An investigation into pro-apoptotic targets in experimental glaucoma and the neuroprotective effects of Ginkgo biloba in retinal ganglion cells

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Declaration

I, Abeir Baltmr, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Ginkgo biloba has been advocated as a neuroprotective agent for several years in glaucoma. In this study, immunohistochemistry was used to identify known potential molecular targets of Ginkgo biloba related to retinal ganglion cell (RGC) apoptosis in experimental glaucoma, including amyloid precursor protein (APP), Aß, cytochrome *c*, caspase-3 and tumor necrosis factor receptor-1 (TNF-R1). Furthermore, using apoptotic inducers related to mechanisms implicated in glaucoma, namely Dimethyl sulphoxide (DMSO), ultraviolet C (UVC) and Sodium Azide (NaN_3), the effects of the terpenoid fraction of Ginkgo biloba (Ginkgolide A, Ginkgolide B and Bilobalide) were investigated separately in cultured retinal ganglion cells (RGC-5). Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and morphological analysis of DMSO treated RGC-5 was performed using Hoechst 33342 stain.

Immunohistochemistry showed a strong inverse correlation between Aß and APP in ocular hypertension (OHT) animals, with APP and Aß accumulation peaking at 1 and 12 weeks after intraocular pressure (IOP) elevation respectively. Cytochrome *c* and TNF-R1 expression peaked at 3 weeks, and active caspase 3 activity at 12 weeks after IOP elevation. 1% DMSO, UV40, 1mM NaN_3 and 50µM Aβ25-35 dose dependently reduced RGC-5 survival at 24 hours by 27%, 20%, 35% and 27% respectively. These effects were inhibited by Ginkgolide A, Ginkgolide B and Bilobalide in different assays at different levels. In these experiments, all three compounds showed a dose-related response although some intrinsic toxicity was observed with Ginkgolide A.

Ginkgolide B had the most profound neuroprotective effects in the majority of assays at a concentration range of 0.5-5µg/ml, whereas Ginkgolide A and Bilobalide had variable activity.

Although the effect of simultaneous administration of all three fractions was not assessed, work in this thesis suggest that Ginkgolide B can be neuroprotective to RGCs in preventing apoptosis and cell death, therefore may be of use as a neuroprotective strategy in glaucoma management.

Key Word: glaucoma, neuroprotection, retinal ganglion cell apoptosis, Ginkgo biloba

Dedication

For the spirit of my parents
You will live forever in my heart

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List of Abbreviation

AAVs	Adenoassociated viruses
AAV-CBA	Adeno-associated viral vector using chicken-β-actin
Aβ	Amyloid β
ACh	Acetylcholine
ACh-R	Acetylcholine Receptors
AD	Alzheimer's disease
Ads	Adenoviruses
AIF	Apoptosis inducing factor
Akt	Serine/threonine protein kinase
ALS	Amyotrophic lateral sclerosis
AMD	Age Related Macular Degeneration
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP1	Activator protein-1
Apaf-1	Apoptotic protease-activating factor-1
APP	Amyloid precursor protein
AS	Atherosclerosis
ATM	Alzheimer transgenic mouse
BDNF	Brain-Derived Neurotrophic Factor
Bil	Bilobalide
BP	Blood pressure
BSI	β-secretase inhibitors
Ca^{2+}	Calcium
CAT	Catalase
CCT	Central corneal thickness
C/D	Cup to disc
CNS	Central nervous system
CNTGS	Collaborative Normal Tension Glaucoma Study
Cop-1	Copolymer-1
CoQ_{10}	Coenzyme Q_{10}
CP 205	Flavonoid fraction
CPP32	Caspase 3 activators
CTGF	Connective tissue growth factor
D	Diopter
DAPI	4′, 6-Diamidino-2-phenylindole dihydrochloride
DBA/2J	Mouse model of Glaucoma
DDS mouce	Dextran sulfate sodium mouse model of colitis
DEX t	Dexamethasone treatment
DM	Diabetes Mellitus
DMEM	Dulbecco's modified Eagle's medium
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulphoxide
DNQX	6,7-Dinitroquinoxaline-2,3-dione
DPP	Diastolic perfusion pressure
DXM	Dextromethorphan

ECM	Extracellular matrix
EDV	End diastolic velocity
EGCG	Epigallocatechin Gallate
EMGT	Early Manifest Glaucoma Trial
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
Erk1/2	Extracellular signal-regulated kinases
FBS	Fetal Bovine serum
FCI mouse	Focal cerebral ischemia mouse model
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FIV	Feline immunodeficiency virus
GABA	ɣ-aminobutyric acid
GBB	Ginkgo biloba extract with higher level of terpene
GBE	Ginkgo biloba extract
GGA	Geranylgeranylacetone
GA	Ginkgolide A
GB	Ginkgolide B
GSH-Px	Glutathione peroxidase
HE 208	Terpene and flavonoid-free EGb 761
HIV	Human immunodeficiency virus
HLECs	Human lens epithelial cells
HR	Heart rate
HSPs	Heat shock proteins
HSVs	Herpes simplex viruses
6-HKA	6-hydroxykynurenic acid
IAP	Inhibitors of apoptosis protein
IL	Interleukin
IFN-γ	Interferon-gamma
IGF-1	Insulin-like growth factor-1
iNOS	Inducible nitric oxide synthase enzyme
IOP	Intraocular pressure
IP	Intraperitoneal
IS	Immune Stimulated
JNK	C-Jun N-terminal kinases
K^+	Potassium
kDa	Kilo Dalton
LGB	Lateral Geniculate Body
LIRD	Light-induced retinal damage
LoGTS	Low Pressure Glaucoma Treatment Study
LPs	Liposomes
LPS	Lipopolysaccharide
LVs	Lentiviruses
MAO	Monoamino Oxidase
MAP	Mean arterial pressure.
MCAO	Middle Cerebral Artery Occlusion

MDA	Malondialdehyde
MK801	5-methyl-10, 11-dihydro-5H-dibenzo [a, d]cyclohepten-5, 10-imine maleate
MHC 9	Major Histocompatibility Complex 9
MMP	Mitochondrial membrane potential
MPTP	1methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple Sclerosis
MYOC	Myocilin
mtDNA	Mitochondrial DNA
mPTP	Mitochondrial permeability transition pores
MTT	3-4,5-dimethyithiazol-2-yl-2,5-diphenyl-tetrazolium bromide
Na^+	Sodium
NaN_3	Sodium Azide
NF-κBp65	Nuclear factor- kappa Bp65
NF-κB	Nuclear factor-kappa B
NFL	Nerve Fiber Layer
NGF	Nerve Growth Factor
NMDA	N-methyl-D-aspartic acid
NO	Nitric oxide
NOS	Nitric Oxide Synthase
NT	Neurotrophine
O^{2-}	Superoxide anion
OA	Ophthalmic artery
OAG	Open-angle glaucoma
OBF	Ocular blood Flow
OD	Occulus dexter
OHT	Ocular Hypertension
ONOO-	Peroxynitrite
OPP	Ocular perfusion pressure
OS	Occulus sinister
PAF	Platelet activating factor
PAF-R	Platelet activating factor receptor
PBS	Phosphate buffered saline
PC 12	Pheochromocytoma cell line
PGE	Prostaglandin
PKC	Protein Kinase C
PI	Propidium iodide solution
PI3K	Phosphoinositide-3-kinases
POAG	Primary open angle glaucoma
PS	phosphatidyl serine
PSD	Pattern standard deviation
PTP	Mitochondrial permeability transition pore
PVCRD	Peripheral vitreochoreoretinal dystrophies
RAW 264.7	Macrophage cell line
RGC	Retinal ganglion cells
RD	Retinal detachment
ROS	Reactive Oxygen Species

RPE	Retinal pigment epithelium
rtp	Room temperature
SD	Sprague-Dawley
SBP	Systolic blood pressure
SHRSP	Stroke-prone spontaneously hypertensive rats
SH-SY5Y	Human neuroblastoma cells
Sig	Significant
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SPP	Systolic perfusion pressure
SS	Serum Starvation
SSP	Staurosporine
TNBS	2,4,6-trinitrobenzene sulfonic acid
TGF-ß1	Transforming growth factor-ß1
THP-1	human monocytic macrophage like cells
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor-alpha
TNF-R1	Tumor necrosis factor receptor 1
TNF-R2	Tumor necrosis factor receptor 2
Trk A	Neurotrophic tyrosine kinase receptor type 1
Trk B	Neurotrophic tyrosine kinase receptor type 2
Trk C	Neurotrophic tyrosine kinase receptor type 3
T-SOD	Total superoxide dismutase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UEG	Unilateral experimental glaucoma
UV	Ultraviolet
UVB	Ultraviolet Subtype B
UVC	Ultraviolet Subtype C
UV40	40 mj/cm^2 UVC
V/F	Visual field
Z-VLL-CHO	N-benzyloxycarbonyl-Val-Leu-leucinal

Chapter one:

1. Introduction

1.1 Glaucoma

1.1.1 Overview

Glaucoma is a distinctive group of progressive optic neuropathies characterized by gradual degeneration of neuronal tissue due to RGC loss, with accompanying loss of visual field over time (Quigley et al., 1995;Gupta and Weinreb, 1997). It is a leading cause of irreversible blindness estimated to affect 79.6 million people worldwide by 2020 (Quigley and Broman, 2006). Glaucoma can be classified into congenital (developmental) or acquired, and based on the mechanism of aqueous outflow obstruction, it is further subdivided into open-angle glaucoma (OAG) and closed-angle glaucoma types. Each classification can be primary, when it is not associated with another cause and secondary, when there is an ocular or non-ocular disorder affecting aqueous dynamics.

1.1.2 Glaucoma risk factors

Risk factors have been identified both for developing OAG, and for its progression. Although intraocular pressure (IOP) is the most important risk factor in the pathogenesis of glaucoma, optic nerve cupping and visual field loss can progress despite successful reduction in IOP via medical and surgical approaches (Leske et al., 1999;Oliver et al., 2002;Rossetti et al., 2010). In the Early Manifest Glaucoma Trial (EMGT) around 27% of treated patients with 30% reduction in IOP showed progression even after 6 years of follow up (Leske et al., 2003;Leske et al., 2007). Moreover, 12% of treated patients recruited in the Collaborative Normal Tension Glaucoma Study (CNTGS) revealed progression in the course of the disease after 7 years of follow up (Group, 1998). Table 1.1 shows risk factors for developing

glaucoma and table 1.2 shows risk factors for glaucoma progression.

Table 1.1 Risk Factors for developing Glaucoma

Parameter	Risk factor	References
Age	> 40 years	(Quigley and Vitale, 1997;Mukesh et al., 2002;de Voogd et al., 2005;Boland and Quigley, 2007;Leske et al., 2008)
Ancestry	African Caribbean	(Tielsch et al., 1991;Wormald et al., 1994;Leske et al., 2008)
Family History	1st degree relatives	(Tielsch et al., 1994;Boland and Quigley, 2007)
IOP	>21 mm Hg	(Kass et al.;Bengtsson et al., 2007;Leske et al., 2007)
C/D Ratio	> 0.7	(Le et al., 2003)
Myopia	> 2 D	(Mitchell et al., 1999;Ramakrishnan et al., 2003)
CCT	< 555 µm	(Leske et al., 2008)
Lower Ocular SPP, DPP & MPP	SPP=SBP-IOP DPP=DBP-IOP MPP= 2/3MAP-IOP	(Leske et al., 2008)

IOP= intra ocular pressure, C/D= cup to disc, D= diopter, CCT= central corneal thickness, SPP= systolic perfusion pressure, DPP= Diastolic perfusion pressure, MPP= mean perfusion pressure, MAP = mean arterial pressure.

Table 1.2 Risk factors for Glaucoma Progression

Parameter	Risk factor	References
Age	> 68 years	(Gordon et al., 2002;Leske et al., 2007)
IOP	> 21 mmHg	(Gordon et al., 2002;Heijl et al., 2002;Bengtsson et al., 2007;Leske et al., 2007)
Bilateral involvement	positive	(Leske et al., 2003;Leske et al., 2007)
CCT	< 555 µm	(Brandt, 2004;Leske et al., 2007;Miglior et al., 2007;Brandt et al., 2008)
Greater V/F PSD per 0.2 dB	positive	(Gordon et al., 2002;Miglior et al., 2007)
Disc haemorrhage	positive	(Leske et al., 2003;Leske et al., 2007;Bengtsson et al., 2008)
Pseudoexfoliation	positive	(Leske et al., 2003;Grodum et al., 2005;Leske et al., 2007)
Vertical C/D ratio	> 0.7	(Gordon et al., 2002;Miglior et al., 2007)
Vertical C/D ratio asymmetry	positive	(Miglior et al., 2007)
SPP	≤125 mmHg	(Leske et al., 2007)
SBP	≤160 mmHg	(Leske et al., 2007)
Diabetes mellitus	positive	(Lichter et al., 2001)
Cardiovascular diseases	positive	(Leske et al., 2007;Miglior et al., 2007;Brandt et al., 2008)

V/F= visual field, PSD=Pattern standard deviation, SPP=systolic perfusion pressure, SBP=systolic blood pressure.

1.1.3 Cell Death Mechanisms

Cellular breakdown can be categorized into three widely identified forms: apoptosis, necrosis and autophagic cell death (Jellinger, 2001;Elmore, 2007;Kroemer et al., 2009;Levine and Kroemer, 2009).

1.1.3.1 Apoptosis

Apoptosis, a gene-directed programmed form of cell death, is an energy-dependent process essential in embryogenesis and tissue homeostasis (Wenzel et al., 2005). Inappropriate apoptosis is heavily implicated in the pathogenesis of many neurodegenerative diseases (Thompson, 1995;Perry et al., 1998) including glaucoma (Quigley et al., 1995;Cordeiro et al., 2004). A specific series of morphological features including formation of apoptotic bodies, chromatin condensation and DNA fragmentation within an intact cell membrane are characteristically seen (Kerr et al., 1972;Kerr, 2002). Apoptosis is brought about by the intrinsic (mitochondrial) pathway mediated via cytochrome *c* release (Ekert and Vaux, 2005) or the extrinsic (the death receptor) pathway involving transmembrane receptors which are members of tumor necrosis factor (TNF) receptor superfamily (Locksley et al., 2001). The intrinsic apoptotic pathway can be either caspase-dependent or caspase-independent. In the dependant pathway the proteolytic activity of caspases, aspartate-specific cysteine proteases, appears to be central with initiators (caspase-8 and 9) and the executors (caspase-3, 6 and 7). A caspase-independent pathway can be activated by an increased intracellular calcium level and is mediated via the activity of other effectors such as calpain-mediated release of apoptosis inducing factor (AIF) and calpain-mediated release of caspase-12 (different from traditional caspases). AIF and caspase12 both translocate to the nucleus to participate in DNA damage (Tezel and Yang, 2004;Sanges et al., 2006;Sanges and Marigo, 2006). However, it has becoming increasingly apparent that the caspase-dependent and the caspase-

independent pathways are not separate entities but that they are linked together at certain points (Igney and Krammer, 2002). Nonetheless, it is believed that each pathway has different mechanisms. So far, there are a variety of proposed triggers for apoptosis including abnormal aggregation of subcellular proteins, oxidative stress, inflammation and immune system modulation, and withdrawal of neurotrophic support, which will be discussed in further detail later.

1.1.3.2 Necrosis

In comparison with apoptosis, necrosis, which is considered to be a passive pathological process of cellular breakdown, involves swelling of the cytoplasmic membrane with formation of cytoplasmic vacuoles, loss of plasma membrane integrity and subsequently loss of intracellular contents in the absence of DNA fragmentation (Kerr et al., 1972;Nicotera et al., 1999a;Nicotera et al., 1999b). Although the causative factors of necrotic cell death are still unspecified, several interconnecting elements have been implicated including energy depletion and direct injury to the cell membrane (Kroemer et al., 2009). An activation of death domain receptors such as tumor necrosis factor receptor 1 (TNFR1) and Toll-like receptors such as TLR3 have been observed to provoke necrotic cell death (ibid).

1.1.3.3 Autophagic cell death

The term "autophagy" from Greek means "self-eating", and autophagic cell death, another form of cellular breakdown, is characterized by sequestration and vacuolization of cytoplasm and organelles and degradation by the cell's lysosomes. It is an energy-dependent process in which chromatin condensation, DNA fragmentation and phagocytosis are not seen (Kroemer and Levine, 2008;Levine and Kroemer, 2008;Kroemer et al., 2009;Levine and Kroemer,

2009). Activation of autophagy in RGCs has been demonstrated after optic nerve transection, and suggested to have a cell-protective role in neurodegenerative diseases (Kim et al., 2008).

1.1.4 Mechanisms of RGC death in glaucoma

In chronic glaucoma retinal ganglion cell (RGC) apoptosis appears to be an early event (Quigley et al., 1995;Kerrigan et al., 1997;Cordeiro et al., 2004). It is estimated that at least 25% to 35% RGCs in human eye with glaucoma (Kerrigan-Baumrind et al., 2000) and up to 50% RGCs in the animal model of experimental glaucoma (Harwerth et al., 2002) must have been lost before a visual filed defect can be detectable.

Several damaging mechanisms have been implicated in the pathogenesis of apoptotic RGC death in glaucoma; although a single causative mechanism has yet to be identified. Even though IOP may be viewed as a direct inducer of RGC stress and apoptosis (Guo et al., 2005a;Kwon et al., 2009), damage to RGCs can occur even in the presence of a low level of IOP (Sommer, 1989;Drance, 2004). In conditions of elevated IOP, mechanical stress on the lamina cribrosa can lead to initial axonal degeneration, as suggested by Howell et al (2007) using the DBA/2J mouse model of glaucoma. This study also suggested that it is possible to protect RGCs against such damage using the Wallerian degeneration slow allele, which allowed functional protection from glaucoma in the DB2-J2.Wld^{s} mouse model (Howell et al., 2007). Glaucoma appears to be of a multifactorial nature with complex genetic and environmental factors (Fingert et al., 1999;Ray et al., 2003;Libby et al., 2005;Mabuchi et al., 2007). In addition to IOP, several other stress inducer factors have been identified such as tissue hypoxia (Kaur et al., 2008;Tezel et al., 2010), and glial cell activation (Lebrun-Julien et al., 2009).

The various proposed mechanisms of RGCs death in glaucoma have been investigated

through the use of a variety of *in vitro* and *in vivo* studies include: oxidative stress (Neufeld et al., 1999;Ko et al., 2005;Tezel, 2006), protein misfolding (McKinnon et al., 2002a;Yoneda et al., 2005;Guo et al., 2007a), inflammation (Tezel et al., 2001;Tezel et al., 2007), mitochondrial dysfunction (Mittag et al., 2000;Tatton et al., 2001;Tezel and Yang, 2004), and excitotoxicity (Dreyer et al., 1996;Osborne et al., 1999;Guo et al., 2006;Salt and Cordeiro, 2006). These mechanisms will be discussed further below:

1.1.4.1 Oxidative stress

Oxidative stress is a pathological condition in which the rate of reactive oxygen species (ROS) production exceeds the body's anti-oxidative capacity. ROS are partially reduced, highly reactive metabolites of molecular oxygen, containing an unpaired electron. ROS is generated primarily via the electron transport chain at relatively low levels during aerobic metabolism and plays an integral part in signal transduction. Ischemia potentially by vascular dysregulation and reperfusion injury to cells are critical inducers for oxidative stress (Flammer et al., 1999), leading to further ROS generation with ATP depletion and mitochondrial failure, triggering the caspase-dependent and caspase-independent mitochondrial cell death pathways (Murphy, 1999).

The increased levels of ROS enhance lipid peroxidation, protein peroxidation (Siu and To, 2002) and single strand breaks in nucleic acids (Finkel, 1998;Finkel and Holbrook, 2000). ROS have also been found to induce Muller cell activation and dysfunction, generating further oxidative material (Yuan and Neufeld, 2001;Neufeld and Liu, 2003;Tezel et al., 2003).

