are being impacted upon by neurodegenerative diseases. In the

developed world, about 2% of the population is afflicted at any time (Hardy & Orr, 2006; Mattson, Chan, & Duan, 2002). It forecasted that overall number of neurodegenerative disease drug market will make a notable growth from around US$9 billion in 2005 to more than US$17 billion by 2010 (Fung, 2007; Huss, Spoerri, Egger, & Röösli, 2009). Consequently, the overall neurodegenerative diseases market has been making the so-called grow at over 12% per year. Therefore, with an increase in life expectancy and the number of old people, alongside with advances in treatments of neurodegenerative diseases, the increases look set to be continued.

Central nervous system trauma and neurodegenerative disorders trigger a cascade of cellular events resulting in an extensive damage to neurons (Figure 1-1) (Koeppen, 2008; Magharious, D'Onofrio, & Koeberle, 2011). Regenerative failure is truly a critical endpoint to these destructive triggers which are increasingly culminating in neuronal apoptosis (Magharious et al., 2011; Mattson, 2006) and inhibition of functional recovery.

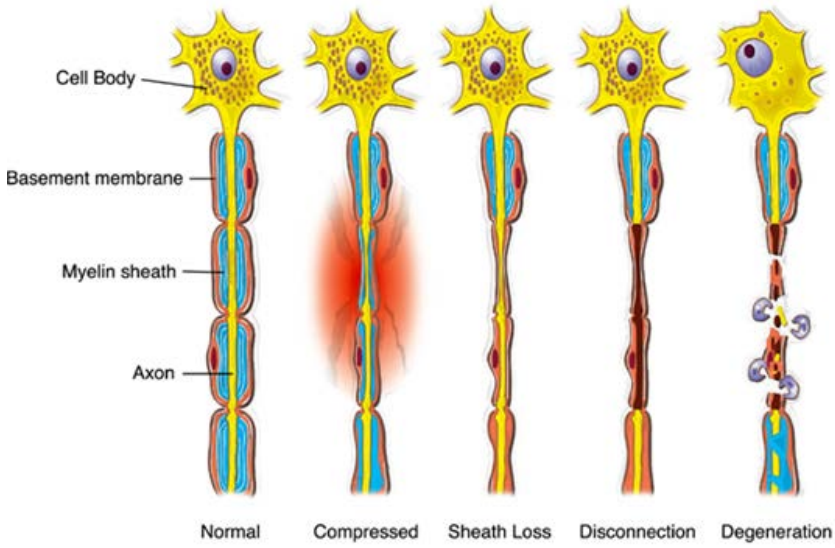

Figure 1-1 Process of Neuron Degeneration

Source: (Koeppen, 2008)

The non-permissive regenerative environment is due to expression of inhibitory cues (Magharious et al., 2011; Winzeler et al., 2011), glial scarring (Silver & Miller, 2004), slow clearance of axonal debris, and CNS inflammation (Jaerve & Müller, 2012). Neuronal susceptibility to apoptosis and regenerative failure are crucial endpoints of CNS trauma (Figure 1-2).

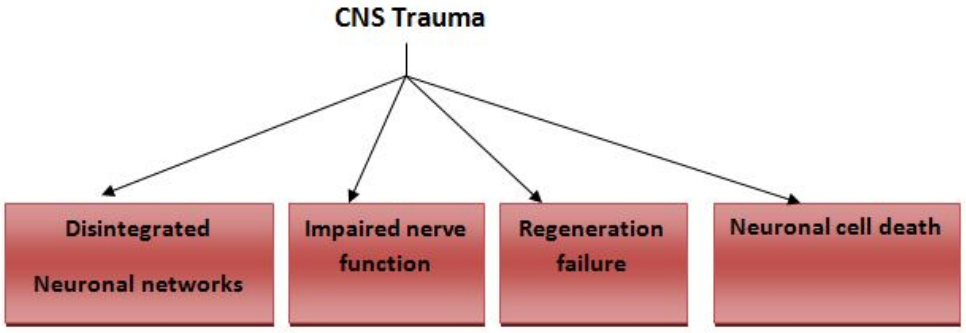

Figure 1-2 Trauma to the central nervous system

Source: (Burnett & Zager, 2004)

Trauma to the central nervous system causes axonal injury leading to disintegrated neuronal networks, impaired nerve function, regeneration failure and cell death, resulting in long-term neuronal disability.

The retina is a division of the central nervous system (CNS), with various types of neurons, including photoreceptors, bipolar cells, horizontal cells, amacrine cells and retina ganglion cells (RGCs) (Figure 1-3) (Arendt, 2003; Kolb, Nelson, Ahnelt, & Cuenca, 2001). The retinal ganglion cells (RGCs) are considered as the output neurons of the retina (Boya, 2012). The axons of RGCs, which constitute the last stop of the relay, will also be merged to form the optic nerve (Lamb, Collin, & Pugh, 2007). They transmit signals to the visual centers of the brain (Kolb et al., 2001). These visual signals are specifically transmitted from the retina to the lateral geniculate nucleus plus superior colliculus (SC) within the brain. Therefore, retina

and optic nerve could be affected by similar degenerative processes during neurodegeneration (Tezel, 2006; Zhai et al., 2003).

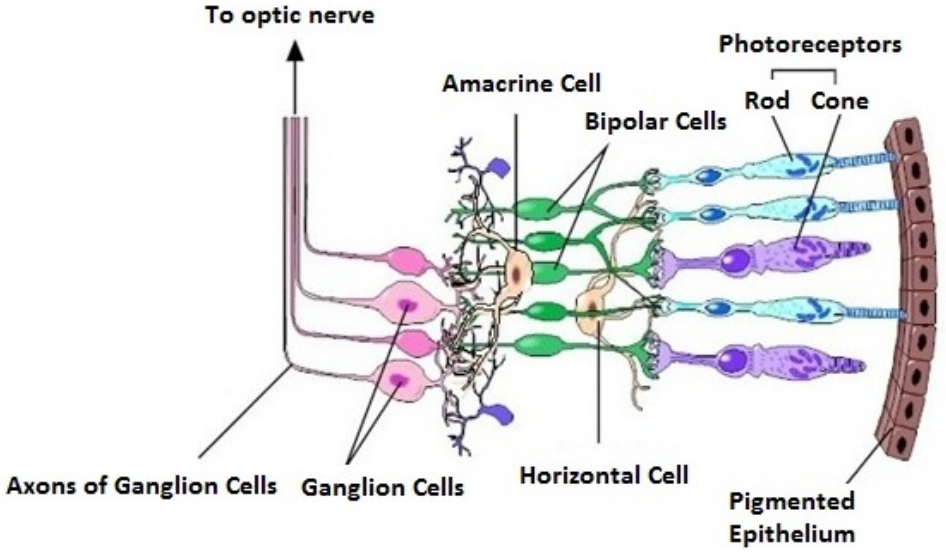

Figure 1-3 Retinal Cellular Structure
Source: (Hagerman & Johnson, 1991)