ROS and NO pathways

ROS generation has been implicated in the pathogenesis of glaucoma (Bonne et al.,

1998;Levin, 1999), inducing remolding and excavation of the lamina cribrosa, damaging the trabecular meshwork and facilitating glutamate–dependant RGC death (Fern et al., 1993;Chidlow et al., 2007;Cheung et al., 2008a). In glaucomatous stress, excessive production of ROS enhances the production of nitric oxide (NO) (Neufeld, 1999;Neufeld and Liu, 2003), which can have direct cytotoxic effects on the RGCs in a caspase-dependent and caspase-independent manner.

The extent of the oxidative damage appears to depend on the type and reactivity of ROS, the rate of their production and the cellular antioxidant defense mechanism. The retinal protection against the damaging effects of ROS is composed mainly of glutathione peroxidase, glutathione reductase, glutathione S-transferase, ascorbic acid, catalase, and superoxide dismutase (Ferreira et al., 2004). An increased level of autoantibodies against glutathione S-transferase (Yang et al., 2001), and a compromised antioxidant capacity (Gherghel et al., 2005) have been documented in glaucoma patients. Corresponding data in the rat model of raised IOP displayed increased levels of antioxidant enzymes (Moreno et al., 2004). Excessive production of ROS enhanced the production of NO in the rat model of glaucoma (Siu et al., 2002) and in astrocytes and glial cell at the optic nerve head of glaucoma patients, enhancing activation of the N type Ca^{2+} channels in RGCs (Hirooka et al., 2000). NO can further interact with a superoxide anion ($O2^{-}$) to form the highly potent oxidant peroxynitrite ($ONOO^{-}$) (Luthra et al., 2005), as shown in figure 1.1

Methods of reducing ROS production have been shown to enhance RGC survival following axotomy (Geiger et al., 2002), whilst intravitreal injection of a nitric oxide donor has been shown to induce RGC death (Oku et al., 1997). Therefore, blocking the production of NO

could arrest the development of glaucomatous optic neuropathy (Liu and Neufeld, 2000;Yuan and Neufeld, 2001). Complications, however, arise from the potential antiapoptotic action of ROS through the phosphorylation of nuclear factor (NF)- κB, via reduction of its inhibitory protein IκBα, and subsequent inhibition of caspases (Mattson et al., 2000). ROS has also been shown to up-regulate the expression of the antiapoptotic member of Ras proteins, which are phosphoinositide-3-kinases (PI3K) that enhance cell survival by inactivating bad proteins and Caspase-9 (Rebollo and Martinez, 1999;Mattson et al., 2000).

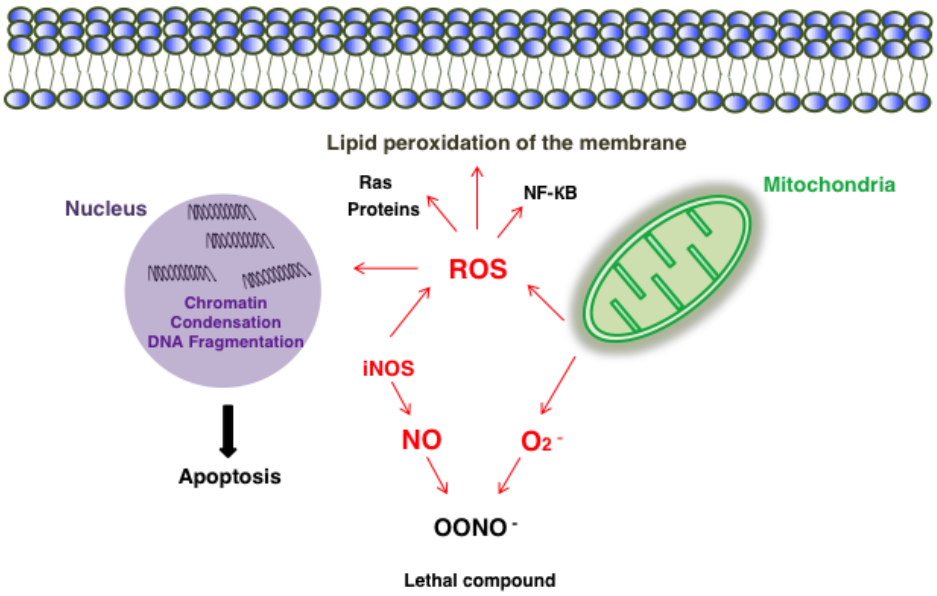


Figure 1.1 Oxidative stress in RGCs

In oxidative stress excessive production of ROS enhances the production of NO by the iNOS enzyme. NO will interact with a superoxide anion (O2-) to form the highly potent oxidant peroxynitrite (ONOO). ROS will also activate the phosphorylation of NF-κB and Ras proteins. The increased levels of ROS enhance cell membrane lipid peroxidation and the nuclear DNA fragmentation.

1.1.4.2 Protein Misfolding

Amyloid deposits, consisting of aggregates of Aβ, are a characteristic feature of several neurodegenerative diseases such as Alzheimer's (Pepys, 2006), Parkinson's disease (Bayer et al., 2002) and mild cognitive impairment (Attems and Jellinger, 2006;Verwey et al., 2008;Villemagne et al., 2008). They have also been recently implicated in the pathogenesis of retinal damage (Shimazawa et al., 2008), Age Related Macular Degeneration (AMD (Johnson et al., 2002), and glaucoma (McKinnon et al., 2002a;Yoneda et al., 2005;Goldblum et al., 2007;Guo et al., 2007a).

Amyloid-β pathway

Amyloid precursor protein (APP) is a trans-membrane protein expressed by many cells, including CNS neurons and RGCs (Morin et al., 1993). APP cleavage involves three proteases: α-secretase generates the soluble non-pathogenic αAPP that serves trophic functions inside cells (Li et al., 1997), whereas β and γ secretase produce Aβ.

The β-secretase enzyme is believed to be the primarily responsible initiator of the amyloidogenic processing of APP (Pastorino and Lu, 2006), with cleavage of APP by β-secretase generating the βAPP and C99 fragments. Further proteolysis of βAPP by **γ** secretase enzyme yields the insoluble Aβ and P6 fragment (Augustin et al., 2009) as summarized in figure 1.2. Cleavage of the C-terminal cytoplasmic tail of APP to yield Delta C-APP, further potentiating Aβ production has been shown in neuronal cells and in apoptotic RGCs in a rat model of ocular hypertension (Gervais et al., 1999;McKinnon et al., 2002a).

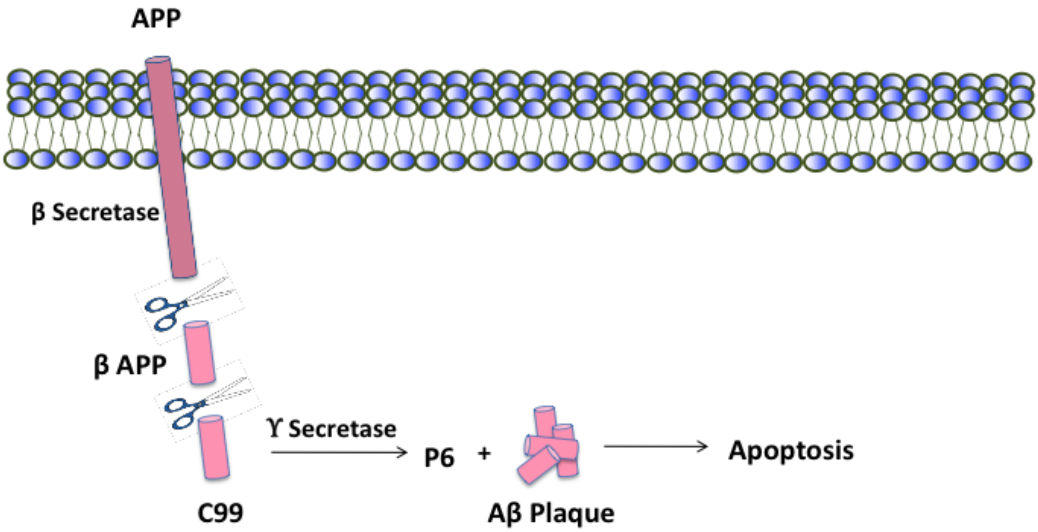


Figure 1.2 The pathways for AB formation

The production of Aβ, an amino-acid peptide, derived from the proteolytic processing of a larger plasma membrane bound protein known as amyloid precursor protein APP. APP is cleaved by β-secretase enzyme to produce βAPP and a C99 fragment. Further cleavage of C99 by γ-secretase will produce Aβ and P6.

Heat shock proteins (HSPs)

Also of interest with regards to protein misfolding is Heat shock proteins (HSPs), a group of specialized molecular chaperons that mediate various physiological functions inside cells. HSPs are up-regulated in stressful conditions to restore normal structural integrity (Soti et al., 2005). Several families of HSP have been implicated in glaucoma and other neurodegenerative diseases (Tezel et al., 1998;Tezel et al., 2000;Pepys, 2006) with increased levels of circulating autoantibodies to alpha-crystallins and HSP27 (Tezel et al., 1998), and increased immunostaining of HSP-60, HSP-27 in RGCs and the retinal blood vessels in glaucoma patients (Tezel et al., 2000). Systemic administration of Geranylgeranylacetone, an

anti-ulcer agent, in the rat glaucoma model has been shown to increase the expression of HSP-72 with a marked reduction in RGC loss (Ishii et al., 2003), possibly through interaction with different protein kinases such as Akt kinase, and the inhibiting NF-κB (Thomas et al., 1998;Neckers, 2007).

1.1.4.3 Inflammation

Growing evidence in clinical and experimental studies strongly suggests the involvement of the immune system in glaucoma (Tezel, 2009). The sustained neuronal damage in glaucoma and other ischemic neurodegenerative conditions can trigger immune responses, leading to an excessive production of T-cells. The activated T cell subsequently attacks the antigen presented to its receptor by the major histocompatibility complex 9 (MHC 9). Up-regulation of the MHC class II molecules on rat glial cells and stimulation of T cell activation in cultured retinal and optic nerve tissue have been demonstrated (Tezel et al., 2007). In a rat model devoid of T cells due to thymectomy there was increased RGC death after optic nerve crush (Yoles et al., 2001). Furthermore, several research studies showed that, augmentation of immune system by passive transfer of T cells directed against myelin basic proteins or active immunization with the myelin derived peptide, reduces RGC loss after optic nerve injury (Schwartz, 2001). A similar finding has been reported in rats injected with activated anti-myelin basic protein T cells after partial optic nerve crush (Moalem et al., 1999;Moalem et al., 2000) . The release of TNF-α, a potent proinflammatory cytokine, and its subsequent binding to the death receptor, TNF-α Recpor-1 (TNR-R1), triggers a caspase-dependent and a caspase-independent component of mitochondrial death promoting pathways. The TNF-α –R complex is able to recruit adaptor proteins that activate caspase 8, which in turn activates caspase 3 (Pastorino et al., 1996;Tezel et al., 2004).

TNF-α

TNF-α, also known as cachectin, is a cytokine that plays an important role in the regulation of immune cells, inflammation, as well as the induction of the caspase-dependent and caspase independent mitochondrial apoptotic pathways. TNF-α binding with TNF Receptor 1 (TNF-R1) triggers a series of intracellular events initiated by recruitment of key adaptor proteins leading to activation of NF-κB, a redox sensitive transcription factor that is heavily implicated in apoptosis (Lu et al., 2010;Meinel et al., 2010). TNF-α-R1 complex upregulates IκB Kinases that activates the phosphorylation of the inhibitory protein IκB releasing activated NF-κB. The activated NF-κB can then translocate to the nucleus to exert its apoptotic effects (Karin and Delhase, 2000). Excessive expression of TNF-α has been documented on RGCs *in vitro* and *in vivo* (Fuchs et al., 2005;Kitaoka et al., 2006). Similar results were shown in cultured glial cells exposed to ischemic conditions and high IOP as well as in human glaucomatous eyes (Tezel and Wax, 2000;Tezel et al., 2001). Conversely, TNF-α is also proposed to have a neuroprotective action mediated through upregulating NF-κB. TNF-α Receptor apoptotic pathway is shown in Figure 1.3

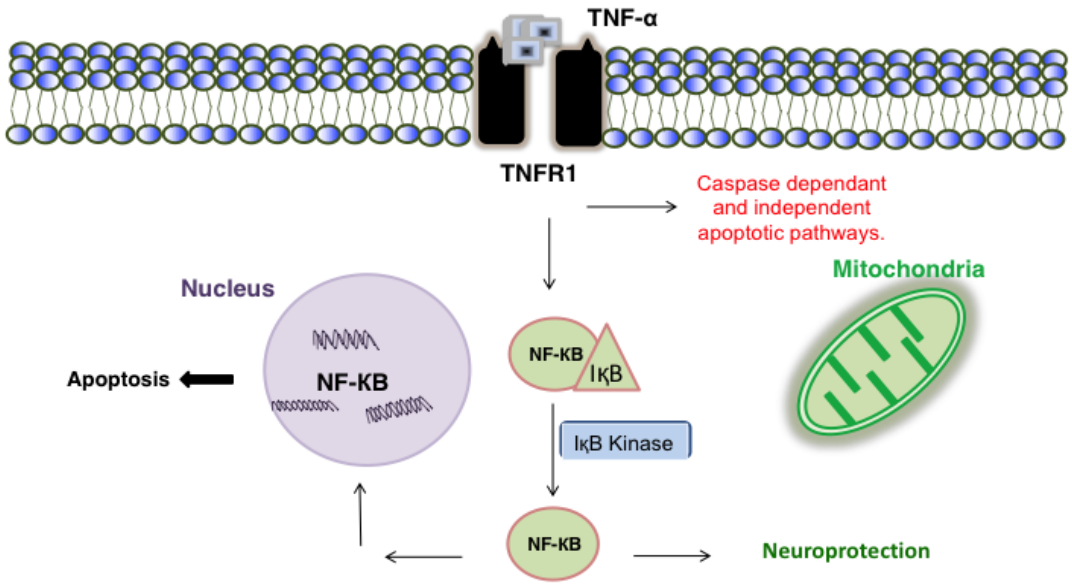


Figure 1.3 TNF-α death receptor pathways

TNF-α binding with the death receptor TNFR1 has been implicated in the induction of caspase-dependent and caspase independent mitochondrial apoptotic pathways. It also up-regulates IκB Kinases that activates the phosphorylation of IκB to release active NF-κB. The activated NF-κB will then translocate to the nucleus to exert its apoptotic effects. Furthermore, activated NF-κB could exerts its proposed neuroprotective effects.

TNF Receptor 1 and TNF Receptor 2

TNF-α activities are mediated via interaction with two distinct receptors, the death domain-containing TNF-receptor 1 (TNF-R1) and the non-death domain-containing TNF-receptor 2 (TNF-R2) (Wajant and Scheurich, 2001). TNFR1 has been confirmed to mediate majority of TNF-α biological activity (Chen and Goeddel, 2002) and has been suggested to be involved in the neurodegenerative process of glaucoma (Tezel et al., 2001), neuronal cell loss and retinal ischemia (Fontaine et al., 2002), whereas TNF-R2 showed neuroprotective activity and reduced retinal ischemia (ibid).

1.1.4.4 Mitochondrial Dysfunction

Mitochondrial dysfunction is believed to play a major role in cell death due to changes in oxidative phosphorylation, ATP synthesis and ROS production. Mitochondria are the main energy source inside cells and the primary site of ROS production, making it a major target for reducing oxidative stress. A decrease in mitochondrial membrane potential and an increase in the membrane permeability have been implicated as a causative factor for RGC apoptosis in glaucoma (Mittag et al., 2000;Tatton et al., 2001;Tezel and Yang, 2004). Glaucoma-related stimuli such as hypoxia, TNF-α and oxidative stress can trigger the mitochondrial-mediated RGC death pathway. In glaucomatous stress, mitochondria buffer excess cytosolic Ca^{+2} leading to intra-mitochondrial accumulation of Ca^{2+}, mitochondrial membrane depolarization and the production of ROS (Kristian and Siesjo, 1998). This has been shown to trigger the opening of the mitochondrial permeability transition pore (PTP) with subsequent release of apoptosis-inducing proteins, such as cytochrome *c* (Nickells, 1999), and CPP32 (caspase 3 activators) (Marchetti et al., 1996;Hirsch et al., 1997).

Released cytochrome *c* from the mitochondria, complexes with apoptotic protease-activating factor-1 (Apaf-1) and procaspase 9 to form the apoptosome, which in turn activates caspase 3, leading to chromatin condensation and DNA fragmentation. Furthermore, cytochrome *c* release has been implicated in inducing mitochondrial dysfunction and ROS production (Ricci et al., 2003), creating a positive feedback loop.

The release of cytochrome *c* is regulated by the competitive actions, at the surface of the mitochondria of a family of cell death regulators, the Bcl-2 protein family (Ow et al., 2008). This family of proteins includes both pro as well as anti-apoptotic molecules and the ratio between these molecules is crucial in a cell's final decision to live or die (Gross et al., 1999a). The members of BCL-2 family of proteins have been found to have up to four BCL-2

homology (BH) domains that correspond to α-helical segments and nominated as BH1, BH2, BH3 and BH4 (Gross et al., 1999a). In figure 1.4 it can be seen that the BCL-2 family of proteins can be divided into the pro-survival Bcl-2 subfamily which includes Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1, the pro-apoptotic Bax subfamily including Bax, Bak and Bok, and the pro-apoptotic BH3 subfamily that includes Bad, Bid, Bik, Blk, Hrk, BNIP3 and Bim (Gross et al., 1999a).

The Bcl-2 protein can prevent apoptosis induced by various stimuli and has a vital role in maintaining mitochondrial integrity, whereas conversely, Bax has been implicated in mitochondrial-mediated apoptosis in various neuronal cells by promoting the release of cytochrome *c* from the mitochondria (Merry and Korsmeyer, 1997;Gross et al., 1999a). It is believed that a high Bax/Bcl-2 ratio affects mitochondrial outer membrane permeability via multiple mechanisms (Scorrano and Korsmeyer, 2003) and stimulates mitochondrial-induced death pathways (Deckwerth et al., 1996;Wei et al., 2001) as summarized in figure 1.4

Mitochondria are also involved in the exacerbation of extrinsic or death receptor mediated apoptosis. Bid, a proapoptotic protein member of the Bcl-2 family is cleaved by death receptors and caspase-8 to yield a truncated Bid (Li et al., 1998), which is able to translocate to the mitochondria and mediate cytochrome *c* release.