The optic nerve head appears to be more vulnerable than the rest of the retina, probably because the tissue is softer and the inelastic sclera does not cover the outside of the eye in this area (Lönngren, 2008). The unproblematic accessibility of the optic nerve and the reproducibility of optic neurodegeneration would make it a highly effective tool to study CNS trauma and also help in understanding the ensuing traumatic events that activate neuronal apoptosis (Lönngren, 2008; Srejić, 2009).

1.1. Mechanism of Neurodegeneration

1.1.1. Reduced Neurotrophin

The neurotrophic hypothesis holds the idea that both mammalian neuronal growth and maintenance depend sharply upon the viability of retrograde axoplasmic transport of soluble growth factors which are; scientifically speaking, called neurotrophins (Agarwal, Gupta, Agarwal, Saxena, & Agrawal, 2009; Bennett,

Gibson, & Lemon, 2002; Kaushik, Pandav, & Ram, 2003; Linker, Gold, & Luhder, 2009; Nave, 2010). It has been shown that increased neurotrophic factor concentrations delay or attenuate cell death following injury. The neurotrophins supplied to the retinal ganglion cells are small peptides that function in order to regulate cellular metabolism by having them attached to neuronal target-cell receptors (Gao, Qiao, Cantor, & WuDunn, 2002; Kido et al., 2000; Osborne et al., 2004). From there, a cascade of molecular enzymatic events and maintain cellular homeostasis are being initiated. Ganglion cells appear to be particularly dependent upon the brain-derived neurotrophin factor which necessarily is vital for their continued survival (Nave, 2010; T. Su, 2010). This factor is able to promote survival; furthermore, prevent neuronal death after axotomy in the optic nerve (Koeberle, Tura, Tassew, Schlichter, & Monnier, 2010). Further support for the neurotrophic hypothesis comes from the findings of Gao et al showing that an enhanced expression of brain-derived neurotrophin factor in the RGC layer takes place after the optic nerve injury (Kaushik et al., 2003; Kuehn, Fingert, & Kwon, 2005; Seki et al., 2005).

1.1.2. Excitotoxicity and Glutamate Release

In the pathology of a list of neurodegenerative disorders regarding the brain, excitotoxicity has been implicated as forth, Alzheimer's, Parkinson's, Huntington's disease and amyotrophic lateral sclerosis (Mattson, 2008; Swartz, Linn, & Linn, 2013). Neurodegenerative diseases of the eye, considering glaucoma, retinal ischemia, and diabetic retinopathy, also have pathologies associated with excitotoxicity (K.-G. Schmidt, Bergert, & Funk, 2008; Seki & Lipton, 2008; Swartz et al., 2013). Excitotoxicity is the excessive stimulation of neurons by excitatory amino acids such as glutamate, aspartate and quisqualate through postsynaptic

receptors. Glutamate serves as a neurotransmitter at most CNS synapses. Ironically, in addition to being the main neurotransmitter involved in excitotoxicity in mammals, glutamate is also the most prevalent neurotransmitter in the mammalian brain, being utilized in varying degrees by nearly all neurons of the vertebrate central nervous system and exposure to high concentrations has shown to have apoptosis of cells brought forththrough a process of excitotoxic cell death in a variety of model systems, giving rise to the very term which is called the glutamate-induced excitotoxicity (Mattson, 2003; Swartz et al., 2013). This so-called matter occurs when the oxygen and glucose supply in the brain has been dramatically decreased in cases where blood flow ceases; for instance, during a stroke.

Regarding a number of reports indicating that the neurotoxic effects of ischemia and hypoxia on the brain and retinal tissue result directly from an increase in the concentration of amino acid neurotransmitters, most notably to name glutamate. It has been confirmed that in both brain and retina, the levels of glutamate are being increased due to experimentally induced ischemia or hypoxia.

Certain pathological conditions can cause excessive glutamate to be released, however under normal circumstances glutamate from the synaptic cleft is reaccumulated immediately into the nerve terminal or into adjacent glia, which holds glutamine synthetase that would have glutamate converted into glutamine. Possessing a similar plasma membrane glutamate uptake carrier, glial cells and neurons keep the extracellular glutamate concentration below levels that damage neurons (Inage, Itoh, Wada, Hoshika, & Takashima, 2000).

The self-reinforcing deleterious action of glutamate receptor stimulation also produces an influx of sodium and calcium ions plus further membrane depolarization.

The influx of Na^+ is accompanied with the influx of chloride and the osmotically-driven influx of H_2O, resulting in cellular swelling. This early phase of excitotoxic injury is followed by activation of several types of cell receptors, including N-methyl-D-aspartate receptors that can allow entry of excessive amounts of calcium. In ischemic neuronal injury, the loss of calcium homeostasis plays a critical role in neuronal death. Abnormally calcium overload leads to sustained activation of a number of calcium-dependent enzymes, including protease, endonucleases and lipid peroxidases, they straightly attack cell constituents which leads to the generation of highly reactive free radicals plus the activation of the nitric oxide pathway (Naskar & Dreyer, 2001). DNA nitrosylation, fragmentation and activation of the apoptotic program turn out of the resulting interaction between intermediate compounds and free radicals (Wei et al., 2000; Z. Zheng, Lee, & Yenari, 2003).