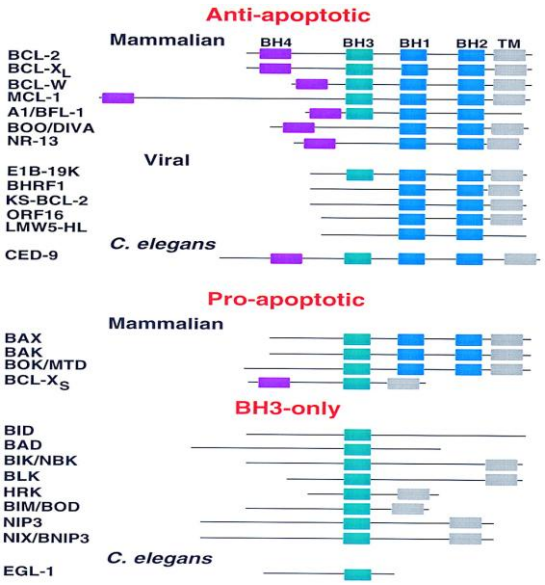


Figure 1.4 Summary of anti and pro-apoptotic BCL-2 family members

Taken from (Gross et al., 1999a)

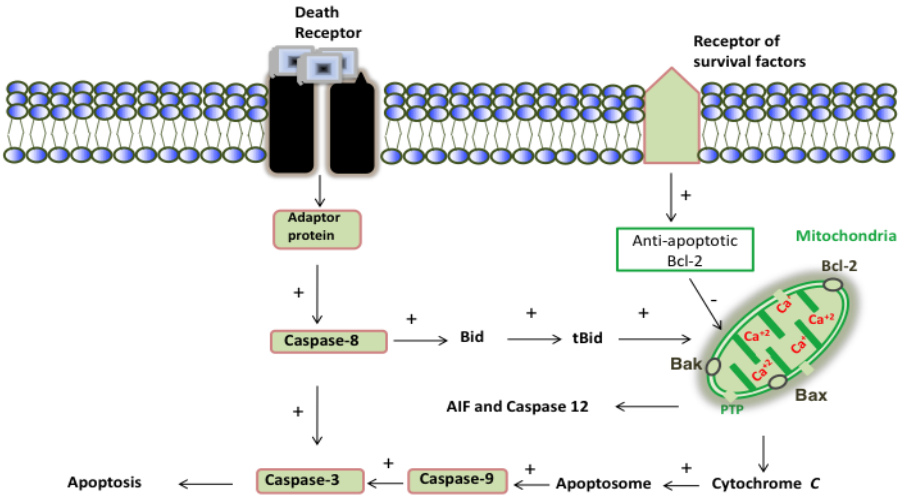


Figure 1.5 Mitochondrial death pathways

The caspase-dependent mitochondrial death pathway is initiated by cytochrome *c* release inducing activation of caspase-9, and caspase-3, which is the primary activator of DNA fragmentation, whereas the caspase-independent pathway, activated by calpain, will induce release of AIF, which will translocate to the nucleus to enhance DNA damage. Apoptotic stimuli, such as stress or activation of death receptors, converts Bid to tBid, which affects mitochondrial membrane permeability and leads to release of death inducing proteins such as cytochrome *c*. A high Bax/Bcl-2 ratio can also affect mitochondrial outer membrane permeability and stimulate the intrinsic mitochondrial pathway and cytochrome *c* independently of tBid. Trophic factor receptors and the antiapoptotic members of Bcl-2 family have inhibitory effects on cytochrome *c* release preventing activation of mitochondrial death pathways.

1.1.4.5 Excitotoxicity

Excitotoxicity is the pathological process by which RGCs (Osborne et al., 1999;Casson, 2006) and other neuronal cells die as a result of excessive extracellular glutamate (Olney, 1969;Choi, 1992;Doble, 1999). As outlined in figure 1.5 glutamate released from the apoptotic cell can trigger necrotic death of surrounding cells that have been spared from the original insult initiating a cascade of autodestruction, further cellular injury and death (Osborne et al., 1999;Casson, 2006;Cheung et al., 2008a). Several studies have confirmed the neurotoxic effect of glutamate in the retina (Hyndman, 1984;Gross et al., 1999b;Kawasaki et al., 2000;Luo et al., 2001), whilst others have suggested that glutamate-mediated RGC injury and death contributes to glaucoma (Dreyer et al., 1996;Brooks et al., 1997;McIlnay et al., 2004;Moreno et al., 2005;Guo et al., 2006;Salt and Cordeiro, 2006).

Mechanism of Excitotoxicity

Glutamate binding of NMDA receptors depolarizes the RGCs, permitting the influx of Ca^{2+}. Excess Ca^{2+} further increases Ca^{2+} entry through voltage-gated Ca^{2+} channels, enhancing Ca^{2+} release from the endoplasmic reticulum, stimulating Ca^{2+}/Na^{+} exchange and further increasing the Ca^{2+} dependent release of vesicular glutamate from the nerve terminal. This glutamate will induce further Ca^{2+} influxes through NMDA, AMPA and Kainate receptors (Casson, 2006). The high intracellular Ca^{2+} triggers a cascade of events including disruption of mitochondrial function, leading to increased reactive oxygen species (ROS) production and the release of cytochrome *c* and apoptosis inducing factor (AIF) in mitochondria ((Marigo, 2007). Subsequently AIF translocates from the cytoplasm to the nucleus where it initiates nuclear chromatin condensation and DNA fragmentation (Figure 1.5 a), thereby

triggering the caspase-independent apoptotic pathway (Zhang et al., 2002). Release of mitochondrial cytochrome *c* (Figure 1.5 b) triggers a range of events including the activation of caspase-9, and the subsequent activation of caspase-3. In addition, raised intracellular calcium as illustrated in figure 1.5, also stimulates calcium dependent proteases such as calpain (Zhang and Bhavnani, 2006), proteolytic enzymes such as lipases, proteases, and nucleases (Casson, 2006), and the inducible form of nitric oxide synthase enzyme (iNOS) generating nitric oxide and reactive oxygen species (ROS).

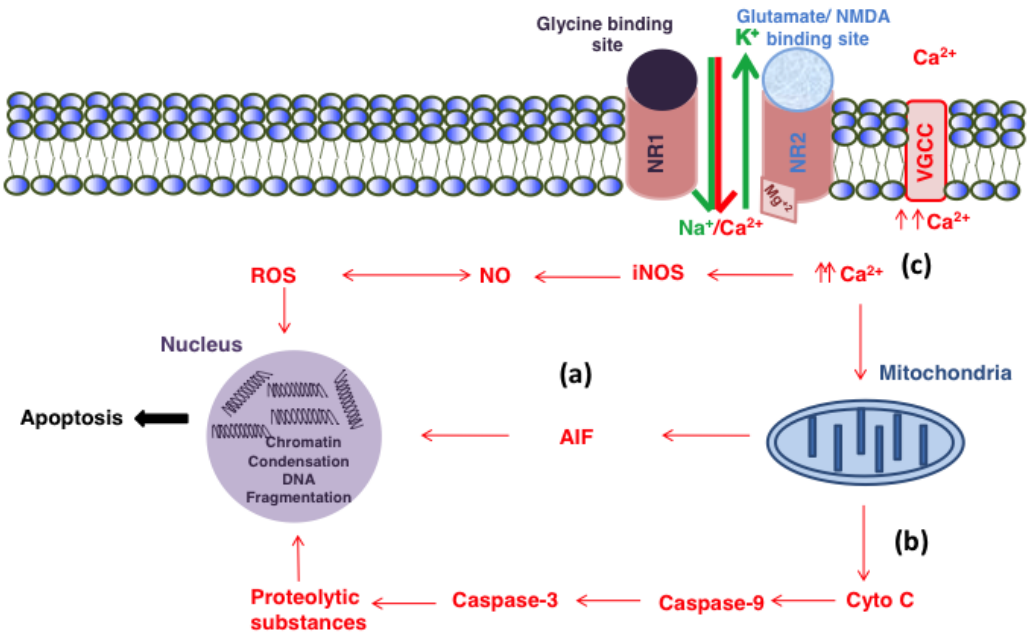


Figure 1.6 Excitotoxicity in RGCs

In excitotoxicity, the high intracellular Ca+2 levels lead to release of the mitochondrial apoptotic inducing factor (AIF) (a), which translocates to the nucleus and initiates nuclear chromatin condensation and DNA fragmentation thereby triggering the caspase-independent apoptotic pathway. Release of mitochondrial cytochrome *c* (b) induces activation of caspase-9, and caspase-3. Caspase-3 is the primary activator of DNA fragmentation, as well as stimulation of the calcium dependant protease. High Ca^{+2} levels (c) will also induce the production of the inducible form of Nitric Oxide Synthase enzyme (iNOS) generating free radicals such as nitric oxide.

1.1.4.6 Neurotrophin Deprivation

Neurotrophic factors are small molecular weight peptides that are widely expressed in the RGCs (Jelsma et al., 1993) and have an indispensible role in growth, differentiation and survival. They include: nerve growth factor (NGF) which bind with one of the tropomyosin-related kinase receptors known as neurotrophic tyrosine kinase receptor type 1 (Trk A) (Kaplan et al., 1991), brain-derived neurotrophic factor (BDNF), Neurotrophine 4 and 5 (NT4 and NT5) which exert their action via neurotrophic tyrosine kinase, receptor, type 2 (Trk B) (Barde et al., 1982;Berkemeier et al., 1991) and NT3 which binds neurotrophic tyrosine kinase receptor type 3 (TrkC) (Hohn et al., 1990;Rosenthal et al., 1990). Several research studies demonstrated that the flow of the neurotrophic factors from the superior colliculus in the CNS to the RGCs is markedly reduced in the animal model of glaucoma, where both the retrograde and the anti-retrograde axonal transport are compromised (Anderson and Hendrickson, 1974;Hayreh et al., 1979;Rudzinski et al., 2004). This leads to a reduction in neuronal trophic support, which in turn compromises neuronal survival and triggers a series of molecular events, stimulating the apoptotic cascade, as seen in RGCs following transection of the optic nerve (Berkelaar et al., 1994).

1.1.5 Neuroprotection in glaucoma

Neuroprotection can be defined as a therapeutic approach aiming at directly preventing, hindering and, in some cases, reversing neuronal cell damage (Society, 2008) Neuroprotection in glaucoma is becoming a key research area as traditional strategies of lowering IOP have been shown to be unable to prevent progressive vision loss in all glaucoma patients, where some patients can continue deteriorating in spite of an apparently controlled IOP (Group, 1998).

Research studies have shown that RGC damage is not confined to the primary insulted neurons, but that secondary injury follows which affects the neighboring neurons as well (Chidlow et al., 2007). It is therefore believed that in glaucoma, treatment modalities that directly target both primary and secondary degeneration of the RGCs are required. This makes the prospect of discovering alternative therapeutic approaches independent of IOP reduction highly sought after, as IOP lowering therapy is indirect and not completely effective at preventing RGC loss.

RGCs and the optic nerve are integral parts of the central nervous system (CNS) and a link has been demonstrated between mechanisms of cell death in glaucoma and Alzheimer's disease (AD) (Weber et al., 2000;Guo et al., 2006;Gupta et al., 2006;Guo et al., 2007b). Therefore, glaucoma may be viewed as a neurodegenerative disease of the CNS, and neuroprotective agents that have been approved for the treatment of neurodegenerative diseases such as AD (Reisberg et al., 2003) and Amyotrophic lateral sclerosis (ALS) (Bensimon et al., 1994;Lacomblez et al., 1996), are being assessed for the treatment of glaucoma (Cheung et al., 2008a).

1.1.6 Targets for neuroprotection in glaucoma

The various proposed mechanisms of RGC cell death which have been detailed previously section (1.1.4); have been investigated through the use of both *in vitro* and *in vivo* models, as shown in Table 1.3. The advantages, disadvantages and how closely these different models correlate to primary open angle glaucoma (POAG) in humans have recently been the focus of some comprehensive reviews (Johnson and Tomarev, 2010). It is important to recognize that there is still no perfect model of glaucoma, and translating results from preclinical to clinical studies is often problematic.

The possible role of ROS in glaucoma has led to the investigation of multiple anti-oxidants as potential neuroprotective agents on RGCs both *in vivo* and *in vitro* as summarized in Table 1.3 including Aminoguanidine, Brazilian Green Propolis, Carotenoids such as Lutein and Zeaxanthin, beta-carotene and Docosahexaenoic acid. Other drugs in this group are: Trolox, 15d-PGJ2 and troglitazone.

Ginkgo biloba is perhaps the most promising antioxidant compound and will be discussed in detail in the second section of this chapter. Another compound with high neuroprotective potential within this group is Melatonin, a potent, naturally occurring antioxidant with free radical scavenging activity, which displays a critical role in aqueous humour circulation (Wiechmann and Wirsig-Wiechmann, 1994;Dubocovich et al., 1997;Sugden et al., 1997;Wiechmann et al., 1999). Its neuroprotective actions are believed to be mediated via multiple mechanisms including reducing single and double strand breaks in DNA (Sun et al., 2002), increasing Akt phosphorylation (Kilic et al., 2005a;Lee et al., 2006;Tajes Orduna et al., 2009), reducing NO-induced apoptosis (Siu et al., 2004) and inhibiting the mitochondrial

transition pores and cytochrome *c* release (Andrabi et al., 2004;Jou et al., 2004;Kilic et al., 2004). Melatonin demonstrated a neuroprotective effect on RGCs *in vivo* (Siu et al., 2004;Tang et al., 2006), it also protected rabbit retinal neurons *in vitro* (Cazevieille and Osborne, 1997).

Modulation of NMDA receptor has constituted a major area of research in glaucoma neuroprotection (Guo et al., 2006;Dong et al., 2008). *In vivo* and *in vitro* studies have suggested that blocking both the NMDA and the non-NMDA receptors simultaneously offers optimal protection against ischemic neurodegeneration (Mosinger et al., 1991;Leinders-Zufall et al., 1994). There are several anti-excitotoxic drugs that have been investigated *in vivo* and *in vitro*, that exert their neuroprotective actions by overcoming the glutamate-induced excitotoxicity on the NMDA receptors such as the Ifennprodil, Eliprodil, Flupirtine and dextromethomethorphan as in Table 1.3 but the most prominent amongst them are MK801 and memantine. MK801, also known as (+)-5-methyl-10, 11-dihydro-5H-dibenzo [a, d]cyclohepten-5, 10-imine maleate, is a non-competitive antagonist of the NMDA receptor and has demonstrated neuroprotective potential in the CNS for many years (Foster et al., 1988;el-Asrar et al., 1992;Tamura et al., 1993).

MK-801 has also been found to protect RGCs both *in vitro* (Tsuda, 2004) and *in vivo* in the optic nerve injury model (Russelakis-Carneiro et al., 1996), the laser-induced retinal injury rat model (Solberg et al., 1997), and in the OHT models (Chaudhary et al., 1998;Guo et al., 2006). Work on the experimental model of high IOP-induced retinal ischemia verified the neuroprotective effect of MK801 to be mediated through decreasing Bad expression (Russo et al., 2008). The effect of MK-801 *in vivo* on RGC apoptosis in a staurosporine-induced retinal toxicity model showed a reduction in the number of apoptotic RGCs in comparison to

the controls (Guo et al., 2006). Unfortunately, MK801 is not used clinically because of its neurotoxic effect (Olney et al., 1989;Fix et al., 1993), which is believed to be due to high affinity to the NMDA receptors and its long stay time in the channel (Lipton, 1993).

Another interesting drug is Memantine, also known as 1-amino-3, 5-dimethyl-adamantane, is a three-ringed structural derivative of the anti-influenza drug, amantadine (Cheung et al., 2008a). The additional amine (–NH2) and two methyl (–CH3) side groups are thought to be responsible for the increased residency with, and affinity for, NMDA receptors in relation to amantadine (Lipton, 2006). Memantine, however, exhibits strong voltage dependency with rapid blocking/unblocking kinetics, displaying weak potency during the normal synaptic transmission (Johnson and Kotermanski, 2006).

The Food and Drug Administration (FDA) approved Memantine, for treating moderate to severe Alzheimer's disease (Reisberg et al., 2003). It is the only neuroprotective agent that has completed a phase III clinical trial in patients with OAG. Memantine's efficacy as a neuroprotectant for glaucoma, however, was shown to be ineffective, with the variable mechanisms of retinal ganglion apoptosis being offered as an explanation (Osborne, 2008), although an inadequate design of study and an inappropriate end point could be the reasons for this result.

Potential neuroprotective agents aimed at targeting AMPA/Kainite receptors has been widely studied, DNQX, 6,7-Dinitroquinoxaline-2,3-dione, is an AMPA receptor antagonist that has shown greater enhancement of RGC survival than MK-801 (Schuettauf et al., 2000), whilst Riluozole has been shown to decrease pressure induced apoptosis and enhance ERG wave recovery, highlighting the benefits of targeting multiple receptors in excitotoxic cell death.

Another potential neuroprotective pathway is through the mediation of acetylcholine (ACh) receptors, as has been seen in studies on the ACh esterase inhibitor, Galatamine, a drug clinically used in the treatment of AD. Galatamine potentially activates muscarine AChR, leading to RGC protection independent of IOP level (Almasieh et al., 2010).

Drugs designated to target β-Amyloid (Aβ) include β-secretase inhibitors (BSI) such as *N*-benzyloxycarbonyl-Val-Leu-leucinal (Z-VLL-CHO) which has been found to reduce RGC apoptosis *in vitro* and *in* vivo (Yamamoto et al., 2004;Guo et al., 2007a), as well as Congo red and Anti-Aβ antibodies (Lorenzo and Yankner, 1994;Guo et al., 2007a). Triple therapy, targeting different stages of the Aβ pathway using BSI, Anti-Aβ antibodies and Congo red, has a superior neuroprotective effect on RGC apoptosis in a rat ocular hypertension, both *in vitro* and *in vivo* in relation to singular treatments (Guo et al., 2007a).

Several compounds, outlined in Table 1.3, have been proposed to enhance the available energy within the cell and prevent mitochondrial depolarization. Coenzyme Q_{10} (CoQ_{10}), also known as Ubiquinone, plays an indispensable role in energy metabolism. It serves as a co-factor within the respiratory chain, carrying electrons and facilitating ATP production. It has been found to be highly effective as a neuroprotectant in animal models of neurodegenerative diseases such as Parkinson's disease, Huntington's disease and Friedreich's ataxia (Beal, 2003). Its neuroprotective effect on RGCs both *in vivo* and *in vitro,* is believed to be multifactorial (Nucci et al., 2007b;Nakajima et al., 2008), exerted not only through mediation of electron transport from complex I and II to complex III within the electron transport chain but also through its antioxidant properties, regulation of gene expression, and inhibition of the PTP (Papucci et al., 2003;Cheung et al., 2008a).

Anti-inflammatory drugs, which target the TNF-α signalling pathway and displayed neuroprotective activity has become an area of increasingly active investigation including as can be seen in Table.1, Agmatine, Curcumin, Pitavastatin, and GLC756.

The most promising anti-inflammatory drug is Copolymer-1 (Cop-1), also known as glatiramer acetate, which was approved by the FDA to treat Multiple Sclerosis (MS). Cop-1 is a low affinity synthetic non-encephalitogenic analogue to myelin basic protein, triggering a neuroprotective autoimmune response, by binding to MHC proteins and cross reacting with various T cell and CNS myelin. Cop-1 displayed neuroprotective activity on RGCs *in vivo* in the rat model of optic nerve crush (Kipnis et al., 2000), in animal models of high IOP (Bakalash et al., 2003;Ben Simon et al., 2006), and against glutamate-induced excitotoxicity (Schori et al., 2001). This neuroprotective effect is believed to be mediated by increasing the number of T-Lymphocytes in a rat model of glaucoma (Li et al., 2008).

Various growth factors have successfully been investigated as a neuroprotectant, as seen in Table 1.3 Ocular application of the NGF has demonstrated neuroprotective effects both experimentally in the Morrison's glaucoma model (Lambiase et al., 2009;Colafrancesco et al., 2010) and clinically in patients with progressive visual field defects despite controlled IOP (Lambiase et al., 2009). However, sustainability remains a limitation. Following prolonged treatment with neurotrophins, RGCs exhibited a decrease in neurotrophin receptor expression reducing the effectiveness of the treatment. To overcome this a combination of neurotrophin and TrkB gene transfer has been performed to up regulate the receptors and protect RGCs *in vivo* in the axotomy model (Cheng et al., 2002).

The field of gene therapy in neuroprotection has been recently investigated and it is growing rapidly (Wax and Patil, 1994;Liu et al., 2009). In glaucoma it is becoming a highly accessible approach (Harvey et al., 2006), because trabecular meshwork, ciliary epithelium, ciliary muscle, Muller cells and RGCs are all appropriate target structures for gene therapy, with various delivery systems having been tested including: adenoviruses (Ads), adenoassociated viruses (AAVs), herpes simplex viruses (HSVs), lentiviruses (LVs; feline immunodeficiency virus (FIV) human immunodeficiency virus (Kroemer et al.), liposomes (LPs), and naked DNA(Borras et al., 2002).

A promising agent for glaucoma therapy is BIRC-4, also known as XIAP (IAP: inhibitors of apoptosis protein). Intravitreal injection of adeno-associated viral vector using chicken-β-actin (AAV-CBA) coding for human BIRC4 in the rat model of chronic glaucoma resulted in marked reduction in RGC apoptosis which lasted for 12 weeks. This neuroprotective effect is believed to be mediated either via direct inhibition of caspase-3 and caspase-8, or indirectly by maintaining the neurotrophin production from Muller cells and influencing aqueous humour circulation or a combination of both (McKinnon et al., 2002b).

The last group in Table 1.6 includes compounds with multiple mechanisms of action: Estrogens, Brimonidine and Cannabinoids. Estrogens, cholesterol derived steroid hormones, maintain the normal function of various organs, with estrogen receptors ERα and ERβ widely expressed in human and animal retinal tissues (Kobayashi et al., 1998;Ogueta et al., 1999). Estrogen has demonstrated neuroprotective effects on animal models of Alzheimer's (Simpkins et al., 2005) and other neurological diseases (Hoffman et al., 2006) The neuroprotective action of estrogen is believed to be mediated via multiple mechanisms; the

binding with estrogen receptors ERα and ERβ (Singer et al., 1996;Dubal et al., 2001;D'Astous et al., 2004), activation of antiapoptotic genes such as Bcl-2 and Bcl-xl (Garcia-Segura et al., 1998), inhibition of mitochondrial oxidative stress (Razmara et al., 2007), inhibition of β-amyloid induced neuronal death, as well as stimulation of Akt/PI-3k pathway (Honda et al., 2000;Zhang et al., 2001). 17β-estradiol of rat cortical neurons exposed to glutamate demonstrated increased neuronal integrity and function mediated possibly via a reduction in the levels of caspase-3 and calpain (Sribnick et al., 2004).

An Estradiol analogue has also demonstrated protective effects on the retinal pigment epithelium (RPE) (Dykens et al., 2004;Yu et al., 2005), and on the RGCs *in vitro* (Kumar et al., 2005) and *in vivo* (Nakazawa et al., 2006;Zhou et al., 2007).

Brimonidine tartrate 0.2% is known also as UK-14, 304, is a third generation α_2 adrenergic agonist that draws the interest of many researchers in the field of neuroprotection. The neuroprotective effect of Brimonidine on RGCs has been demonstrated *in vivo* (Wheeler et al., 1999;Donello et al., 2001;WoldeMussie et al., 2001) and *in vitro* (Knels et al., 2008). The mode of action for Brimonidine however remains unclear with various proposed mechanisms. The positive effect of Brimonidine on RGC survival, that includes a reduction in their soma size in a rat model of ocular hypertension, is believed to be mediated through the attenuation of glutamate toxicity and the up regulation of brain-derived neurotrophic factors (Hernandez et al., 2008). However, in a rat model of pressure-induced retinal ischemia, Brimonidine's neuroprotective effect was suggested to be mediated via induction of anti-apoptotic genes Bcl-2 and Bcl-x, as well as extracellular-signal-regulated kinases and phosphatidylinositol-3′ kinase/protein kinase Akt pathways (Lai et al., 2002). Whilst, Brimonidine's effect on RGCs in isolated rat retinas, as well as *in vivo* in rat and rabbit glaucoma models was shown to be mediated through the reduction of α_2-adrenoceptor

mediated reduction of intracellular cAMP (Dong et al., 2008).

A clinical trial assessing the non-IOP-related effects of Brimonidine, demonstrated a reduction in visual field deterioration in comparison to 360° laser trabeculoplasty (Gandolfi et al., 2004), whilst the promising result of Brimonidine treatment in the Low-Pressure Glaucoma Treatment Study are now published (Krupin et al., 2011).

Table 1.3 Summary of potential neuroprotective agents and their pro-apoptotic targets

Pro-Apoptotic Mechanism	Target	Compound	Model	References
Oxidative stress	NOS	Ginkgo biloba	*in vitro* Alloxan, Glutamate Dexamethsone. *in vivo* OHT	(Thiagarajan et al., 2002) (Hirooka et al., 2004)
		Aminoguanidine	*in vitro* explant *in vivo* OHT	(Katsuki et al., 2004) (Neufeld et al., 1999;Neufeld, 2004)
	ROS	Brazilian green Propolis	*in vitro* H_2O_2, SSP, Oxygen-Glucose deprivation/Reoxygenation in vivo NMDA	(Inokuchi et al., 2006;Nakajima et al., 2009a) (Inokuchi et al., 2006)
		Carotenoids	*in vitro* H_2O_2, SS *in vivo* OHT, Ischemia	(Nakajima et al., 2009b) (Neacsu et al., 2003;Li et al., 2009)
		Melatonin	*in vitro* Ischemia, Kainate *in vivo* Ischemia, Kainate	(Cazevieille and Osborne, 1997) (Siu et al., 2004;Tang et al., 2006)
		Tocopherol	*in vitro* H_2O_2 *in vivo* ischemia	(Nakajima et al., 2008) (Aydemir et al., 2004)
		PPAR-g agonists	*in vitro* glutamate	(Aoun et al., 2003)
Protein Misfolding	Aß	Congo Red	*in vitro* *in vivo* OHT, Aß	(Yamamoto et al., 2004) (Lorenzo and Yankner, 1994;Guo et al., 2007a)
		Anti-ß-amyloid	*in vitro* *in vivo* OHT, Aß	(Yamamoto et al., 2004) (Lorenzo and Yankner, 1994;Guo et al., 2007a)
	ß-secretase	Z-VLL-CHO	*in vitro* *in vivo* OHT, Aß	(Lorenzo and Yankner, 1994) (Guo et al., 2007a)
	HSP	GGA	*in vivo* OHT	(Ishii et al., 2003)
Mitochondrial Dysfunction	ROS	FK506	*in vivo* Optic Nerve Crush *in vitro* Ischemia	(Huang et al., 2005) (Chidlow et al., 2002)
		Lipoic Acid	*In vivo* (Aged, Ischemia)	(Hagen et al., 1999;Chidlow et al., 2002;Liu et al., 2002)

Pro-Apoptotic Mechanism	Target	Compound	Model	References
	ROS, NF-kB	Creatine	*in vitro* Glutamate *in vivo* Animal Model, MPTP	(Klivenyi et al., 1999;Juravleva et al., 2005) (Matthews et al., 1998;Klivenyi et al., 1999;Matthews et al., 1999)
	Unknown	EGCG	*In vitro* Ischemia, Light insult, H_2O_2 *in vivo* Ischemia.	(Yang et al., 2007;Zhang et al., 2007;Zhang et al., 2008) (Negishi et al., 2004;Zhang et al., 2007;Zhang et al., 2008)
	PI3-Akt, NF-kB	Erythropoietin	*in vivo* Ischemia, Optic Neuritis, Axotomy, Cytokines, DBA/2J	(Digicaylioglu and Lipton, 2001;Junk et al., 2002;Sattler et al., 2004;Kilic et al., 2005b;Zhong et al., 2007)
	Unknown	Nicotinamide	*in vitro* Ischemia, Light insult	(Ji et al., 2008)
	PTP	CoQ10	*in vitro* SS, Antimycin A, Ceramide, UVC, H_2O_2 *in vivo* Ischemia, NMDA	(Papucci et al., 2003;Nakajima et al., 2008) (Nucci et al., 2007b;Nakajima et al., 2008)
Inflammation and immunological strategies	Multiple	Curcumin	*in vitro* IS, NMDA, H_2O_2 *in vivo* ATM	(Mandal et al., 2009;Teiten et al., 2009) (Lim et al., 2001)
	ROS	Pitavastatin	*in vivo* NMDA	(Nakazawa et al., 2007)
	Myelin Basic Protein	Cop-1	*in vivo* OHT,Optic Nerve Crush, Glutamate	(Kipnis et al., 2000;Schori et al., 2001;Bakalash et al., 2003;Ben Simon et al., 2006;Li et al., 2008)
	TNF-α	GLC756	*in vitro* IS *in vivo* IS	(Laengle et al., 2006)
	Unknown	Agmatine	*in vitro* Hypoxia, NMDA, TNF-α	(Wang et al., 2006;Hong et al., 2007;Hong et al., 2009)

Pro-Apoptotic Mechanism	Taeget	Compound	Model	References
Excitotoxicity	NMDR	Memantine	*in vitro* NMDA *in vivo* OHT, Optic nerve crush, UEG, DBA/2J, Ischemia	(Pellegrini and Lipton, 1993) (WoldeMussie et al., 2002;Yucel et al., 2006;Ju et al., 2009)
		MK801	*in vitro* Hypoxia, Glutamate	(Tsuda, 2004) (Russelakis-Carneiro et al., 1996;Solberg et al., 1997;Chaudhary et al., 1998;Guo et al., 2006;Russo et al., 2008)
		Flupritine	*in vitro* NMDA *in vivo* Ischemia	(Nash et al., 2000) (Nash et al., 2000)
		DXM	*in vitro* Hypoxia *in vivo* Laser, OHT, SSP, Subdural Hematoma	(Goldberg et al., 1987) (Duhaime et al., 1996;Calzada et al., 2002;Guo et al., 2006)
		Eliprodil	*in vitro* Glutamate *in vivo* NMDA, Ischemia	(Kapin et al., 1999;Pang et al., 1999) (Kapin et al., 1999)
		Ifenprodil	*in vitro* Glutamate *in vivo* OHT,SSP	(Tamura et al., 1993) (Guo et al., 2006)
		P38 inhibitor	*in vivo* Axotomy	(Kikuchi et al., 2000)
	AMPA/Kainate-R	Topiramate	*in vitro* Glutamate, Kainate *in vivo* Ischemia	(Gibbs et al., 2000;Skradski and White, 2000;Yoneda et al., 2003) (Yoneda et al., 2003)
		DNQX	*in vitro* Glutamate *in vivo* Optic Nerve Crush	(Otori et al., 1998) (Schuettauf et al., 2000)
		Arachidonic Acid	*in vitro* Glutamate *in vivo* Kainate	(Miller et al., 1992;Kovalchuk et al., 1994;Kawasaki et al., 2002) (Cunha et al., 2004)
	mGluR2/mGluR3	LY354740	*in vivo* Ischemia, OHT, SSP	(Guo et al., 2006)

Pro-Apoptotic Mechanism	Target	Compound	Model	References
	Ca+2	Flunarizine	*in vitro* NMDA *in vivo* Axotomy, Ischemia, NMDA	(Osborne et al., 2002) (Eschweiler and Bahr, 1993;Osborne et al., 2002)
		Diltiazem	*in vitro* Glutamate *in vivo* Ischemia	(Vallazza-Deschamps et al., 2005) (Vallazza-Deschamps et al., 2005)
		Riluzole	*in vivo* Axotomy	(Ettaiche et al., 1999)
		TRPV1 agonist	*in vitro* hydrostatic pressure *in vivo* DBA/2 mouse	(Sappington et al., 2009) (Sappington et al., 2009)
	Na+ channels	Phenytoin	*in vivo* Optic Nerve Crush, OHT	(Naskar et al., 2002;Hains and Waxman, 2005)
	ACh-R	Galantamine	*in vivo* Axotomy, OHT	(Tamura et al., 1993)
Neurotrophin withdrawal	TrkB, p75	BDNF	*in vitro* BDNF withdrawal *in vivo* Axotomy, Superior Colliculus remova, OHT	(Johnson et al., 1986;Rodriguez-Tebar et al., 1989) (Mansour-Robaey et al., 1994;Cui and Harvey, 1995)
		NT-1	*in vivo* Optic Nerve Crush, NMDA	(Senaldi et al., 1999;Schuettauf et al., 2005)
	TrkB	NT-4/5	*in vivo* Superior Colliculus removal	(Cui and Harvey, 1995)
	CNTF receptor	CNTF	*in vivo* OHT	(Ji et al., 2004;Pease et al., 2009)
	GFRA-1,GFRA-2	GDNF	*in vivo* OHT	(Jing et al., 1996;Naskar et al., 2000;Jiang et al., 2007;Ward et al., 2007)
	TrkA	NGF TrkA agonist	*in vivo* OHT, axotomy	(Lambiase et al., 2009;Lebrun-Julien et al., 2009;Colafrancesco et al., 2010)
Gene therapy	RrkB, BIRC-4, GDNF	Viral Vectors	*in vivo* OHT, axotomy	(Cheng et al., 2002;McKinnon et al., 2002b;Pease et al., 2009)

Pro-Apoptotic Mechanism	Target	Compound	Model	References
Multiple mechanisms	Multiple	Estrogen	*in vitro* Glutamate *in vivo* Ovariectomy, DBA/2J	(Sribnick et al., 2004;Kumar et al., 2005) (Nakazawa et al., 2006)
		Brimonidine	*in vitro* NMDA, Glyoxal, H_2O_2 *in vivo* Ischemia, Optic Nerve Crush, OHT	(Knels et al., 2008) (Wheeler et al., 1999;Donello et al., 2001;WoldeMussie et al., 2001;Lai et al., 2002;Dong et al., 2008;Hernandez et al., 2008)
		Cannabinoids	*in vitro* Potassium Chloride, Ischemia *in vivo* NMDA, Ischemia	(El-Remessy et al., 2003;Nucci et al., 2007a) (Opere et al., 2006)

NMDA= N-methyl-D-aspartic acid, OHT= Ocular Hypertension, UEG= Unilateral experimental glaucoma, DBA/2J= Mouse model of Glaucoma, MCAO= Middle Cerebral Artery Occlusion, SSP= staurosporine, DXM= Dextromethorphan, DNQX= 6,7-Dinitroquinoxaline-2,3-dione, ROS=Reactive Oxygen Species, MPTP= 1methyl-4-phenyl-1,2,3,6-tetrahydropyridine, EGCG= Epigallocatechin Gallate, PTP= Mitochondrial Permeability Transition Pore, SS= Serum Starvation, UVC= Ultraviolet Subtype C, Aß = Amyloid-ß, HSPs= Heat Shock Proteins, GGA= Geranylgeranylacetone, NOS= Nitric Oxide Synthase, IS= Immune Stimulated, TNF-α = Tissue Necrosis Factor α , ATM=Alzheimer transgenic mouse, SHRSP= stroke-prone spontaneously hypertensive rats, ACh-R= acetylcholine receptors. Taken from (Baltmr et al., 2010)

1.2 Ginkgo biloba

Ginkgo biloba has been integral part of traditional medicine for centuries, for treating a wide range of neurodegenerative diseases including Alzheimer's disease (Kanowski et al., 1996;Ahlemeyer and Krieglstein, 2003;Yancheva et al., 2009), AMD (Lebuisson et al., 1986) and low-tension glaucoma (Quaranta et al., 2003). Ginkgo biloba has attracted considerable interest because it is believed to have several biological actions which combine to make it a potentially significant agent in neurodegenerative diseases including improvement of central and peripheral blood flow, reduction of vasospasm, reduction of serum viscosity, antioxidant activity, platelet activating factor inhibitory activity, inhibition of apoptosis, and inhibition of excitotoxicity (Ahlemeyer and Krieglstein, 2003;Chan et al., 2007).

1.2.1 Plant Description and the available forms

Ginkgo biloba tree is believed to be one of the oldest existing species, which has been used in non-conventional Chinese medicine since 3000BCE (Ritch, 2000) and it is amongst the most extensively studied plant–based drug nowadays (van Beek and Montoro, 2009).

The Ginkgo biloba tree, which is also known as Fossil tree, Kew tree and Maidenhair tree (Diamond et al., 2000), has short branches with two lobed, fan shaped leaves around 3 inches long with indigestible fruits. The fruit contains an inner seed (ibid).

Figure 1.7 Ginkgo biloba leaves and fruits

Taken from (RAKSA THAI HERBS CO., 2010)

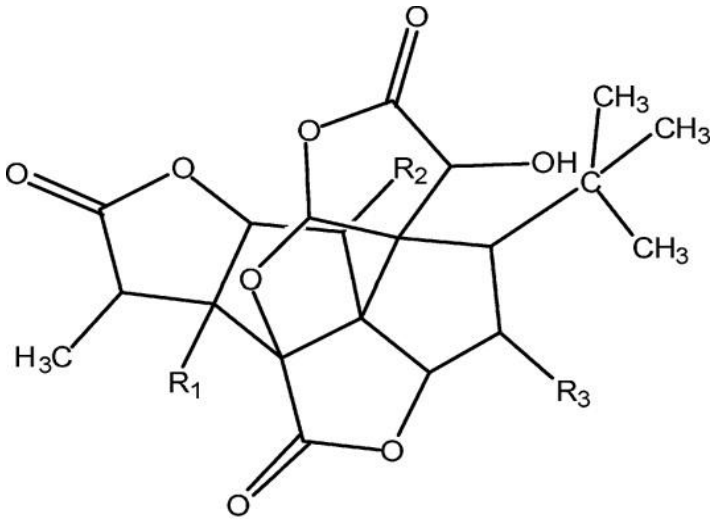
The leaves of Ginkgo biloba are subjected to multistep extraction procedure to form a concentrated standardized extract, Ginkgo biloba extract (EGb761) (Chan et al., 2007). EGb761 is a brown powder with a characteristic odor (van Beek and Montoro, 2009), which contains two major pharmacologically active groups of compounds: 24% flavone glycosides and 6% terpene trilactones (van Beek, 2002). Flavone glycosides are composed of quercetin, kaempferol and isorhamnetin glycosides (1–3) and the terpene trilactones are composed of A, B, C, and J ginkgolides and bilobalide. In addition, the extract also contains non-flavonol glycosides, proanthocyanidins, carboxylic acids that can be divided into non-phenolic and phenolic acids. The phenolic acid including ascorbic, D-glucaric acid, quinic acid and shikimic acid, and the phenolic acids includes 6-hydroxykynurenic acid (6-HKA). EGb 761 also contains alkylphenols, glucose, rhamnose and various other constituents (Ahlemeyer and Krieglstein, 2003;van Beek and Montoro, 2009). EGb761 constituents are illustrated

in table 1.4 and the chemical structure of the terpene trilactones fraction of Ginko biloba: Ginkgolides and Bilobalide, principle constituents of Ginkgo biloba, which have been used in this study are given in Figure 1.2

Ginkgo biloba is available as the standardized extract EGb 761, in the form of 40-240 mg tablets, 40 mg eye drops, fluid extract, alcoholic extract with 40-60% ethanol (tinctures), or fluid extract made with glycerites and as a dried leaf for tea (Chan et al., 2007). The recommended daily dose of EGb761 is 120-240 mg/day, with the 240 mg having superior therapeutic effect (Diamond et al., 2000;Le Bars and Kastelan, 2000).