1.1.3. Free Radical Generation

Being unstable, and having highly reactive molecules are distinctive features of free radicals characterized by the presence of unpaired electrons in their outermost shells (Halliwell, 2005). Usually, electrons associated with atoms or molecules are paired because this makes atoms relatively stable and unreactive. The loss of an electron leaves a molecule much more reactive than its paired counterpart. In case two radicals react, both radicals are eliminated; while if a radical reacts with a non-radical, another free radical is; thus, produced. Allowing free radicals to participate in chain reactions, this characteristic may be thousands of events long. According to Williams and Jeffrey (Si et al., 2011; G. M. Williams & Jeffrey, 2000), reactive free radicals or reactive oxygen species, such as superoxide anion, hydroxyl radical and peroxyl radical are particularly reactive and known to be a biological product of reducing molecular oxygen or metabolic processes.

Ischemia causes a net increase in free radicals, overwhelming the antioxidant capabilities and contributing to ischemia/reperfusion injury in the retina (Shibuki, Katai, Yodoi, Uchida, & Yoshimura, 2000), particularly in the early reperfusion period when flow is restored to energy-depleted tissue. Generation of free radicals are not only through the activation of glutamate receptors but it also is an inevitable by-product of normal oxidative mechanisms (Halliwell, 2001). Specially, this is true in the retina which owns a very high metabolic rate. These free radicals are normally being inactivated by endogenous antioxidants such as superoxide dismutase, Vitamins E and C, and glutathione. However, worthy to be mentioned, when not inactivated sufficiently, the so-called free radicals can react detrimentally with most macromolecular cellular constituents resulting in one: the disruption of membrane fluidity, two: protein denaturation, three: lipid peroxidation, four: oxidative DNA and finally: alteration of platelet functions (D.-O. Kim, Jeong, & Lee, 2003).

1.1.4. Oxidative Stress

Oxygen is essential to life and it plays an essential role in variety of biological phenomena, however, it can also provoke damaging oxidative events within cells. There is a variety of free radicals given rise to by molecules, those that are produced from molecular oxygen have received the most investigative interest, though (Apel & Hirt, 2004). Due to their higher reactivity relative to molecular oxygen, partially reduced metabolites of molecular oxygen (O_2) are referred to as "reactive oxygen species (ROS)" (Tezel, 2006). ROS includes molecules such as superoxide anion, hydrogen peroxide (H. A. Jung et al., 2008), hydroxyl radical, nitric oxide, peroxyl radical and singlet oxygen (S. Jung et al., 2008), are generated as by-products of normal aerobic metabolism or, in other words, as secondary messengers (Choi, Kim, Kim, Yoo, & Chon, 2006) in various signal transduction

pathways (Choi et al., 2006). An increase over physiological values in the intracellular concentrations of ROS is defined as oxidative stress. At the time when there are changes in the endogenous activity of antioxidant enzymes, this situation is being initiated (e.g. catalase, glutathione, superoxide dismutase, metallothionein) and/or at the time of the concentrations regarding certain vitamins (S. Jung et al., 2008; Mostafa, 2006).

Excessive ROS are cytotoxic, as they disturb intercellular homeostasis and impair the mitochondrial transport chain, thus inducing cell death. Mitochondria comprises the greatest amount of ROS, since the mitochondrial transport chain consumes 85% of oxygen that the cell utilizes. Once generated, ROS inhibit complex enzymes in the electron transport chain, resulting in an enhancement of ROS production and a shutdown of mitochondrial energy production. Energy depletion causes failure of many ATP-reliant processes in the cell and destroys cell integrity. Moreover, ROS activates mitochondria-mediated apoptosis pathway, leading to DNA breakdown (Bayr, 2005; Sena & Chandel, 2012). Oxidative stress has been suggested to have the pathogenesis of age-related macular disease contributed to (Jarrett & Boulton, 2012; Virgili, Do, Bressler, & Menchini, 2007). Not only because of photochemical reactions the photoreceptors of the retina are particularly susceptible to oxidative damage (Chrysostomou, Rezania, Trounce, & Crowston, 2013) but also because of their high content of polyunsaturated fatty acid (B. Zhang & Osborne, 2006) and rich supply of oxygen from the choroidal circulation (B. Zhang & Osborne, 2006). Support for the involvement of oxidative stress in age-related macular degeneration (AMD) springs from the finding which notices that daily administration of antioxidant vitamins and/or zinc significantly decelerates the rate of progression in specially patients from high risk atrophic age-related macular degeneration to

neovascular age-related macular degeneration (Fawcett & Osborne, 2007). Another compelling piece of evidence, moreover, stems from mass spectroscopy of drusen (deposits) from postmortem eyes of patients with age-related macular degeneration, which showed many oxidized proteins (Crabb et al., 2002; Fawcett & Osborne, 2007). A causative role in glaucoma has also been suggested to be played by oxidative stress (Mozaffarieh, Grieshaber, Orgül, & Flammer, 2008). As the neurons in glaucoma are at a low energetic state, the scavenging system may not be efficient enough to remove cell metabolites, thus accumulating waste products and generating ROS. Also, substantial evidence exists to bear the suggestion that oxidative stress plays a major part in the pathogenesis of glaucoma or glaucomatous optic neuropathy (Izzotti, Bagnis, & Saccà, 2006).

1.1.4.1. GSH is a Key Endogenous Antioxidant

To name, one of the crucial modulators of cell survival, under both normal and pathological conditions, that would be the endogenous antioxidant tri-peptide glutathione (GSH, γ-glutamyl-L-cysteinyl-glycine) (Circu & Aw, 2012; Franco, Schoneveld, Pappa, & Panayiotidis, 2007). The involvement of GSH exists in many essential cell processes, for instance, DNA synthesis and cell metabolism, yet acting as an antioxidant and scavenger of ROS is its primary function (G. Wu, Fang, Yang, Lupton, & Turner, 2004). There is a reduced and active state GSH exists and is also oxidized to glutathione disulfide (GSSG) upon unstable molecules being reduced, such as ROS. The reduction of ROS by GSH is facilitated by the enzyme GSH peroxidase (GPx) (Dringen, Gutterer, & Hirrlinger, 2000; Takahashi, 2012). The enzyme, glutathione reductase (GR), then returns GSSG to its reduced form GSH (Figure 1-4).