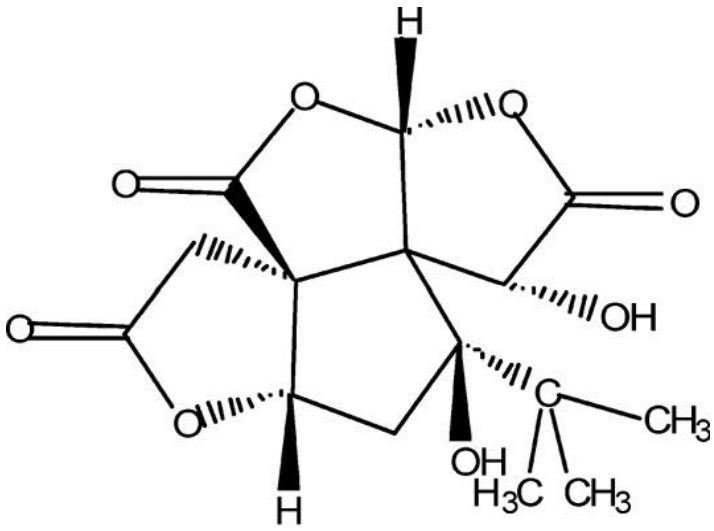
Table 1.4 Constituents of Ginkgo biloba extract EGb761Table

Compound	**Percentage %**
Flavone glycosides: Quercetin, kaempferol and isorhamnethin	24%
Terpene trilactones: 1. Bilobalide 2. Ginkgolide A, B, C and J	6% 2.9% 3.1%
Carboxylic acids	13%
Proanthocyanidins	7%
Non-flavonol glycosides	20%
Water, solvent	3%
Inorganic constituents	5%
Various	3%
Unknown	13%
Alkylphenols	≤ 5 mg/kg



Ginkgolides

	R1	R2	R3
Ginkgolide A	OH	H	H
Ginkgolide B	OH	OH	H
Ginkgolide C	OH	OH	OH
Ginkgolide J	OH	H	OH
Ginkgolide M	H	OH	OH



Bilobalide

Figure 1.8 Chemical structure of Ginkgolide A and Bilobalide

Taken from (Chan et al., 2007)

1.2.2 Pharmacokinetics of Ginkgo biloba

The half-life of Ginkgo biloba extract after oral administration is around 4.5 hours (Diamond et al., 2000;Chan et al., 2007). The pharmacological effect of EGb 761 is believed to be mediated via its two main chemical constituents, flavone glycosides and terpene trilactones. Nevertheless, the poor lipid solubility of flavone glycosides remains the main reason for the poor bioavailability of EGb 761 (Chen et al., 2010), to overcome this obstacle a recent development of a novel formulation of flavone glycosides has been made by Chen et al (2010). This was through preparing Ginkgo biloba extract phospholipid complexes and Ginkgo biloba extract solid dispersions. The authors have shown that these formulation had superior bioavailability compared with the standardized EGb 761 (Chen et al., 2010).

In an animal models, 21% of GBE is excreted in the urine and 29% is excreted in faeces, with an absorption estimated at 60% (Diamond et al., 2000;Chan et al., 2007). A summary of some of the pharmacokinetic studies that have been carried out *in vitro* and *in vivo* in experimental animals or in human is provided in table 1.5

Table 1.5 Pharmacokinetic studies on Ginkgo biloba

Compound	Model	Half-life t 1/2	References
Ginkgolide A, B, C and Bilobalide	in vivo I/V, rat	0.97, 1.02, 0.67, 1.13	(Xie et al., 2008)
Ginkgolide B	in vitro rat urine, rat liver microsomes	-	(Wang et al., 2008)
EGb 761	in vivo oral, humans, analysis of urine	-	(Wang et al., 2003)
Quercetin, Kaempferol and Isorhamnetin	in vitro human breast cancer cells	-	(Wang et al., 2005a)
EGb761, bilobalide, Ginkgolide A and Ginkgolide B	in vivo oral, humans, analysis of plasma	2.33, 2.31, 2.34	(Woelkart et al., 2010)
Ginkgolide B 40mg/80mg	in vivo oral, humans, analysis of plasma	11.64 /4.31	(Drago et al., 2002)

1.2.3 Pharmacological activities of Ginkgo biloba

Ginkgo biloba has a broad spectrum of pharmacological activities, which are derived from its various constituents. The administration of each of these alone is expected to exert a different action, as compared to when it is given together with the other constituents of the extract (DeFeudis and Drieu, 2000). This range of pharmacological activities includes, effects on nitric oxide (Kobuchi et al., 1997;Bastianetto et al., 2000b;Ahlemeyer and Krieglstein, 2003) and modulation of ROS (Szabo et al., 1993;Oyama et al., 1996;Zhou and Zhu, 2000;Thiagarajan et al., 2002), preservation of mitochondrial function (Chandrasekaran et al., 2001;Tendi et al., 2002;Eckert et al., 2003;Wang et al., 2005b;Abdel-Kader et al., 2007) and platelet activating factor (Kobuchi et al., 1997) as well as the inhibitory action on some apoptosis related caspases (Luo et al., 2002;Smith et al., 2002;Massieu et al., 2004). Furthermore, Ginkgo biloba has demonstrated effects on cellular peptide such as APP and Aβ (Yao et al., 2001;Bastianetto and Quirion, 2002;Gong et al., 2005;Wu et al., 2006;Augustin

et al., 2009;Shi et al., 2009) and on inflammatory mediators (Jiao et al., 2005;Park et al., 2006;Zhou et al., 2006;Kotakadi et al., 2008;Tsao et al., 2008;Zhou et al., 2010). Ginkgo biloba appears also to influence an ionic channel such as the glutamate-gated cation channel (NMDA) (Zhu et al., 1997;Wang et al., 2005b;Xu et al., 2010;Li et al., 2011), GABA-gated chloride channels (Kiewert et al., 2007), with modulatory action on neurotransmitters such as glycine (Kiewert et al., 2008).

The flavone glycosides including quercetin, kaempferol and isorhamnetin have the potential to modulate oxidative metabolism, and are believed by some authors to be responsible for the free radical scavenging effects of Ginkgo biloba. Oyama et al (1994) have investigated the antioxidant action of myricetin, quercetin and kaempferol, on oxidative metabolism of neuronal cells, and the authors found a significant reduction in oxidative metabolism in both resting and calcium loaded neurons in comparison with EGb 761, possibly via a reduction in the cellular content of superoxide anion (Oyama et al., 1994). Flavone glycosides have also been shown to protect and rescue rat hippocampal cells from nitric oxide-induced toxicity, via free radical scavenging effect, and blocking nitric oxide-induced stimulation of protein kinase C (Bastianetto et al., 2000b).

The terpene trilactone fraction of Ginkgo biloba: Ginkgolides and Bilobalide is believed to mediate its anti-inflammatory effect. Park and colleagues (2006) compared the effect of EGb 761 and another Ginkgo biloba extract with a higher level of terpene, a lower level of flavonol glycosides, and none detectable proanthocyanidins, known as (GBB) on lipopolysacharide-induced nitric oxide (NO)

and prostaglandin (PGE_2) release in the macrophage cell line RAW 264.7. They have shown that GBB was more potent than EGb 761 in blocking lipopolysacharide and TNF-α-induced expression of iNOS and COX-II via suppression of NF-κB (Park et al., 2006). Furthermore, treatment with terpene fraction extracted from EGb 761, showed a therapeutic effect parallel to treatment with indomethacin, an anti-inflammatory agent, on Candida albicans-induced arthritic inflammation and suppressed NO production in lipopolysacharide-treated macrophages (Han, 2005). Ginkgo biloba extract enriched 10 times in terpene trilactones have been found to be more effective than EGb 761 in rescuing hippocampal neurons from Aβ-induced synaptic dysfunction and this protection is believed to be mediated mainly by Ginkgolide J (Vitolo et al., 2009). Another proposed mechanism for terpene trilactones is via antagonistic effects on the inhibitory glycine and GABA receptors (Ivic et al., 2003).

Ginkgolides, particularly Ginkgolide B (GB), in addition to its anti-inflammatory action are selective agonists on platelet activating factor receptor (PAF-R), where it competes with the platelet activating factor (PAF) and acts as effective anti-platelet factor antagonists, therefore preventing platelet aggregation and thrombus formation (van Beek and Montoro, 2009;Chen et al., 2010). Ginkgolide B has also been shown to protect primary cultured neurons from glutamate-induced excitotoxicity. This protection was more pronounced than EGb 761 and inferior to MK-801 (Xu et al., 2010). Another study has shown that Ginkgolide A (GA) has an inhibitory effect on Aβ oligomerization and Aβ-induced paralysis in transgenic caenorhabditis elegans (Wu et al., 2006).

The Bilobalide (Bil), which constitutes 2.9% of terpene trilactones (Chan et al., 2007), has been investigated by several *in vivo* and *in vitro* studies, and is believed to have anti-ischemic and anti-edematous effects on cerebral neurons, and to mediate the neuroprotective effects of ginkgo biloba (Defeudis, 2002). Kiewert et al (2007; 2008) investigated the interaction between bilobalide and neuronal transmission mediated by gamma-aminobutyric acid and glycine, in rat hippocampal slices exposed to NMDA (Kiewert et al., 2007;Kiewert et al., 2008). Other research studies have shown that bilobalide can modulate gene expression by enhancing expression of mRNA-encoded COX III subunit of cytochrome oxidase in the resistant hippocampal CA1 neurons, which are challenged by global brain ischemia (Chandrasekaran et al., 2001) and enhancing the level of mRNA for the mtDNA-encoded subunit 1 of NADH dehydrogenase in PC12 cells (Tendi et al., 2002). In addition, simultaneous treatment of PC12 cells with ROS and bilobalide has resulted in a dose dependent reduction in the apoptotic rate via reducing ROS-induced elevation of Bax and caspase-3 activation (Zhou and Zhu, 2000). In another experiment, bilobalide, ginkgolides A and J have demonstrated anti-apoptotic effect in cultured chick embryonic neurons and in a mixed culture of hippocampal neurons subjected to staurosporine and serum deprivation, with more protection being observed with bilobalide (Ahlemeyer et al., 1999).

Although flavone glycosides and terpene trilactones fractions of EGb 761 are believed to be the neuroprotective constituents of Ginkgo biloba (Defeudis, 2002;Ahlemeyer and Krieglstein, 2003), other constituents are not devoid of pharmacological activity, and even though their precise contribution to the Ginkgo biloba neuroprotective effect

are not clear in literature, future research studies should be directed to elucidate their neuroprotective properties. Those include proanthocyanidins, which are antioxidant oligomers and polymers of monomeric flavans, forming approximately 7% of EGb 761 and they occur in Ginkgo leaves as well in the standardized extract (van Beek, 2002).

The phenolic acid 6-HKA, a derivative of kynurenic acid, is among the carboxylic acids occurring in EGb 761. It is believed to have low affinity, antagonistic activity at the NMD and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (Weber et al., 2001). In comparison to other derivatives of kynurenic acid, 6-HKA displayed superior affinity to AMPA receptors in hippocampal CA1 neurons. In addition its affinity to the NMDA receptors was half that of the parent compound, which reduces the side effects of the 6-HKA as compared with the high affinity antagonist, and adds to its therapeutic potentials (Weber et al., 2001).

There are six different types of alkylphenols that have been reported to occur in leaves of Ginkgo biloba (ginkgolic acids, ginkgols, bilobols, urushiols, isourushiols and α-hydroxycardanols), which constitute only less than 5 part per million of EGb 761 because of their allergic, cytotoxic and mutagenic side effects (van Beek, 2002;van Beek and Montoro, 2009).

Ginkgo biloba is generally a safe compound; however, there are some reported adverse effects including spontaneous hyphema (Rosenblatt and Mindel, 1997), spontaneous subdural hematoma (Rowin and Lewis, 1996)mild GIT upset and headache.

1.2.4 Ginkgo biloba in clinical trials

Ginkgo biloba has been advocated for the potential treatment of acute mountain sickness (Bartsch et al., 2004), tinnitus (Smith et al., 2005), diabetic nephropathy (Lu and He, 2005), bleeding disorders (Jiang et al., 2005) and intermittent claudication (Pittler and Ernst, 2000). However, there has been no substantial evidence of its therapeutic efficacy in these conditions.

The potential efficacy of Ginkgo biloba in alleviating memory impairment and other cognitive disorders associated with Alzheimer's and age related dementia, are widely suggested (DeFeudis and Drieu, 2000;Chan et al., 2007). However, clinical trials showed contradictory results about the benefits of Ginkgo biloba supplement in treating those conditions. A randomized clinical trial conducted for 6 weeks on patients with memory impairment, indicated that the Ginkgo biloba group did not differ from the controls, and that Ginkgo biloba supplement provided no enhancement in memory or related cognitive function (Solomon et al., 2002). Conversely, Le Bars et al (2002) documented improvement in the cognitive and social function in patients with mild and moderate Alzheimer's disease (Le Bars et al., 2002). These conflicting results in fact necessitate the need for clinical trials with large sample sizes and long follow-up periods.

Compared with the large number of clinical trials conducted to evaluate the effect of EGb 761 in other neurodegenerative diseases, only a few trials were conducted to evaluate its ophthalmic potential. EGb 761 has a demonstrated vasomodulatory effect on blood vessels, where it increases the ocular blood flow velocity by a mean of 24% compared to placebo (Chung et al., 1999). However, a randomized, double-masked,

placebo-controlled study was conducted over 2 years to investigate the effect of oral administration of 240 mg EGb 761 on ocular blood flow. This study on 15 healthy male volunteers failed to confirm a significant association between administration of a single dose of EGb 761 and the ocular blood flow (Wimpissinger et al., 2007), however, this result should not preclude its use in conditions with impaired ocular perfusion such as glaucoma, because it could be due to the small sample size, or the selected dose, or another possible neuroprotective mechanism for Ginkgo biloba. This is supported by an earlier prospective, randomized, placebo-controlled, double-masked trial, which was conducted in a sample of 27 patients with normal tension glaucoma, to evaluate the effect of EGb 761 on preexisting visual field damage, and reported improvements in automated visual field indices, with no significant changes in IOP, blood pressure or heart rate (Quaranta et al., 2003).

A summary of clinical trials that have demonstrated the effects of Ginkgo biloba supplementation in Alzheimer's dementia and in the eye is provided in Table 1.6

Table 1.6 Clinical trials on Ginkgo biloba

Neurodegenerative diseases	Main outcome measure	Outcome	Dose/Route of administration	Duration/ No of participant	References
Alzheimer's	Psychopathological and cognitive function tests	Improvement of dementia and cognitive impairment were same as cholinesterase inhibitors.	160 mg EGb 761 per day, 5mg donepezil 1x1/ oral	24 weeks, 76 patients	(Mazza et al., 2006)
	Cognitive function and activity of daily living tests	Decrease risk of developing Alzheimer's dementia.	-	7 years, 1462 elderly women	(Andrieu et al., 2003)
	Neuropsychological tests of cognitive functions	Improve cognitive and social functions.	40 mg EGb 761, 1x 3/oral	52 weeks, 168 patients	(Le Bars et al., 1997;Le Bars et al., 2002)
	Neuropsychological tests of cognitive functions	No beneficial effects were observed.	40 mg EGb 761, 1x 3/ oral	6 weeks, 230 elderly volunteers	(Solomon et al., 2002)
	Neuropsychological tests	Sig. improvement in memory and congnation.	120mg per day/ oral	3o days, 61 participants	(Stough et al., 2001)
	Psychopathological neuropsychological, and electrophysiological tests	Sig. improvement of attention and memory function.	40 mg EGb 761, 2x 3/ oral	3 months, 20 patients	(Maurer et al., 1997)
	Psychopathological and neuropsychological tests of cognitive and behavioral functions	Sig. improvement of attention and memory performance.	120 mg EGb 761, 1x 2/ oral	24 weeks, 216 patients	(Kanowski et al., 1996)

Ocular disease	Main outcome measure	Outcome	Dose/Route of administration	Duration/ No of participant	References
Glaucoma	VF and any ocular or systemic complications	Sig. improvement in VF parameters, no Sig. changes in IOP, BP or HR.	40 mg EGb 761, 1x 3/ oral	4 weeks/27 patients	(Quaranta et al., 2003)
	Doppler imaging to measure EDV in OA	Sig. increase in EDV in OA.	40 mg EGb 761, 1x 3/ oral	2 days/11 volunteers	(Chung et al., 1999)
	Doppler imaging to measure OBF	No Sig changes in ocular and systemic hemodynamics compared to controls.	240 mg EGb 761,1x 1/ oral	1 day/ 15 healthy volunteers	(Wimpissinger et al., 2007)
PVCRD, RD	Plasma and tear lipid peroxidation and antioxidative activity, Visual function	Decrease lipid peroxidation and increase antioxidant activity in tear and plasma and improve visual function.	-	-/33 Patients with PVCRD, 135 with operated RD, 32 with non-operated dystrophic RD, 22 healthy volunteers.	(Karazhaeva et al., 2004)
AMD	Visual function	Improve long distant visual acuity.	-	-/10 patients	(Lebuisson et al., 1986)
DM	Anatomical and Visual function	Improve colour vision.	40 mg EGb 761, 1x 3/ oral	3 months/ 15 patients.	(Bernardczyk-Meller et al., 2004)

SKT (Syndrome Kurz test), CGI (Clinical Global Impression), MMSE (Mini-Mental State Examination), Visual field (V/F), Significant (Sig.), intraocular pressure (IOP), blood pressure (BP), heart rate (HR), end diastolic velocity (EDV), ophthalmic artery (OA), Ocular blood Flow (OBF), peripheral vitreochoreoretinal dystrophies (PVCRD), retinal detachment (RD), Age related macular degeneration (AMD), Diabetes Mellitus (DM).

1.2.5 Neuroprotective effects of Ginkgo biloba and its potential mechanisms of action

1.2.5.1 In the Central Nervous System (CNS)

Several of the above-mentioned clinical trials in section 1.2.4 as well as recent experimental evidence suggest neuromodulatory effect of Ginkgo biloba in the CNS and reported its benefit in improving the symptoms of Alzheimer's disease and other cognitive disorders (Wu and Zhu, 1999;Le Bars et al., 2002;Mazza et al., 2006;Augustin et al., 2009).

Many research studies attributed the neuroprotective effect of Ginkgo biloba to its outstanding antioxidant capacity (DeFeudis and Drieu, 2000). This was observed in aged brain and liver where EGb 761 protected mitochondrial DNA from free radical attack (Sastre et al., 2002), and from nitric oxide induced toxicity (Bastianetto et al., 2000b). The neuroprotective effect has also been observed in cerebellar neurons stressed by H_2O_2 (Oyama et al., 1996). Bastianetto et al have shown that 100 µg/ml EGb 761 protects rat primary mixed hippocampal culture from sodium nitroprusside (SNP) induced neuronal loss which is believed to be mediated through blockade of Protein Kinase C (PKC) activity and superoxide scavenging properties (Bastianetto et al., 2000b).

Aβ-induced neuronal death has been attributed in part to increased ROS production by Aβ and the resultant oxidative damage (Shi et al., 2009). An *in vitro* study looking at Aβ-induced apoptosis in a pheochromocytoma neuronal cell line (PC12) demonstrated that exposure to $A\beta_{1-42}$ has increased ROS levels and that simultaneous administration EGb 761 completely prevented the toxic effect of Aβ (Yao et al.,

2001). Furthermore, in the same experiment, higher concentrations of EGb 761 were associated with increased cell viability measured using 3-4,5-dimethyithiazol-2-yl-2,5-diphenyl-tetrazolium bromide (MTT) and trypan blue assays. The mechanism of this neuroprotection of EGb 761 is thought to be through its inhibitory effect on Aβ derived diffusible neurotoxic ligands, presumably dimmer and tetramer of Aβ oligomer. This experiment also pointed out that flavone glycosides and terpene trilactones-free extract prepared from EGb 761 did not confer any protection against Aβ-induced apoptosis (Yao et al., 2001).

The effect of EGb 761 and its constituents, the flavone glycoside quercetin, and the terpene trilactone ginkgolide B, were tested against 100 µg $A\beta_{1-42}$-induced ROS accumulation and mitochondrial dysfunction in human neuroblastoma (SH-SY5Y) cells (Shi et al., 2009). The result showed mitochondrial protection mainly by EGb 761 and to a lesser extent by ginkgolide B. This reduction in Aβ-induced mitochondrial dysfunction is believed to be mediated via modulation of downstream effectors in the apoptotic pathway such as JNK, ERK1/2 and Akt signalling pathways. Moreover, it was found that, EGb761 prevented both H_2O_2 and platelet activating factor-induced apoptosis (Shi et al., 2009). Constitutes of Ginkgo biloba have shown protective effects even on $A\beta_{25-35}$ induced cytotoxicity. PC12 cells treated with 100 µM/l $A\beta_{25-35}$ for 24 hours were associated with cell loss, which was attenuated in a dose dependent manner by adding 25-100 µM/l bilobalide (Zhou et al., 2000). Furthermore, the inhibitory effect of ascending concentrations from 0.01-1 µM $A\beta_{25-35}$, on cholinergic transmission in a rat hippocampal slices, was examined by Lee

et al. (2004). This inhibition was blocked by addition of Ginkgolide B 0.01-10 μM, possibly via suppressing potassium-evoked acetylcholine release (Lee et al., 2004).

Augustin et al (2009) have investigated APP as a molecular target of Ginkgo biloba neuroprotective effects. The authors examined the effect of dietary supplementation with 300 mg/kg of EGb 761 for 1 and 16 months, on APP protein levels in a transgenic human APP (Tg2576), model of Alzheimer's disease. Up to a 50% reduction in the APP level in the cortex of the 16 months group was reported by this group, whereas no such reductions were observed in the hippocampus or in young mice (Augustin et al., 2009). To examine the effect of EGb 761 on the α-secretase pathway, another study using a daily dose of 80 and 150mg/kg/day EGb 761 was administered orally for 5 days to normal Sprague-Dawley (SD) rats. 5 days after commencement of treatment, western blot analysis was used to measure soluble α APP in the cortex and hippocampus. EGb 761 at 150mg/kg/day was found to significantly increase α APP secretion (Colciaghi et al., 2004). Furthermore, the authors examined the effect of increasing concentration from 5-200 μg/ml of EGb 761 on acute hippocampal slice for 30 min. They found that the dose response curve to increasing EGb 761 dosage was bell shaped, where immunoreactivity of α APP observed with the lowest concentrations and was not seen at higher doses (ibid).

Accumulating evidence suggests a link between Aβ deposition, oxidative stress, and apoptosis. Luo et al have looked at the antioxidative, antiamyloidogenic and antiapoptotic effects effect of EGb 761 on Aβ accumulation and caspase-3 activation using a neuroblastoma cell line (Luo et al., 2002). In this experiment, 40μM $A\beta_{1-40}$ was incubated either alone or in the presence of 100g/ml of EGb 761.

Immunohistochemical labelling of Aβ showed that EGb 761 prevented Aβ accumulation both *in vitro* and in medium of Aβ producing cells. Furthermore, using Mitosensor, the authors have shown that EGb 761 attenuated caspase-3 activity (ibid). The effect of EGb 761 on cochlear caspase level has also been investigated as a possible mechanism in age related hearing loss. EGb 761 administered orally at a dose of 100mg/kg daily to 4 and 12 months old SD rats were associated with a significant reduction in age related caspase-3 and caspase-9 activation, an increase in ATP levels, and modulation of superoxide dismutase activity in rat cochlea (Nevado et al., 2010).

Ginkgo biloba is proposed to have multiple protective effects on mitochondrial function and apoptotic pathways, including stabilization of mitochondrial membrane potential, and enhancing respiratory chain energy production as well as down regulation of executor caspases. Abdel-Kader et al have investigated the effects of EGb 761 *in vitro* on mitochondrial functions in the PC12, dissociated mice brain cells, as well as on isolated mitochondria exposed to serum deprivation, SNP and complex inhibitors such as Sodium Azide (NaN_3) (Abdel-Kader et al., 2007). They used ATP levels and mitochondrial membrane potential as indicators of mitochondrial function; and showed enhancement of mitochondrial function even with the lowest EGb 761 concentration 0.01 mg/ml. Additionally *in vivo*, the authors found that treating two different age groups of mice with EGb 761 100mg/kg for 14 days was associated with beneficial effects on complexes I, IV and V of the mitochondrial respiratory chain (Abdel-Kader et al., 2007). Furthermore, Eckert and associates have demonstrated that EGb 761 protected PC12 cells mitochondria from H_2O_2, antimycin (complex III I

inhibitor) and Aβ-induced MTT reduction, and that 2 weeks treatment with EGb 761 reduced ROS induced apoptosis in mice lymphocytes (Eckert et al., 2003).

To examine the effect of Ginkgo biloba on the mitochondrial respiratory chain, one study looked at the effect of oral administration of 25-100mg/kg/day EGb 761 and 3 and 6 mg/kg/day bilobalide on the level of mitochondrial DNA (mtDNA)-encoded cytochrome Oxidase COX subunit III in hippocampal CA1 neurons. The result showed that both EGb 761 and bilobalide fraction confer protection against ischemia-induced reduction in COXIII mRNA and neuronal loss (Chandrasekaran et al., 2001). Furthermore Tendi et al reported that 100 µg/ml EGb 761 and 10 µg/ml bilobalide increases NADH Dehydrogenase (complex I) mRNA level and mitochondrial respiratory control ratio in PC12 cells (Tendi et al., 2002).

The anti-inflammatory effect of Ginkgo biloba and its modulation of key-inflammation related molecules in the CNS have been investigated by several research studies. Jiao et al (2005) studied the effect of Ginkgo biloba extract on protein and mRNA expression of pro and anti-inflammatory cytokines in the brain of a rat model of atherosclerosis. This group found that 100mg/kg/day EGb 761 for 8 weeks inhibited the production of pro-inflammatory cytokines: IL-1β and TNF-α and up regulate the anti-inflammatory cytokines: IL-10 and IL-10R (Jiao et al., 2005).

Another experiment looked at activated human T lymphocytes, isolated from whole blood and was conducted by Tsao and associates in 2008. This research reported that pretreatment with 25-100 µg Ginkgo biloba extract protected human T lymphocyte from TNF-α and H_2O_2 induced damage (Tsao et al., 2008).

Recent studies have looked at the anti-inflammatory activity of Ginkgo biloba in inflammatory bowel disease, chronic relapsing inflammatory conditions mediated by overactive immune system, and have shown that EGb 761 suppresses the activation of inflammatory markers (iNOS, Cox-2 and TNF-α) and reduces the number of effector T cells (Kotakadi et al., 2008;Zhou et al., 2010).

EGb761 has been reported to prevent glucose induced accumulation of ECM in rat mesengial cells cultured in hyperglycemic conditions, and this is believed to be mediated via multiple mechanisms including decreasing the level of transforming growth factor-ß1 (TGF-ß1), insulin-like growth factor-1 (IGF-1), connective tissue growth factor (CTGF) and decreasing the expression of collagen IV and laminin (Ji et al., 2009).

The effect of Ginkgo biloba on excitotoxicity has also been investigated, and EGb761 has been shown to reduce glutamate-induced elevation of calcium concentrations, enhance neuronal viability in primary cultures from mouse cerebral cortex (Zhu et al., 1997), and prevent impairment of the Na/K-ATPase activity in a mouse model of focal cerebral ischemia (Pierre et al., 1999;Pierr et al., 2002).

Xiao et al (2006) investigated the effect of EGb 761 pretreatment against excitotoxicity induced by NMDA receptor over-activation and focal cerebral ischemia. In this study EGb 761 enhanced cell viability and showed lower affinity to NMDA receptors compared with MK-801 (Xiao et al., 2006). Furthermore, Li et al (2011) have investigated the modulatory effects of Ginkgo biloba on the NMDA receptor in acutely isolated hippocampal neurons. The authors showed that in addition

to antioxidation and free radical scavenging effect, Ginkgo also inhibited calcium influx via the NMDA receptors (Li et al., 2011).

The potential neuroprotective effect of EGb 761, ginkgolides A and B administration in Parkinson's disease has also been investigated in a 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced mouse model. The result showed that EGb 761, compared to Ginkgolides A and B, effectively protects against MPTP-induced nigrostriatal dopaminergic neuronal death, possibly via inhibition of Monoamine Oxidase (MAO) (Wu and Zhu, 1999).

Ginkgo biloba has also been used to improve cerebral insufficiency and enhances the cerebral blood flow, through inhibition of NO, PAF and catechol-O-methyltransferase (Diamond et al., 2000), and to recover the peripheral circulation and ameliorate peripheral vascular diseases such as intermittent claudication (Kleijnen and Knipschild, 1992).

1.2.5.2 In the Eye

There are several reported ophthalmic beneficial effects of Ginkgo biloba both in experimental models and in patients, which encourage its use in the treatment of ischemic and neurodegenerative ocular diseases.

As seen in the previous section (1.2.5.1), in studies on neuronal cells both *in vivo* and *in vitro*, mechanisms of oxidative stress, inhibition of the inducible pathological form of the NO synthase enzyme (iNOS), possible effect on the caspase-3 and Aβ pathway have all been proposed. In fact, *in vivo* and *in vitro* experiments conducted on Ginkgo biloba in the CNS thus far (summarized in table 1.7) appears suggestive of its potential in targeting mechanisms implicated in RGCs apoptosis in glaucoma.

Ginkgo biloba has been shown to increase the ocular blood flow velocity in young healthy volunteers compared to a placebo (Chung et al., 1999) and improves visual field parameter in patients with normal tension glaucoma and preexisting visual field damage (Quaranta et al., 2003). Furthermore, a case report published in 2007, on a patient with primary OAG, reduced visual field and acuity, an IOP between a 15-32 mmHg and on maximal tolerated medical therapy, showed that EGb 761 dramatically improved visual acuity over months, from counting finger occulus dexter (OD) and 20/50 occulus sinister (OS), with -8.00 sphere OD and -7 sphere Os to 20/40 OD and 20/30 OS after 30 months of follow-up. Although no improvement was observed in the visual field, the improvement in visual acuity was believed to be mediated either through enhancement of the ocular blood flow or via an EGb 761 neuroprotective effect (Dorairaj et al., 2007).

To investigate whether Ginkgo biloba usage could confer any protection against development of glaucoma over a 12 month period, the National Health Interview

study was conducted, in which self-reported information was collected from 30,964 participants over the age of 50 years and at risk of glaucoma. EGb 761 supplementation was not associated with reducing risks of developing glaucoma in the study group, where patients who reported having glaucoma were 26% less likely to report EGb 761 usage (Khoury et al., 2009). However, no clear association between EGb 761 and glaucoma was demonstrated, possibly because of the cross sectional design and the lack of information on the duration of Ginkgo biloba use and glaucoma (ibid), which necessitate the need for prospective randomized clinical trial to see if prophylaxis supplement of EGb 761 could prevent glaucoma development.

In a rat model of unilateral chronic glaucoma, Hirooka et al (2001) investigated the neuroprotective effects of EGb 761, and demonstrated that pretreatment and early post treatment with 100 mg/kg/day EGb 761 twice weekly protected and rescued RGCs. Although the exact mechanism of this protection was not investigated in this study, the authors attributed it to the antioxidant activity of EGb 76 (Hirooka et al., 2004).

In a model of OHT in rabbits, EGb 761 was associated with multiple beneficial effects including: inhibition of dexamethasone-induced IOP elevation, accumulation of extracellular materials within the cribriform layers of the trabecular meshwork as well as improving meshwork cellularity (Jia et al., 2008). The authors further explored its mechanism of action in cultured human trabecular cells; EGb761 was shown to significantly reduce anti-Fas ligand-induced apoptosis and dexamethasone-induced myocilin expression (ibid). This suggests another possible mechanism for Ginkgo biloba via modulation of gene expression.

The effect of a mixture of herbal extracts including Ginkgo biloba have been shown to enhance viability of axotomized RGCs, although separate treatment with any single constituent did not confer such protection (Cheung et al., 2002). To further explore the possible mechanism of this neuroprotection in adult hamsters, the same group has confirmed recently the antioxidant effect of this herbal mixture, where they found a substantial decrease in the endogenous NO content in axotomized RGCs without any effect on the NOS activity, confirming the free radical scavenging activity of this mixture. In addition, this herbal mixture showed inhibitory effect on the caspase-3 independent apoptotic pathway in RGCs (Cheung et al., 2008b).

In a rat model of optic nerve crush, intraperitoneal injections of EGb 761 before and after the injury enhanced RGCs viability in comparison to the controls (Ma et al., 2010). An earlier experiment by the same group in the same animal model showed a dose related enhancement in RGC survival even when EGb 761 was administered intragastrically after the injury (Ma et al., 2009). Using the optic nerve transection model in guinea pigs, intraperitoneal administration of EGb 761 showed anti-apoptotic effects and improved RGCs function on electoretinograms (Xie et al., 2009).

The neuroprotective effect of EGb 761 on RGCs is perhaps due to its broad spectrum of pharmacological activities including its promising antioxidant activity which was advocated by Ritch in 2000 as the basis of its use as anti-glaucoma medication (Ritch, 2000). The observed antioxidant activity of EGb 761 is believed to account for its beneficial effect not only in glaucoma, but also in other neurodegenerative diseases such as AMD (Rhone and Basu, 2008). This beneficial effect was also observed in a

double-blind trial comparing Ginkgo biloba extract with a placebo control in 10 patients with AMD (Lebuisson et al., 1986), and in patients with peripheral vitreo-chorioretinal dystrophies and dystrophic retinal detachment (Karazhaeva et al., 2004).

Ginkgo biloba has been observed to modulate many inflammatory mediators (Jiao et al., 2005;Park et al., 2006;Kotakadi et al., 2008;Tsao et al., 2008;Zhou et al., 2010), and in glaucoma, there are several proposed stress factors for RGC apoptosis (Mittag et al., 2000;Tatton et al., 2001;Tezel and Yang, 2004), including the involvement of the immune system (Tezel, 2009). Glaucoma-related stimuli such as hypoxia, TNF-α and oxidative stress can trigger the mitochondrial-mediated RGC death pathway (Cheung et al., 2008a).

An interesting study looked at the free radical scavenging effect of Ginkgo biloba on aged mitochondria. This study, which was performed on retinal Muller cells from guinea pigs, has confirmed that EGb761 treatment considerably enhanced mitochondrial membrane potential and preserved mitochondrial ultrastructure of the aged cells (Paasche et al., 2000). EGb761 has also been observed to protect primary cultured rat retinal neurons against glutamate-induced ischemic injury and improve mitochondrial membrane potential (MMP), thus making it an effective antioxidant at the mitochondrial level (Wang et al., 2005b). This ability to counteract the glutamate-mediated neurotoxicity further enhances its potential as a possible ophthalmic drug in glaucoma management as these pathways have already been implicated in the apoptotic RGCs death in glaucoma (Osborne et al., 1999;Casson, 2006).

Another interesting study, was conducted in the rat insulinoma cell line and the rabbit corneal cell line by Thiagarajan et al (2002). This group confirmed the potentials of

EGb 761 on alloxan and dexamethasone induced apoptosis and Photo-Fenton reagent induced hydroxyl radicals. Furthermore, *in vivo* EGb 761 halted the progression of selenite-induced cataract in rat through its antioxidant property (Thiagarajan et al., 2002).

Researches on retinal photoreceptor and EGb 761 have also been carried out; intraperitoneal injection of EGb761 protects photoreceptors against light induced injury, possibly by inhibiting apoptosis and preventing oxidative stress in rat retinas (Xie et al., 2007). Similar protection with intragastric administration of EGb761 on photoreceptors after light-induced injury has also been demonstrated using electrophysiological test (Ranchon et al., 1999). EGb761 has been found to significantly reduce the ischemia-reperfusion induced Na^+ and Ca^{2+} accumulation and K^+ loss in ischemic-reperfused retinal tissue (Szabo et al., 1993).

A summary of different molecular targets of Ginkgo biloba that have been identified in literature in CNS and Eye and the experimental work performed thus far are shown in Table 1.7

Table 1.7 Summary of different pro-apoptotic molecular targets of Ginkgo biloba

Pro-Apoptotic Mechanism	Target	Compound	Model	References
Oxidative stress	NOS	EGb 761, Ginkgolide B, Bilobalide and CP 205	*in vitro* LPS, IFN-γ, SNP, 3-morpholinosydnonimine	(Kobuchi et al., 1997;Bastianetto et al., 2000b;Ahlemeyer and Krieglstein, 2003)
	ROS	GBE, EGb 761, Ginkgolides A, B,Bilobalide, quercetin, kaempferol and isorhamnetin EGb 761	*in vitro* H_2O_2, *alloxan or dexamethasone SS, SSP* *in vivo* ischemia-reperfusion, selenite cataract	(Oyama et al., 1994;Oyama et al., 1996;Ahlemeyer et al., 1999;Zhou and Zhu, 2000;Thiagarajan et al., 2002) (Szabo et al., 1993;Thiagarajan et al., 2002)
	MDA, T-SOD, GSH-Px, CAT	EGb 761	*in vivo* LIRD	(Xie et al., 2007)
Mitochondrial Dysfunction	Mitochondrial Respiratory Chain	EGb 761, Bilobalide	*in vitro* H_2O_2, glutamate, NaN_3 SNP, SS, *antimycin*, ischemia *in vivo* young and old mice, gerbil ischemia	(Zhou and Zhu, 2000;Tendi et al., 2002;Eckert et al., 2003;Wang et al., 2005b;Abdel-Kader et al., 2007) (Chandrasekaran et al., 2001;Abdel-Kader et al., 2007)
	Caspases 1,3 and 9	EGb 761, Ginkgolides A, B, C, and J and Bilobalide.	*in vitro* Aß (1-40), SS, SSP *in vivo* aged SD rats, Axotomy in hamster	(Luo et al., 2002;Smith et al., 2002;Massieu et al., 2004) (Cheung et al., 2008b;Nevado et al., 2010)
	ROS	EGb 761, Ginkgolide B, quercetin	*in vitro* Aß (1-42), H_2O_2, PAF	(Shi et al., 2009)
Protein Misfolding	Aß	EGb761, Ginkgolide A, B and J, Bilobalide,HE 208 and CP 205, quercetin	*in vitro* Aß (25-35), Aß (1-40) and (1-42) *in vivo* Aß , transgenic Caenorhabditis elegans, transgenic mice for APP	(Zhou et al., 2000;Yao et al., 2001;Bastianetto and Quirion, 2002;Luo et al., 2002;Shi et al., 2009;Vitolo et al., 2009) (Wu et al., 2006;Augustin et al., 2009)
	APP and caspase-3	GBE	*In vivo aluminum-treated rats*	(Gong et al., 2005)

Pro-Apoptotic Mechanism	Target	Compound	Model	References
	α-secretase	EGb 761	*in vitro* hippocampal slices *in vivo* SD rats	(Colciaghi et al., 2004)
Multiple	MYO anti-Fas ligand-induced apoptosis, HSPs	GBE	*in vitro* DEX treatment *in vivo* DEX induced OHT rabbit	(Jia et al., 2008)
Excitotoxicity	NMDA-R	GBE, EGb 761, Ginkgolide B, Bilobalide GBE	*in vitro* Glutamate, NMDA *in vivo* MCAO model	(Zhu et al., 1997;Wang et al., 2005b;Xiao et al., 2006;Kiewert et al., 2008;Xu et al., 2010;Li et al., 2011) (Xiao et al., 2006)
	Na/K ATPase	EGb 761	*in vivo* mouse model of FCI	(Pierre et al., 1999)
	GABA-R, Glycine-R	Ginkgolide, A, B, C and Bilobalide	*in vitro* GABA, NMDA	(Ivic et al., 2003;Kiewert et al., 2007;Kiewert et al., 2008)
	Unknown	EGb 761, Bilobalide	*in vitro* hypoxia-induced release of choline.	(Klein et al., 1997)
	ACh-R	Ginkgolide A and B	*in vitro* Aß (25-35)	(Lee et al., 2004)
	MAO	EGb761, Ginkgolide A and B	*in vivo* C57 mouse treated with MPTP	(Wu and Zhu, 1999)
Inflammation and immunological strategies	iNOS, Cox-2 TNF-α, IL-1 beta, IL-6, IL10, IL-10R,NF-κBp65, SOD, MDA	GBE, EGb 761	*in vitro* TNF-α *in vivo* TNBS-Induced Colitis, AS model, DDS mouse model	(Kotakadi et al., 2008) (Jiao et al., 2005;Zhou et al., 2006;Kotakadi et al., 2008)
	NO, PGE2	Ecb 761, GBB, terpene	*in vitro* LPS, TNF-α *in vivo* C.albicans-induced inflammation in mouse	(Han, 2005;Park et al., 2006) (Han, 2005)
	AP-1	GBE	*in vitro* H_2O_2, TNF-α	(Tsao et al., 2008)

CP 205= flavonoid fraction, LPS = Lipopolysaccharide, IFN-γ= interferon-gamma, SNP= Sodium nitroprusside, NaN3= sodium azide, GBE= Ginkgo biloba extract, MDA= malondialdehyde, T-SOD= total superoxide dismutase, GSH-Px= glutathione peroxidase, CAT=catalase, LIRD= light-induced retinal damage SS= Serum Starvation, antimycin= complex III inhibitor, SSP= Staurosporin, PAF=platelet activation factor, MYOC= myocilin, HSPs= Heat shock proteins, FCI mouse model= focal cerebral ischemia mouse model, HE 208= terpene and flavonoid-free EGb 761, MAO= Monoamine Oxidase, IL=interleukin, TNF-α= tumor necrosis factor-alpha, NF-κBp65 = nuclear factor-*κ*Bp65, SOD= Superoxide dismutase, MDA= malondialdehyde, TNBS = 2,4,6-trinitrobenzene sulfonic acid, ACh-R= acetylcholine receptors, AS= atherosclerosis, MCAO model= middle cerebral artery occlusion model, FCI= focal cerebral ischemia, GBB= Ginkgo biloba extract with higher levels of terpene and biflavonoid than EGb, PGE2= prostaglandin E , SD rats= Sprague Dawley rats, AP1= activator protein-1, DDS mice model=Dextran sulfate sodium mouse model of colitis, DEX treatment= Dexamethasone treatment.