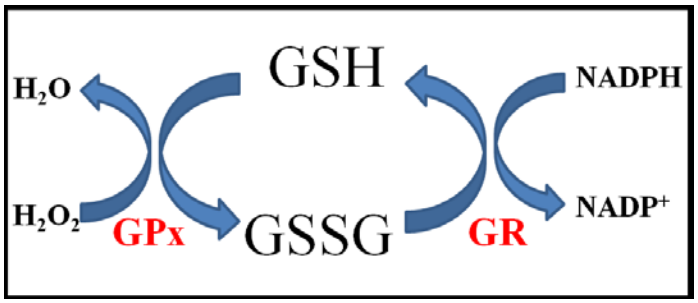

Figure 1-4 Glutathione metabolism
Source: (Circu & Aw, 2012; G. Wu et al., 2004)

There exist two distinct steps in biosynthesis of GSH to happen. The first so-called step occurs when γ-glutamylcysteine ligase (GCL) has glutamate and cysteine combined together. This reaction is, in GSH synthesis, called the rate-limiting step. The second step to be explained in GSH synthesis comes about when GSH synthetase adds up glycine to the C-terminus of the end product of the first step, that is to say, γ-glutamylcysteine. The rate-limiting precursor in GSH synthesis is named Cysteine (Figure 1-5) (G. Wu et al., 2004). Since its potent antioxidant activity plus its neuroprotective capacity have been demonstrated in many *in vitro* and *in vivo* systems, strategies that enhance GSH synthesis draws attention as potentially novel therapies regarding neurodegenerative diseases.

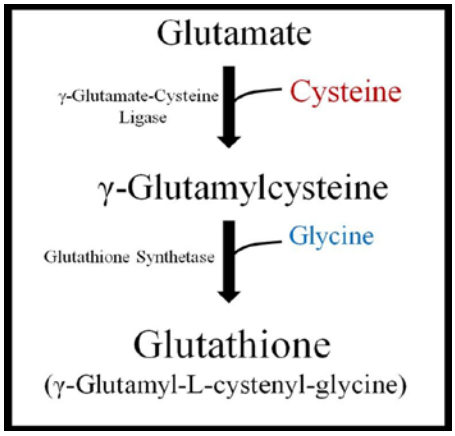

Figure 1-5 Glutathione synthesis
Source: (G. Wu et al., 2004)

1.1.5. Apoptosis

Apoptosis and Necrosis are two distinct mechanisms of cell death, with very different characteristics. In general, necrosis is less organized than apoptosis. Figure 1-6 gives a brief outline of differences between necrosis and apoptosis.

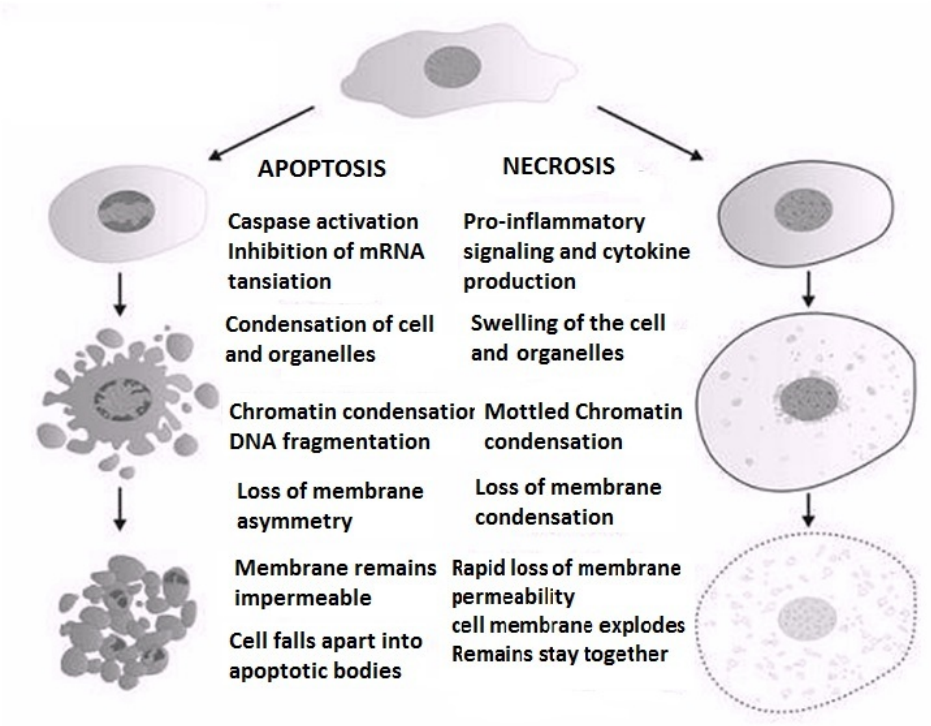

Figure 1-6 Schematic views of the differences between apoptosis and necrosis
Retrieved from: http://www.pasteur.be/pasteur_en
Date of access: July, 10, 2014

Necrotic cell death is caused by the following matters: injury, infection, cancer, infarction and lastly inflammation. In necrosis, cells swell and cytoplasmic organelles are injured, plus random DNA fragmentation which leads to uncontrolled release of enzymes having been stored by lysosomes with a rapid collapse of internal homeostasis. The initiation of cell damage may take place by a reaction due to the membrane lysis and subsequent release of cellular contents that can put surrounding

cells in danger. Unlike apoptosis, the association between necrosis and inflammation is positive (Vanden Berghe et al., 2013).

Apoptosis, or programmed cell death, is energy-dependent and is executed in such a way as to safely dispose of dead cells and their fragments. In contrast to necrosis, apoptosis is carried out in a well ordered process and generally confers advantages during an organism's life cycle. Following an initial insult, the cells try to minimize or buffer the damage done through a variety of processes. Generation of "suicide triggers" could be one of the consequences of these processes and interactions and these molecules may start the process of apoptosis which is characterized by an orderly pattern of inter-nucleosomal DNA fragmentation, chromosome clumping, cell shrinkage and membrane blebbing (Farkas & Grosskreutz, 2001). This is followed by disassembly of cells into multiple membrane-enclosed vesicles that are engulfed by neighboring cells without inciting inflammation.

Up to now two apoptosis pathways have already been described and distinguished, the intrinsic pathway and the extrinsic one. They are initiated by different factors and thus have different biochemical changes, yet both share the same consequences of caspase-3 activation and DNA fragmentation as shown in Figure 1-7.