Aims

Ginkgo biloba has demonstrated neuroprotective effects in animal models of neurodegenerative disease such as Alzheimer's disease, and experimental glaucoma. However, the exact mechanism of this neuroprotection is still unknown. The literature established Ginkgo biloba as targeting several neuroprotective pathways implicated in RGCs apoptosis in glaucoma, including the abnormal processing of amyloid precursor protein (APP) and amyloid-beta (Aß) deposition, abnormal mitochondrial function, modulation of the TNF-α pathway and regulation of apoptosis-related caspases such as caspase-3. The purpose of this study was: firstly to perform histological assessment of known molecular targets of Ginkgo biloba related to RGCs apoptosis in an experimental glaucoma model (specifically the expression of APP, Aß, cytochrome *c*, caspase-3 and TNF-α protein); and secondly, to investigate neuroprotective effects of Ginkgo biloba targets *in vitro*, including assessment of Ginkgolide A, Ginkgolide B and Bilobalide against different apoptotic inducers (namely DMSO, UV40, the mitochondrial toxin Sodium Azide and $Aß_{25-35}$) in cultured RGC-5 cells.

Chapter two:

2 Materials and Methods

2.1 Immunohistochemistry for Pro-Apoptotic target related to RGCs in OHT model

2.1.1 Animals

All conditions and experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines. Animals were housed in a 12 hours light /dark cycle with unlimited access to food and water.

2.1.1.1 OHT Rat Model

Dr Li Guo, Senior Research Associate in the department performed all surgery to achieve elevated IOP in the Ocular Hypertension (OHT) model used in this study. OHT induction was performed in the left eye of 15 Adult Dark Agouti (DA) rats, weighing 150-200 g, using method established in the group, in which IOP was elevated by injecting 1.80 M of hypertonic saline solution into two episcleral veins (Cordeiro et al., 2004;Guo et al., 2005a;Guo et al., 2005b;Guo et al., 2006;Guo et al., 2007a). This OHT rat model is modified from the previously described techniques by Morrison et al (Morrison et al., 1997;Morrison, 2005). The unoperated right eyes served as controls. IOP measurements of both eyes were performed at regular interval using a Tono lab Tonometer (Tiolat OY, Heisinki, Finland). Animals were deeply anaesthetized with intraperitoneal (IP) injection of Ketamix: ketamine (Ketaset 37.5%, Fort Dodge Animal Health Ltd., Southampton, UK)/medetomidine (Dormitor 25%, Pfizer Animal Health, Pfizer limited, Kent, UK)/ sterile water (37.5%, Pfizer Animal Health, Exton, PA) solution (0.75ml ketamine, 0.5 ml medetomidine, and 0.75 ml sterile water) at 0.2 ml/100g. Animals were sacrificed at 1, 3 and 12 weeks

time points after IOP elevation. The eyes were rapidly enucleated and kept in 4% Paraformaldehyde (Sigma-Aldrich, UK).

2.1.2 Preparation of Retinal Slices

Expression of Amyloid Precursor Protein (APP), Aβ, the mitochondrial apoptosis related marker cytochrome-*c*, caspase 3 and TNF Receptor I was studied in OHT rat eyes using immunohistochemistry. Eyes were enucleated by making an incision posterior to the limbus; the cornea, iris and lens were removed. The retina and uveal tissue were dissected from the surrounding tissues and embedded in paraffin. Sequential 5µm thick paraffin-embedded retinal sections from OHT and age matched controls (n=5 per time point) were acquired and cut using the (MSE Ltd, Maidstone, UK) cutting microtome. Three sections were collected from each paraffin block and transferred to onto SuperFrost Plus microscope slides (VWR International bvba) for immunostaining.

2.1.3 Immunohistochemical staining

Retinal sections of OHT and age-matched controls at 3 time points: 1 week, 3 weeks and 12 weeks of IOP elevation were used. Sections were incubated with each of the following primary antibodies: goat polyclonal primary antibody to APP (Abcam ab2084) 1:1000 concentration, rabbit polyclonal to Aβ (Abcam ab68896) 1:750 concentration, rabbit polyclonal to cytochrome *c* (Cell Signaling 4272) 1:400 concentration, rabbit polyclonal to cleaved caspase-3 (Cell Signaling 9661) 1:300 concentration and rabbit polyclonal to TNF-R1 Abcam (ab58436) 1:100 concentration.

The following secondary antibodies directed against each primary were used, Donkey polyclonal anti-goat (FITC) to detect APP, Donkey anti-rabbit IgG (Cy3) to detect Aβ and cytochrome *c*, biotinylated goat anti-rabbit IgG to detect caspase-3 and TNFR1. A list of antibodies used in the study are shown in Table 2.1

The final working concentration of APP, Aβ and cytochrome *c* were determined by prior titration of the antibodies by Shereen Nizari in the group, and the titration of active caspase-3 and TNF-R1 was performed by the writer using the manufacturers recommended dilution range.

Table 2.1 Primary and Secondary antibodies used in the study

Primary antibody	Dilution	Source	Secondary antibody	Dilution	Source
Polyclonal primary antibody to APP	1:1000	Abcam (ab2084)	Donkey polyclonal anti-goat (FITC)	1:100	Abcam (ab6881)
Anti-beta Amyloid	1:750	Abcam (ab68896)	Donkey anti-rabbit IgG (Cy3)	1:100	JacksonImmuno-research Lab
Anti-cytochrome *c*	1:400	Cell Signaling (4272)	Donkey anti-rabbit IgG (Cy3)	1:100	JacksonImmuno-research Lab
Cleaved Caspase-3	1:300	Cell Signaling (9661)	Biotinylated goat anti-rabbit IgG DAB anti-rabbit kit		Abcam (ab64261)
TNF R I	1:100	Abcam (ab58436)	Biotinylated goat anti-rabbit IgG DAB anti-rabbit kit		Abcam (ab64261)

2.1.3.1 Immunohistochemistry on retinal cross sections for APP, Aβ and cytochrome *c* using immunofluorescence staining technique

The same immunohistochemical technique was used to stain retinal section with APP, Aβ and cytochrome *c*. 90 sections of OHT and age matched controls (n=5) were used in the study for each antibody, with 30 section per each time point 1 week, 3 weeks and 12 weeks of IOP elevation.

Retinal sections were de-waxed with Xylene 3 x for 5 minutes each and re-hydrated through a series of ethanol washes with descending concentration (100%-90% and

70%, 5 minutes each) before applying a final wash of running tap water for 5 minutes. Sections were then blocked by incubation in a 3% Hydrogen peroxide solution for 25 minutes at room temperature (rtp). To permeabilize cell membranes for antigen retrieval and to enhance antibody staining, sections were placed in 10 mM Citrate buffer pH 6.0 (10 mM Citric acid, 25 mM Sodium Hydroxide in 1 L distilled water) and irradiated in a microwave at 650 W 2x for 2.5 minutes. Slides were then washed with water and Phosphate buffered saline (PBS) (Sigma-Aldrich, UK) for 5 minute each. Slides were then incubated in a 5% solution of normal donkey serum (Jackson Immuno Research) diluted in PBTA (1000 ml PBS, 0.5% w/v BSA, 0.1% v/v Tween, 0.1% w/v Sodium Azide) for one hour at rtp to block non-specific binding. Sections were then rinsed with PBS containing 0.1% Triton to relieve surface tension and further permeabilize the cytoplasmic membrane and incubated with the primary antibodies.

Goat polyclonal primary antibody to APP (Abcam ab2084), at a dilution of 1:1000, rabbit polyclonal to Aβ (Abcam ab68896) at a dilution of 1:750, and rabbit polyclonal to cytochrome *c* (Cell Signaling 4272) at a dilution of 1:400 were applied to retinal slides of OHT and age matched controls. Sections were incubated with Primary antibodies over night at 4°C in a humidified chamber. Sections were washed in PBS Triton for 5 minutes and incubated with secondary antibodies diluted in PBTA for one hour at rtp. Fluorescein isothiocyanate (FITC) Donkey polyclonal anti-goat antibody (abcam ab6881) at 1:100 concentrations was used as secondary antibody to detect APP, Cy3 donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, INC) at 1:100 concentrations was used to detect Aβ and cytochrome *c*. Sections were then washed with PBS Triton, PBS and water for 5 minutes each.

To stain the nuclear DNA retinal sections were stained with the fluorescent stain 4′, 6-Diamidino-2-phenylindole dihydrochloride (DAPI) (D9542 Sigma-Aldrich) at 1:2500 concentrations for 30 seconds then washed with PBS and water. Sections were then incubated in ascending concentration of ethanol (70%, 90%, 100%) 30 seconds each and then in Xylene for 5 minutes. Sections were then mounted with the glycerol-based mounting medium Citifluor, to avoid possible dehydration –induced changes in retinal sections and dye bleaching (Citifluor Ltd, UK). The slides were then labeled and kept in the fridge, protected from light until imaging.

Leica Microscopic Imaging

Fluorescent images were taken using the Leica Image Capture Microscope and x40 lens. Fluorescent signal of DAPI (excited at 358), Cy-3 (excited at 514 nm) was detected using the blue channel whereas FITC, (excited at 488nm) was detected by the green channel.

2.1.3.2 Immunohistochemistry on retinal cross sections for Active caspase-3 and TNFR1 using Immunoperoxidase staining technique

Retinal sections were de-waxed with three Xylene washes (5 minutes each) before re-hydrating through a descending serious of 5 minute ethanol (100%, 90% and 70%) washes followed by a wash under running tap water for 5 minutes. Sections were then blocked as described in section (2.1.3.1) and incubated with primary antibodies. 30 retinal slides per time point were then incubated with cleaved caspase-3 antibody (Cell Signaling 9661) 1:300 concentration, and 18 retinal slides per time point were stained with TNF R1 Abcam (ab58436) a dilution of 1:100 overnight in a humidified

chamber. Slides were washed 4 times in PBS triton (5 minutes each) before incubating with biotinylated goat anti-rabbit IgG (Abcam ab64261) for 10 min at rtp. Excess IgG was removed by washing a further four times with PBS before; slides were incubated with Streptavidin Peroxidase complex (Abcam ab64261) for 10 min at rtp. Slides were subject to x4 PBS washes before incubating with substrate-chromogen (Abcam ab64261) for 4 min. Finally, the slides were rinsed with water, dehydrated through a graded alcohol series (100%, 90% and 70 %) before staining with Hematoxylin and mounting in DPX. Slides were examined under Nikon Eclipse 80i microscope.

Nikon Eclipse Microscopic Imaging

Light micrographs of the retinal sections were taken using the Nikon Eclipse 80i upright microscope under a x40 objective.

2.1.4 Data Analysis and statistics

Sections were graded for staining by three independent and masked observers on the basis of the presence and the intensity of immunoreactivity, thickness and the homogenous or heterogeneous character of staining as in table 2.2 positive (+) indicated brightest than control slide, and negative (–) stand for no labeling in comparison with the control.

Table 2.2 Grading of Retinal Micrographs

Scale	intensity
+4	76-100% positive control
+3	51-75% positive control
+2	26-50% positive control
+1	1-25% positive control
0	Same as negative control
-1	1-25% negative control
-2	26-50% negative control
-3	51-75% negative control
-4	76-100% negative control

This grading system and the method of analysis was originally described by Shah et al (Shah et al., 1994) and validated by the group (Cordeiro et al., 1999a;Cordeiro et al., 1999b;Cordeiro et al., 2003;Guo et al., 2005a).

Statistical analysis was performed using SPSS 14 software. One-way ANOVA was applied to compare grading among glaucoma and age-matched controls at 3 time points, *$P < 0.05$ was considered to be significant.

2.2 Neuroprotective effects of Ginkgolide A, Ginkgolide B and Bilobalide in vitro against DMSO, UV40, NaN_3, and Aβ toxicity in RGC-5

2.2.1 Retinal ganglion cell (RGC-5) culture

RGC-5, a rat ganglion cell line transformed using E1A virus (Krishnamoorthy et al., 2001), was generously gifted to Prof. M F Cordeiro by Dr. Neeraj Agarwal (UNT Health Science Centre, Fort Worth, TX, USA). This cell line is used extensively in glaucoma research as an alternative to the primary RGCs because of the similarities between both cell lines and the troubles of culturing primary RGCs (Hu and Ritch,

1997). RGC-5 was identified on the basis of the selective expression of RGC markers such as Thy-1 (Krishnamoorthy et al., 2001), RGC-5 was cultured in T75 flasks and was maintained in filtered DMEM containing 10% FBS, 100U/ml penicillin, and 100µg/ml streptomycin under a humidified atmosphere with 5% CO_2 at 37 °C. The RGC-5 was grown to 80-90% confluence before washing with PBS and passaged by trypsinization using 3ml of 0.05% Trypsin-EDTA solution as described previously (Krishnamoorthy et al., 2001). Trypsin was then neutralized with 7 ml filtered DMEM and RGCs were centrifuged at ~200g for 3 minutes to pellet the cells. Pellets were then resuspended in filtered DMEM and split into new T75 flask at a 1×10^6 concentration. These flasks were fed every other day by fresh filtered DMEM (Harper et al., 2009).

2.2.2 Reagents

Dulbecco's modified Eagle's medium (DMEM) from Lonza Walkersville, Inc., Fetal Bovine serum (FBS) from GIBCO invitrogen, Penicilline/Streptomycine by the cell culture company PAA-laboratories Gmbh, Trypsin-EDETA from GIBCO invitrogen, Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N,N-Dimethylformamide (DMF) and Soduim Azide (NaN_3) were purchased from Sigma-Aldrich, Company Ltd. UK, Aβ peptides corresponding to the sequence of human $A\beta_{1-42}$ and $A\beta_{25-35}$ were obtained from Sigma-Aldrich, Company Ltd. UK, Ginkgolide A, Ginkgolide B and Bilobalide were purchased from (Sigma-Aldrich, Company Ltd. UK), Hoechst 33342 and Trypan Blue from invitrogen, ApopTag® Fluorescein Direct In Situ Apoptosis Detection Kit from CHEMICON,

Paraformaldehyde and Propidium iodide solution (PI) from Sigma-Aldrich, UK. Ethanol and Acetic acid were purchased from Fisher Scientific, UK.

2.2.3 Toxicity and pharmacological treatment of RGC-5

For each of the following experiments RGC-5 was seeded at a density of 1×10^5 viable cells per well into 96-well plates. This density was chosen from the published densities in literature (Munemasa et al., 2008) and based on the writer's Preliminary experiments during this cell culture work to give 80-90% confluency in the 96 well plates after 24 hours incubation. Cell population counts were determined using a Haemocytometer with trypan blue exclusion assay. To plate the cells at 1×10^5 viable cells per well into 96-well plates, 10µl Pellet (mass of cells after centrifuging) are mixed with 10µl Trypan Blue dye to mark dead cells which stain dark blue due to Trypan Blue uptake, this 20 µl of cells are then taken onto a hemocytometer, covered with a cover slide and counted under a light microscope. To minimize random errors, cells are counted in the four quadrants of the hemocytometer paying attention to count only cells which intersect two of the boundaries. The total number of counted cells are then divided by 4 and multiplied by 2 (to compensate for trypan blue cell dilution) and this should give the number of cells in 0.1µl of the original pellet, the concentration of the cells (how many cells) in the whole volume of the pellet can be then calculated and using the equation $C_1V_1=C_2V_2$, where C_1= is the concentration of the cells in the original pellet, V_1 is the volume to take from this pellet, V_2 is the volume to be plated into the 96 well plate i.e 9600 µl if the whole 96 well plate are to be used and C_2 is 1×10^5.

Cells were then incubated in humidified atmosphere of 95% air and 5% CO_2 at 37 °C. After 24 hours cells were examined under microscope to check their shape and confluence and pretreated for 2 hours before the insult with ascending concentration (0.5-25 µg) of Ginkgolide A, B and Bilobalide diluted in sterile filtered DMEM. After this pretreatment, the entire medium was replaced with the indicated concentration of the insult (1% DMSO, 1mM NaN_3 and 50µM Aβ25-35 diluted in 0.5-25 µg Ginkgolide A, B and Bilobalide) or exposed to UV40. In every experiment 0.5% Triton was used as a negative control and filtered DMEM as positive control, furthermore, equal number of wells (n=5 in all experiment unless otherwise indicated) have been treated by 0.5-25µg Ginkgolide A, B and Bilobalide alone to make sure no toxicity is encountered from these drugs. RGC-5 viability in all experiment was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

2.2.3.1 DMSO treatment of RGC-5

Dimethyl sulfoxide (DMSO) is a widely used pharmaceutical solvent, has also been used to induce time and concentration dependent apoptotic effect in various cells (Trubiani et al., 1996;Liu et al., 2001). To examine the effects of increasing concentration of DMSO, RGC-5 cells were seeded at a density of 1×10^5 viable cells per well into 96-well plates, then incubated with filtered DMEM in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. After 24 hours the entire media was replaced by filtered DMEM mixed with 1%, 2.5%, 5% and 10% DMSO and incubated for a further 24 hours.

To examine the neuroprotective effect of 0.5-25 µg of Ginkgolide A, B and Bilobalide on the RGC-5 death induced by 1% DMSO, RGC-5 were seeded as described above and incubated in filtered DMEM for 24 hours. Cells were next pre-treated with 0.5-25 µg of Ginkgolide A, B and Bilobalide for 2-hours, following which the media was replaced with ascending concentrations of 0.5-25 µg of Ginkgolide A, B and Bilobalide mixed with 1% DMSO for a period of 24 hours.

2.2.3.2 UV treatment of RGC-5

UV light has been used by several researchers to induce ROS mediated apoptotic damage. It has been shown to produce a dose dependent reduction in the viability of RGC-5 (Dunkern et al., 2001;Balaiya et al., 2010). To investigate the cytotoxic effect of shortwave UV-C energy on RGC-5, cells were plated as described above in four 96 well plates. In one 96 plate unexposed cells were used as control, and the other three plates were exposed to 40, 60 and 80 mj/cm^2 UV-C light (254nm) for 2 minutes using CL-1000 Ultraviolet Crosslinker (Ultra Violet Products Ltd. Cambridge).