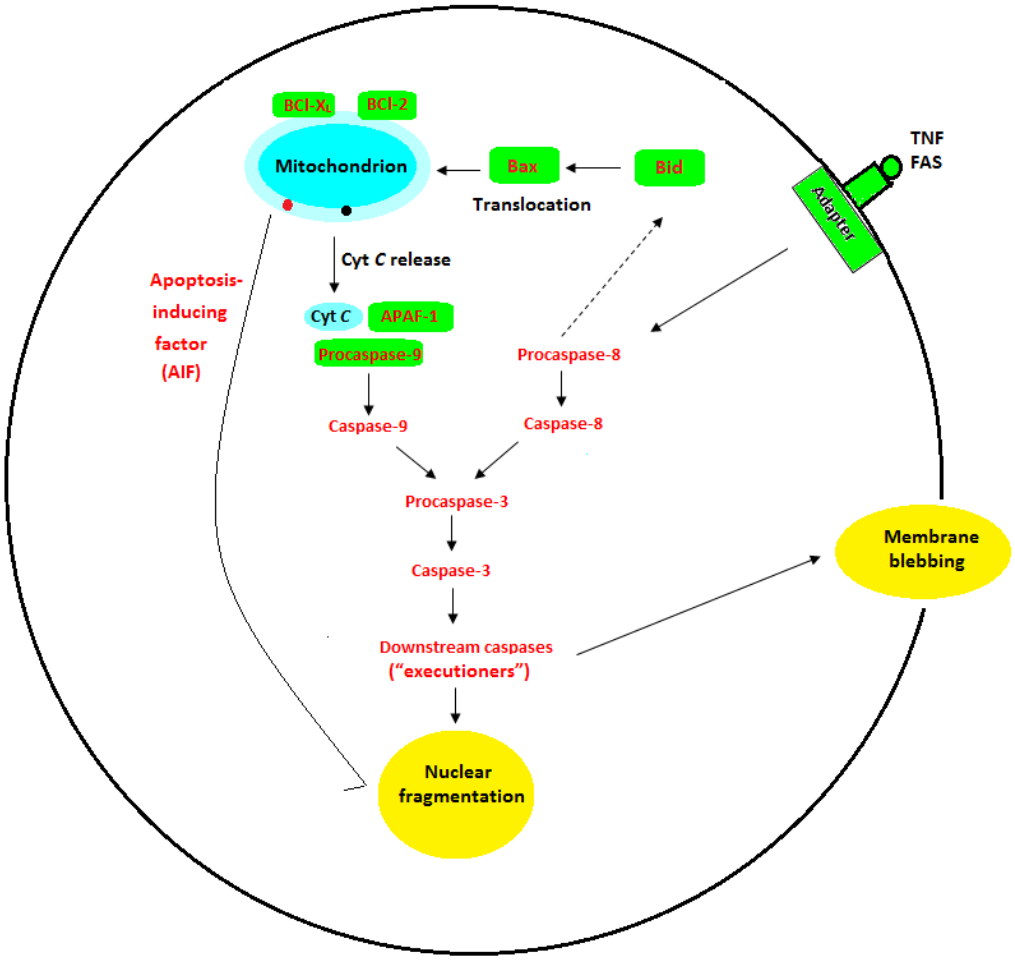

Figure 1-7 Proposed interaction between intrinsic and extrinsic apoptotic pathways
Source: (Schon & Manfredi, 2003)

1.1.5.1. Extrinsic Pathway (Receptor Pathway)

The activation of membrane receptors is the involvement seen in the extrinsic pathway (Figure 1-7). The death signal concerns the binding of extracellular death ligands, such as FAS, glutamate, nitric oxide or tumor necrosis factor-α, which are released by malfunctioning cells, as proposed to be occurred in the region of the optic nerve head (Morgan, 2000; Osborne, Melena, Chidlow, & Wood, 2001), in the initiation of glaucoma.

Such extracellular stimuli can have the intracellular biochemical pathways activated which causes an increase in ROS, then reacts chemically with various targets within the cell in order to elicit a chain of events that is often regulated by the tumour suppressor gene p53. The p53 protein can then alter the expression of several other genes which eventually lead to the catabolic enzymes activation, particularly those that degrade the cellular DNA (nucleases) and proteins (proteases). The death receptors transmit signals to the interior of the cells and recruit procaspase-8, which can be auto-activated by aggregation to caspase-8. Then, this activates procaspase-3 resulting in apoptosis. This process whereby extracellular signals, for instance, glutamate which can stimulate ganglion cell death has been known as the extrinsic pathway of apoptosis (Wajant, 2002, 2003).

1.1.5.2. Intrinsic Pathway (Mitochondria-Dependent Pathway)

In most cell types, however, caspase-8 first cleaves BH3-interacting domain death agonist, the B cell lymphoma 2 family protein, which, in turn, induces the translocation, oligomerization, and also insertion of the other family members, BCL-2-associated X protein and/or BCL-2 antagonist or killer, into the outer mitochondrial membrane (OMM). This is, then, followed by permeabilization of the outer mitochondrial membrane plus the release of several proteins from the mitochondrial intermembrane space, including cytochrome *c*, which forms a cytosolic apoptosome complex with apoptosis having factor-1 (Apaf-1) and procaspase-9 activated (Figure 1-8).

This results in the activation of procaspase-9, which consequently leads to the activation of procaspase-3 and other effector caspases. The distinctive feature regarding this so-called pathway would be the involvement of the mitochondria. Triggering events within the cell to have mitochondria affected can be stress signals

and/or loss of survival signals. The intrinsic pathway would be either caspase-dependent or caspase-independent.

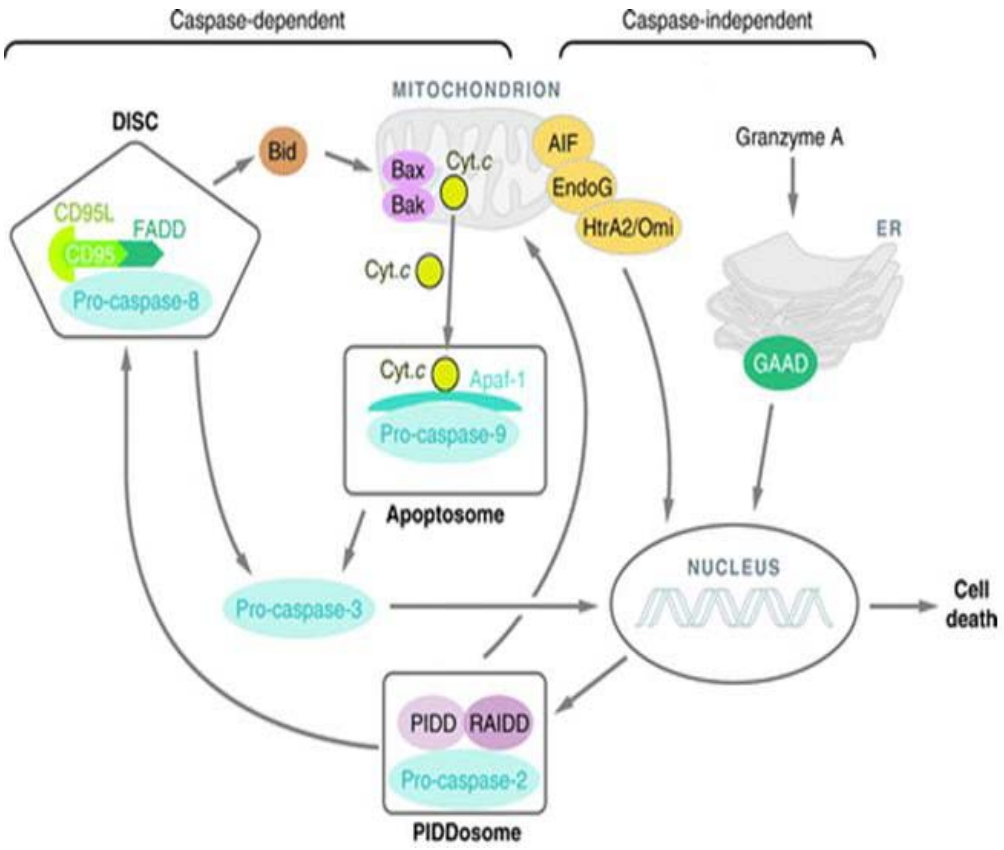

Figure 1-8 General apoptotic pathways
Source: (Orrenius, 2007)

1.1.6. Hypoxia

As the final electron accepter of aerobic respiration, molecular oxygen is essential for the survival of most metazoans. Oxygen deprivation (hypoxia) is often involved in development, homeostasis and many other diseases (Correia & Moreira, 2010; Grimm et al., 2005; Ogunshola & Antoniou, 2009). Multi-cellular organisms, in order to adapt to hypoxia, have evolved complex networks that have metabolic changes regulated in both systemic and cellular levels to mediate changes in

angiogenesis/vascular remodeling, glycolytic metabolism plus cell proliferation (Correia & Moreira, 2010; Grimm et al., 2005; Hopkins & Powell, 2001). The oxygen tension-dependent transcriptional factor, hypoxia inducible factor-1 (HIF-1), holds the responsibility for the induction of genes which is responsible to facilitate the adaption and survival of cells exposed to hypoxia. HIF-1 activation induces a diverse range of target genes, encompassing a wide variety of cellular processes, including angiogenesis, erythropoiesis, energy metabolism, cell proliferation, and cell cycle control (Ke & Costa, 2006; Schumacker, 2005). HIF-1 can improve the redox environment (Guo, Miyake, Liu, & Shi, 2009), increase blood oxygen plus glucose supply, and have an effect on iron metabolism by regulating its target genes. The brain consumes a large quantity of oxygen and demonstrates a high vulnerability at conditions with impaired oxygen supply. It has been noticed that reduced oxygen supply plays an influential role in neurodegeneration during the aging process (Ogunshola & Antoniou, 2009). Oxidative stress, impaired oxygen or glucose supply, and disruption of iron homeostasis would all be considered as pathological processes which are common in neurodegenerative diseases (Benarroch, 2009; Correia & Moreira, 2010; Gironi et al., 2010). This makes up the very possibility that HIF-1 could be a potential therapeutic target for these neurodegenerative diseases.

1.1.6.1. Hypoxia-Induced Factor (HIF)

Simple diffusion of oxygen to metabolizing tissues beyond a size limitation has become inadequate; furthermore, specialized systems of increasing complexity have evolved to meet the demands of oxygen delivery higher in animals (Pugh & Ratcliffe, 2003). One important role in the systems is angiogenesis, to make new vessels sprouting into the location that blood delivery is being needed. Thus, one of the key factors which would lead to the initiation of angiogenesis is ischemia or

hypoxia. Yet how hypoxia exactly induced angiogenesis is however poorly understood. The landmark of hypoxia study in the early 1990s indicated that hypoxia could only induce expression of platelet-derived growth factor (PDGF) mRNA and vascular endothelial growth factor (VEGF) mRNA in tissue culture. Both platelet-derived growth factor (PDGF) and VEGF are thought to be crucial growth factors triggering angiogenesis. A large number of genes are engaged in diverse steps in angiogenesis and they also are independently responsive to hypoxia in tissue culture. Besides platelet-derived growth factor and VEGF, nitric oxide synthase, fibroblast growth factor, angiopoietins, and matrix metalloproteinases are involved. Cell migration or endothelial tube formation which are of the individual phenotypic processes in angiogenesis can be induced by hypoxia tissue culture (Krishnamachary et al., 2003).

Further study of hypoxia-induced angiogenesis leaded to the discovery of a key transcriptional regulator, hypoxia-inducible factor (HIF)-1. The molecular mechanism behind HIF-1 is a pathway which has oxygen availability plus the gene expression of several different growth factors linked, especially VEGF. In normoxia and hyperoxia oxygen-dependent prolyl hydroxylases hydroxylate HIF-1 α proline residues, and this chemical modification leads to a HIF-1 capture by an ubiquitin ligase complex that directs it to the proteasome for destruction. Under hypoxic conditions, HIF-1α is not hydroxylated, escapes ubiquitination, accumulates and directs pro-angiogenic expression (Maxwell & Ratcliffe, 2002).

1.1.7. Role of Neuroinflammation

Neuroinflammation and protein aggregates pathology (e.g. amyloid plaques, Lewy Bodies) are another theory which explains the cause of neurodegeneration (Frank-Cannon, Alto, McAlpine, & Tansey, 2009; Khandelwal, Herman, & Moussa,

2011). Based on this theory, following stimuli, specifically protein aggregates for instance, amyloid plaques, the neuroinflammatory cycle is generated followed by microglia and astrocytic activation (Skaper, 2007). Controlled biosynthesis, maturation, function and terminal breakdown of proteins are factors that play crucial role in maintenance of a healthy organism (Reiser, Adair, & Reinheckel, 2010). Proteolytic enzymes donate all the above processes irreversible cleavage of peptide bonds in a polypeptide chain by a nucleophilic attack on the carbonyl carbon. The proteases are acting in two ways by either cleaving one or a few amino acids at the N or C terminus, which called exopeptidases protease or endopeptidases one, cleaving internal peptide bonds (Barrett & Rawlings, 2007; Puente, Sánchez, Overall, & López-Otín, 2003). Endopeptidases are classified, in terms of their catalytic mechanism, into threonine, aspartic, metallo, serine and cysteine endopeptidases (Vos et al., 2000). The latter, cysteine proteases, form the largest cathepsin family. The human family of cysteine cathepsins comprises cathepsins B, C (also known as cathepsin J and dipeptidyl-peptidase 1), F, H, K (also known as cathepsin O2), L, O, S,W, V (also known as cathepsin L2), and X (also known as cathepsin Z and cathepsin P), which share a conserved active site formed by cysteine, histidine and asparagines residues (Rawlings, Barrett, & Bateman, 2012).

Cysteine cathepsins are synthesized as inactive precursors, normally activated in the acidic environment of lysosomes. Therefore, they were initially considered as intracellular enzymes, responsible for the non-specific, bulk proteolysis in the acidic environment of the endosomal/lysosomal compartments, where they degrade intracellular and extracellular proteins (B. Turk, Turk, & Turk, 2000; V. Turk, Turk, & Turk, 2001). Furthermore, they are involved in proteolytic processing of specific substrates. Cathepsin plays an important role in protein, neuropeptide and hormone

processing (Funkelstein, Toneff, Hwang, et al., 2008; Funkelstein, Toneff, Mosier, et al., 2008), major histocompatibility complex (MHC) classII-mediated antigen presentation (Honey & Rudensky, 2003), bone remodeling (Yasuda, Kaleta, & Brömme, 2005), apoptosis (B. Turk et al., 2002) and to keratinocyte differentiation. Apart their normal physiological roles, cysteine cathepsins are involved in several pathologies, such as tumour development and progression , inflammation (Conus & Simon, 2008), psoriasis , muscular dystrophy, atherosclerosis (J. Liu et al., 2006), rheumatoid arthritis (Yasuda et al., 2005), osteoporosis and other bone disorders (Conus & Simon, 2008; J. Liu et al., 2006; Stoch & Wagner, 2007; Yasuda et al., 2005), acute pancreatitis (Halangk et al., 2000) and neurodegeneration (Nakanishi, 2003b).

It is extensively well-accepted that overexpression, increased enzymatic activity and mis-localization of cysteine cathepsins in cells of the nervous system are associated with pathological processes in neurodegenerative disorders (Hook, 2006; Lynch & Bi, 2003; Yamashima, 2000). Dysfunction of the endosomal/lysosomal system in neurons is closely associated with activation of microglia, which could initiate an inflammatory response to provoke neurodegeneration. Activated microglia also releases certain cathepsins that induce neuronal death through degradation of extracellular matrix proteins (Figure 1-9) (Nakanishi, 2003a; Pišlar & Kos, 2014).

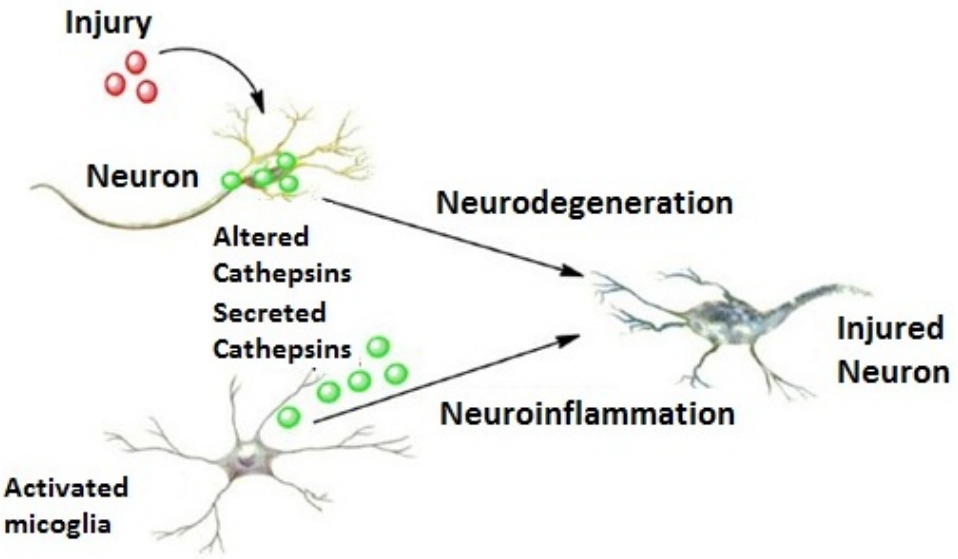

Figure 1-9 Cysteine cathepsins involvement in neurodegeneration and inflammation-induced neurodegeneration

Source: (Pišlar & Kos, 2014)

Lysosomal cysteine cathepsins can be altered by many toxic insults leading to enhanced cathepsin expression and proteolytic activity, which contribute to neuronal injury during neurodegeneration. Activated microglia mediate neuroinflammation by secreting inflammatory cytokines thereby, inducing neuronal death. In addition to cytokines, activated microglia secretes certain cathepsins thus upscaling inflammation-induced neurodegeneration.

During neurodegeneration, cathepsins contribute to neuronal injury induced by excitotoxins, through degradation of axonal and myelin proteins, by converting protein precursor into active peptide neurotransmitters and by amplifying apoptotic signaling (Haque, Banik, & Ray, 2008; Hook, 2006). Furthermore, a central role in the neuronal cell death mechanism has been proposed for cathepsins (Stoka, Turk, & Turk, 2005). In terms of which cysteine cathepsins are specifically involved in neurodegeneration, cathepsins B and L have been investigated most intensively. They have been reported to induce age-related changes that are closely related to neuronal degeneration (Yamashima, 2000). Due to the harmful action of cysteine cathepsins in pathological processes of neurodegeneration (Benchoua, Braudeau,

Reis, Couriaud, & Onténiente, 2004; Chwieralski, Welte, & Bühling, 2006), cathepsin inhibitors constitute a possible tool for therapeutic interventions to inhibit excessive proteolytic activity (Haque et al., 2008; Stoch & Wagner, 2007). Some beneficial effects of cystatins have been demonstrated; however, they are general inhibitors, not selective for particular cathepsins therefore, and off-target side effects are expected if they are used as drugs for treating patients. Instead of cystatins and other endogenous cysteine protease inhibitors therefore, synthetic cathepsin inhibitors have been suggested as agents for treating neurodegenerative disorders (Booth, 2012; Pišlar & Kos, 2014; Tyynelä et al., 2000).

1.2. The Anatomy of the Eye

Optically working like a film camera, the eyes of all the vertebrates are structurally similar. The light enters the eye through the pupil and forms an inverted image on the retina, the light-capturing component that functions like the film in a camera (Drack, 2006). The cornea and the lens help to focus so that the clearest image is presented on the retina. The white outer surface of the eye ball is termed sclera, which consists of tough but flexible fibrous tissue and provides the mechanical support of the entire eye. The choroid is a layer contained within the sclera, and it is a dense meshwork of blood vessels and other tissues. One of the most important functions of the choroid is to provide nutritional and metabolic support for the retina, which is a neuronal sheet that lies within the choroid. The retina is the most inner surface at the back of the eye. Most of the space in the eye is filled with a gelatinous body, called vitreous. It is surrounded by the lens and the retina and the ciliary body. In the ciliary body, the cells secrete the aqueous fluid into the eye, which contributes to the maintenance of the pressure within the eye (Irsch & Guyton, 2014; Purves et al., 2001).

1.2.1. The Retina

The retina, a layer about 0.4 mm in thickness, is primarily composed of neural tissue including five classes of neurons. It spreads out on the interior surface of the back of the eye (Figure 1-10). The visual pathway is initiated when the light stimulates the photoreceptors that are embedded in the outer retinal layers. The signal is transmitted to bipolar cells and then to ganglion cells. The signal then travels along the axon of the ganglion cells lining inner nuclear layer, respectively, while amacrine cells contact with bipolar axons primarily in the inner plexiform layer (Boya, 2012; Rabin, 2013).

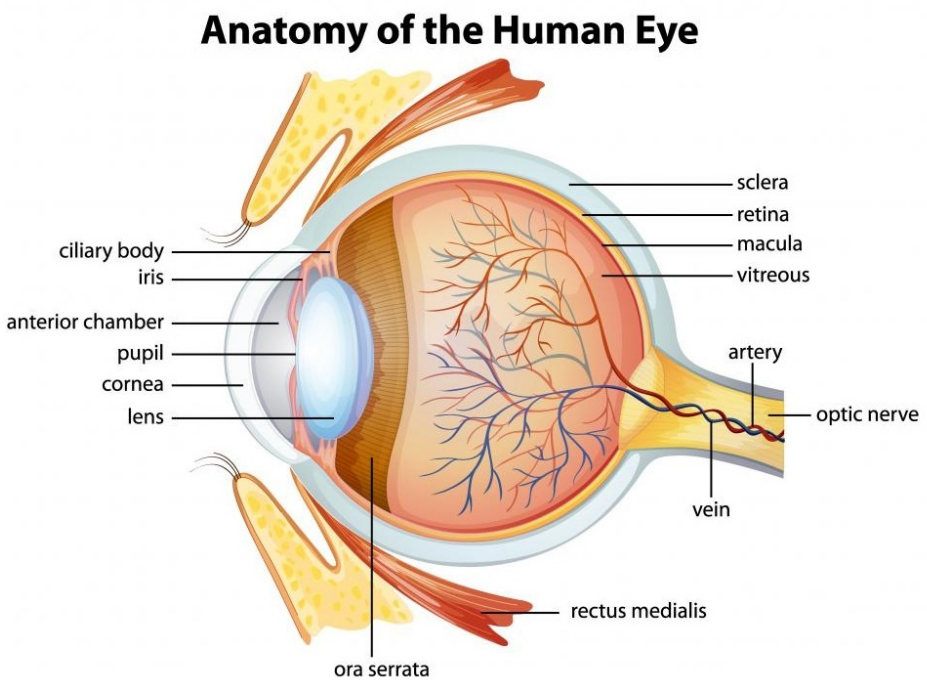

Figure 1-10 Basic structure of human eye
Retrieved from: http://www.nei.nih.gov
Date of access: July, 21, 2014

Light passes through almost the whole thickness of the retina to be captured by photoreceptors, or the outer segments of the photoreceptor in detail, where the visual pigment molecules for light capturing are located. There are two types of

photoreceptors, rods and cones (Boya, 2012). Rods are specialized to convey variations in light intensity in dim conditions, but they are not able to function in bright light. Cones are specialized for bright light conditions, but they are not as sensitive as rods. The retina cross section can be divided into multiple layers. The nuclear layers are basically where cell nuclei are located, and the synaptic layers are the place where cells communicate and transmit electric or chemical signals. The retinal pigment epithelium (RPE) functions as the outer blood-retinal barrier (BRB) that shut off the diffusion of large molecules from choroicapillaries. And the retinal vasculature doesn't grow beyond the inner limiting membranes under normal physiological conditions.

1.2.2. Retinal Ganglion Cells

The innermost neuronal layer of the retina is where the retinal ganglion cells are located in. The axons of the retinal ganglion cells form the nerve fiber layer (stratum opticum) which is situated on top of the retinal ganglion cell soma, next to the side of the vitreous. These axons conjoin in the central retina as optic nerve head and thus form thereafter the optic nerve, which extend after the optic chiasm as optic tract in the optic center of the brain (Provencio, Rollag, & Castrucci, 2002). Different subpopulations of retinal ganglion cells exist (Vaney, Sivyer, & Taylor, 2012) which use different neurotransmitter gamma-aminobutyric acid (GABA), glycine or glutamate (Iuvone, 2012). The retinal ganglion cells, apart from sending photoreceptor derived signal into the optic center of the brain, perform different functions. For instance, photosensitive retinal ganglion cell are involved in the pupillary reaction and circadian rhythm (Lucas, Douglas, & Foster, 2001; T. M. Schmidt, Chen, & Hattar, 2011).