To examine the neuroprotective effect of 0.5-25 µg Ginkgolide A, B and Bilobalide on UV40 stressed RGC-5; cells were pretreated with the indicated concentration of the potential neuroprotective agents for 2 hours before UV40 mj/cm2 exposure. RGC-5 was then incubated for another 24 hours before an MTT assay was conducted.

2.2.3.3 Sodium Azide (NaN_3) treatment of RGC-5

The mitochondrial failure induced by NaN_3 has been used to investigate the mechanisms of ischemic and neurodegenerative diseases, and been advocated to screen potential neuroprotective agents (Selvatici et al., 2009). For this reason, NaN_3

is employed in this study. Stock solution of 20mM of the mitochondrial toxin NaN_3 was prepared by dissolving 13mg of NaN_3 in 10ml DMEM and titrated to prepare the desired concentrations (1mM, 2.5mM, 5mM and 10mM), RGC-5 were treated with the indicated concentration of NaN_3 for 24 hours prior to MTT, based on the result of this experiment 1mM NaN_3 was chosen to stress RGC-5 before Ginkgolide A, B and Bilobalide treatments.

2.2.3.4 $A\beta_{1-42}$ treatment of RGC-5

To assess $A\beta_{1-42}$ induced neuronal cell death RGC-5 was treated with concentration range of 10-100 µM $A\beta_{1-42}$ for 24 hours. Stock solution of 1mM $A\beta_{1-42}$ was prepared by dissolving 0.1mg $A\beta_{1-42}$ powder in 22.15µl DMSO and stored in aliquots at -20 °C. On the day of experiment $A\beta_{1-42}$ aliquots were thawed for 5 minutes in sonicating water bath and centrifuged for 15 minutes at 12.300 RPM then diluted to the final concentration in DMEM. RGC-5 were pre-incubated with DMEM containing the indicated concentration of $A\beta_{1-42}$ (10,25,50 and 100 µM) for 24 hours following which an MTT assay was performed to assess cell viability.

2.2.3.5 $A\beta_{25-35}$ treatment of RGC-5

A stock solution of 5mM $A\beta_{25-35}$ was prepared by dissolving 1mg $A\beta_{25-35}$ in 200µl sterilized distilled water which was then stored at -20 °C and incubated at 37 °C for 3 days to aggregate before usage (Ban et al., 2006c;Tsuruma et al., 2010).

To investigate the toxicity of $A\beta_{25-35}$ on RGC-5 line, cells were treated with 5,10,25,50 and 100µM $A\beta_{25-35}$ for 24 hours before MTT cell viability assay was performed. RGC-5 pretreated for 2 hours with 0.5-25µg Ginkgolide A, Ginkgolide B and Bilobalide were then insulted with 50µM $A\beta_{25-35}$ mixed with the indicated

concentration of Ginkgolide A, B and Bilobalide for 24 hours. MTT assay was then performed.

2.2.3.6 Neuroprotective Drugs

Stock solution of 20mg/ml Ginkgolide A was prepared by dissolving 50mg Ginkgolide A powder in 2500 µl DMF and stored as 50µl aliquots. Ginkgolide B stock solution of 50 mg/ml was prepared by dissolving 10mg Ginkgolide B in 200µl DMSO, aliquoted and stored at -20 °C whereas a stock solution of 25mg/ml Bilobalide was prepared by dissolving 10 mg Bilobalide in 400µl acetone and stored at -20 °C as 40µl aliquots.

2.2.4 Assessment of cell viability and cell injury

2.2.4.1 MTT cell viability assay

24 hours following each of the above experiments MTT assay was conducted. This test is commonly used for assaying cell viability and depends on the reduction of the MTT, a yellow tetrazolium salt into crystalline blue formazan in live cells, by the action of the mitochondrial enzyme oxidoreductase (Ban et al., 2006b). The amount of formazan produced is proportional to the number and activity of the viable cells (Mosmann, 1983). After incubating the cells with the allocated treatment, 10 µl of MTT (0.5mg/ml) were added to each well of the 96 well plate containing 100µl of media. After 2-4 hours incubation period at 37 °C, equal volume of MTT stop solution (5 g SDS+ 50 ml DMF) were added into each well and left for 2 hours at 37 °C to dissolve the formazan crystal. The optical densities (absorbance) of the media in

the 96 well plates were determined using the (Safire II, Tecan, Switzerland) microplate reader at a wavelength of 570 nm with a reference wavelength of 630 nm.

2.2.4.2 In Situ Apoptosis Detection

A cell viability kit, usually referred to as the TUNEL assay, ApopTag® Fluorescein Direct In Situ Apoptosis Detection Kit; Chemicon was used in this study to detect apoptotic RGC-5 cells insulted by DMSO. This assay which is usually known as Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, detect apoptotic cells by specific staining by modifying fragmented DNA utilizing terminal deoxynucleotidyl transferase (TdT). RGC-5 was cultured in five of the 35mm glass bottom microwell petri dishes (MatTek corporation) for 24 hours, after that cells were exposed to 1%, 2.5%, 5% and 10% DMSO, one petri dish was used as a control in which RGC-5 were treated by DMEM only. 24 hours later TUNEL assay was started by fixing the cells by adding 2ml of 1% fresh paraformaldehyde to each petri dish for 10 min at 37°C, then wash twice in PBS for 5 min. RGC-5 cells were then post fixed by adding 2ml of cooled ethanol: acetic acid 2:1 for 5 min at -20 °C. Before applying equilibrium buffer cells washed again twice in PBS for 5 min each wash, aspirate off excess and added 50 µl equilibrium buffers to each petri dish for 10 sec. Excess liquid were gently taped and 30 µl of working concentration of TdT enzyme (105 µl reaction buffer and 45 µl TdT enzyme were applied to the central part of each petri dish and cells then incubated in humidity chamber for one hour at rtp. Later working concentration of stop buffer (1ml stop buffer in 34 ml d H_2O) was applied, cells were agitated for 15 seconds and incubated at rtp for 10 min. The final step in this assay was to counterstain and mounts after fluorescence staining, so

excess liquid gently tapped and 1.5 µl mounting media (1 µl of 1mg/ml PI in 1ml vector shield) was applied, glass cover slip then placed over and petri dishes placed in foil and stored at -20 °C until they were imaged using Leica Image Capture Microscope.

2.2.4.3 Hoechst 33342 staining

Hoechst 33342 is a trihydrochloride trihydrate blue fluorescent dye, capable of penetrating plasma membrane and staining the DNA of the apoptotic cell without needing permeabilization (Ban et al., 2006b). Compared to normal cells, the densely condensed chromatin of the apoptotic cells will uniformly take up the Hoechst 33342 and can be visualized by fluorescent microscopy. In these studies, this vital stain was used to examine DMSO-induced morphological changes in the nuclei of apoptotic RGC-5. After treating the cells in the 12 well plates with 1% DMSO and ascending concentrations of Ginkgolide A, Ginkgolide B and bilobalide for 24 hours as described before, RGC-5 was gently washed with PBS then fixed with fresh 4% Paraformaldehyde for 25 minutes at room temperature. Cells were then stained with Hoechst 33342 dye at a concentration of 5µl/ml for 5 minutes, washed with PBS and stored at -20 °C until they were examined under the Leica Image Capture Microscope and x40 lens. RGC-5 with high fluorescence intensity due to chromatin condensation or nuclear fragmentation were considered to be apoptotic.

2.2.5 Data Analysis and statistics

Data of the MTT reduction were expressed as the mean and 95% CI, statistical significance was further assessed by One-way ANOVA, *P < 0.05, **P < 0.01 were considered to be significant. Dose-response curves of Ginkgolide A, Ginkgolide B and Bilobalide were fitted using Origin 8.5 (OriginLab, Northampton, MA).

Data of Hoechst 33342 staining of DMSO-induced apoptotic nuclei was shown as apoptotic neurons as a percentage of total neurons (Massieu et al., 2004;Ban et al., 2006b;Santiago et al., 2007).

Chapter Three

3 Results:

3.1 Investigation of pro-apoptotic targets in OHT model

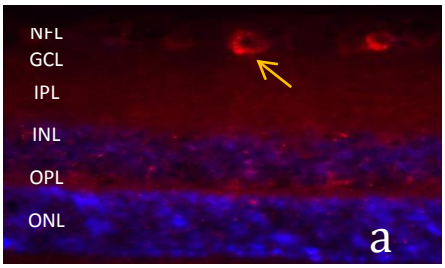
As discussed in section 1.2.5 several possible targets were identified as potentially modifiable by Ginkgo biloba and were investigated. Immunohistochemistry was used in this study to examine several of the potential targets for Ginkgo biloba in OHT rat eyes.

3.1.1 Immunohistochemistry for Cytochrome *c*

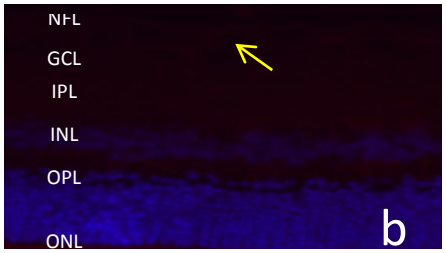
In this study the pattern of cytochrome *c* expression and the effect of IOP elevation was investigated in an OHT model that has been established by our group. OHT and age-matched controls at 1, 3 and 12 weeks after IOP elevation were assessed.

Results of cytochrome *c* deposition on paraffin-embedded retinal sections, as presented in (figure 3.1 a-f, red), showed marked up-regulation of cytochrome *c* immunoreactivity one week post OHT surgery (arrow head), reaching a peak at three weeks ($P < 0.05$). The level of cytochrome *c* activity in normal retina was almost undetectable at all time points. As illustrated in (figure 3.1 g) masked grading of retinal sections showed that cytochrome *c* level in the OHT retina dropped after 3 weeks to reach statistically non-significant level at 12 weeks, which is possibly due to the fact that mitochondrial damage is an early event in the apoptotic process, will lead to cytochrome *c* release, which then potentially contributes to RGCs apoptosis by activating caspases.

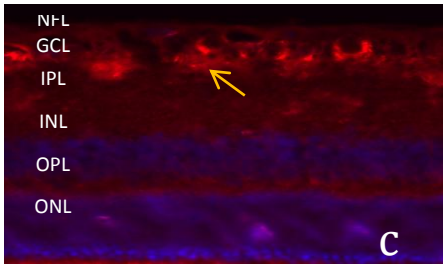
Leica Image Capture of Cytochrome c in OHT model and age–matched controls



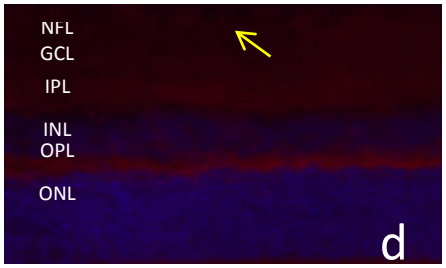
Cytochrome *c* stained OHT retina at 1 week



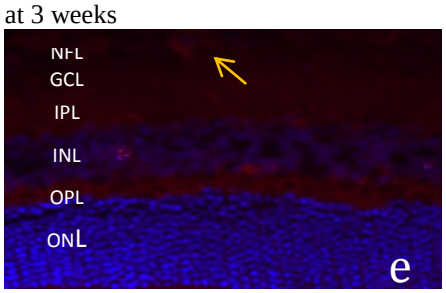
Cytochrome *c* stained control retina at 1 week



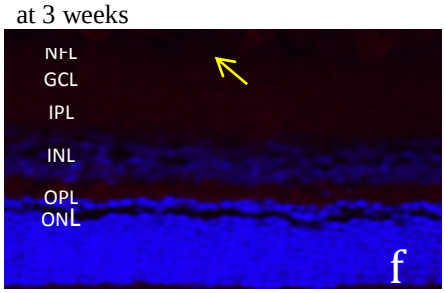
Cytochrome *c* stained OHT retina at 3 weeks



Cytochrome *c* stained control retina at 3 weeks



Cytochrome *c* stained OHT retina at 12 weeks



Cytochrome *c* stained control retina at 12 weeks

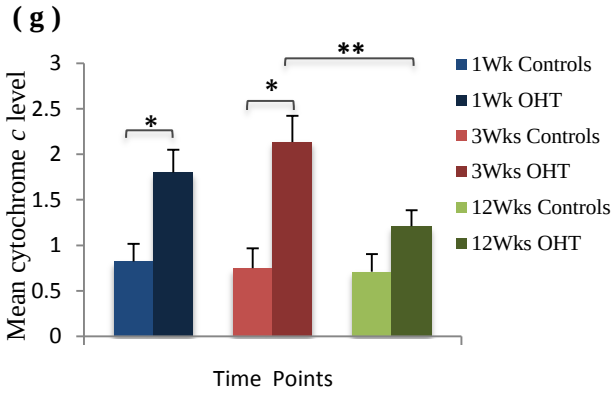


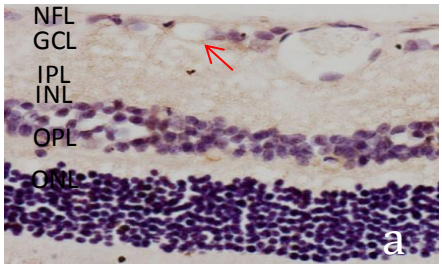
Figure 3.1 Cytochrome *c* Immunohistochemistry

(Figure 3-1 a-f, red): shows cytochrome *c* immunoreactivity in the RGCs and NFL on paraffin-embedded cross sections in the OHT retina and age-matched controls at 3 time points. Note that immunoreactivity was highest at 3 weeks in OHT eyes, with minmal changes in the controls, in (figure 3-1 g): comparison of cytochrome *c* level in OHT and age-matched controls at 3 time points. n=30 slide/time point. (All treatment groups were compared to each other and the control by one-way ANOVA followed by *post hoc* comparisons * $P < 0.05$, * * $P < 0.01$).

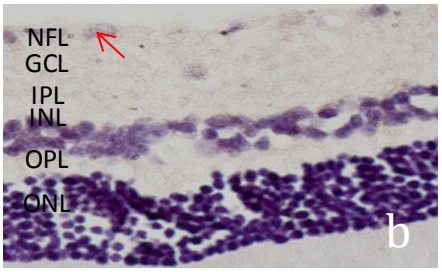
3.1.2 Immunohistochemistry for Active caspase-3 level

Immunoreactivity of activated caspase-3, the ultimate executioner caspase, was next investigated in this study. There was an apparent increase in caspases-3 deposition (figure 3.2 a-f, brown) in the retina of OHT at all of the time points observed (arrow head), compared with age-matched controls, caspase-3 up-regulation in the OHT eyes was statistically significant at 3 and 12 weeks ($P < 0.01$) as illustrated in (figure 3.2 g).

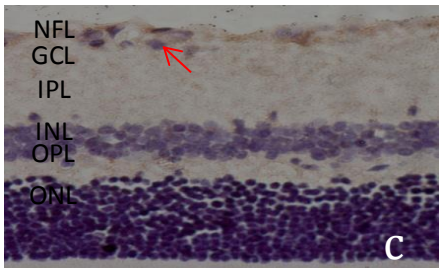
Nikon Eclipse 80i Image Capture of active caspase-3 in OHT model and age–matched controls



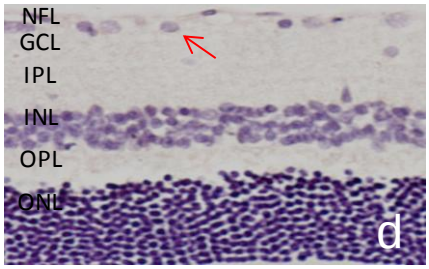
Caspase-3 stained OHT retina at 1 week



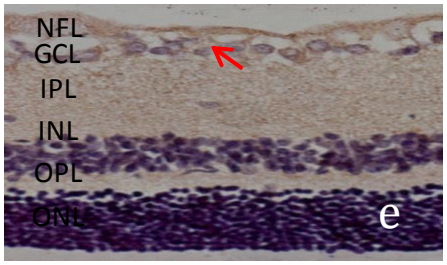
Caspase-3 stained control retina at 1 week



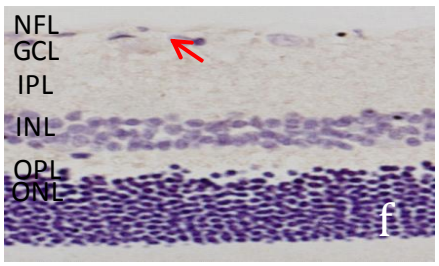
Caspase-3 stained OHT retina at 3 weeks



Caspase-3 stained control retina at 3 week



Caspase-3 stained OHT retina at 12 weeks



Caspase-3 stained control retina at 12 week

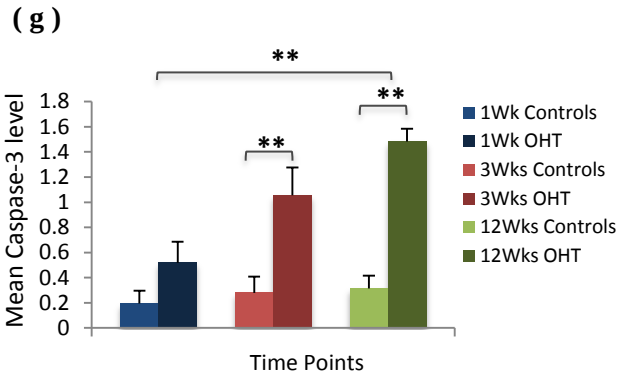


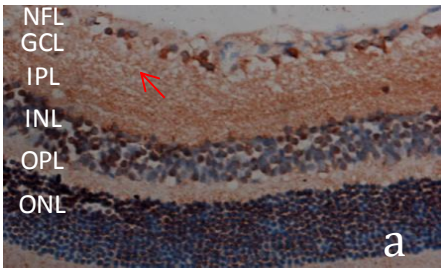
Figure 3.2 Active caspase-3 Immunohistochemistry

(Figure a-f, brown) shows caspase-3 immunoreactivity on paraffin-embedded cross sections in the OHT retina and age-matched controls at 3 time points. Note labeling of the RGCs and NFL which was maximum at 3 weeks in the OHT eyes compared with the controls, (figure 3-2 g) shows histological grading of active caspase-3 level in OHT and age-matched controls at 3 time points. n=18 slide/time point (All treatment groups were compared to each other and the control by one-way ANOVA followed by *post hoc* comparisons * * $P < 0.01$).

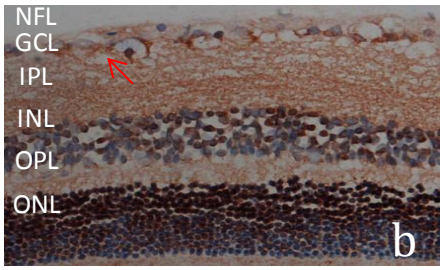
3.1.3 TNF-R1 level in OHT model and age –matched controls

To explore TNF expression in OHT eyes, retinal sections were then stained with TNF-R1 antibodies and the results are displayed in (figures 3.3 a-g, brown). Intense immunostaining of TNF-RI was observed in the ganglion cell layer as well as in the inner plexiform layer (arrow head) in OHT eyes, which peaked at 3 weeks after IOP elevation ($P < 0.05$ or $P < 0.01$) (figure 3.3, g), although positive immunoreactivity was seen in the control eyes as well, the intensity of the staining was lower in age matched controls.

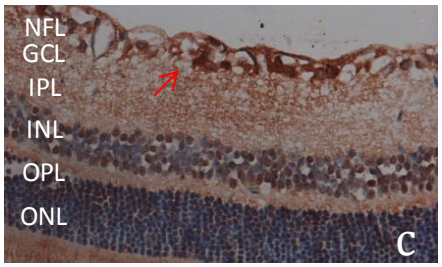
Nikon Eclipse 80i Image Capture of TNF-R1 in OHT model and age–matched controls



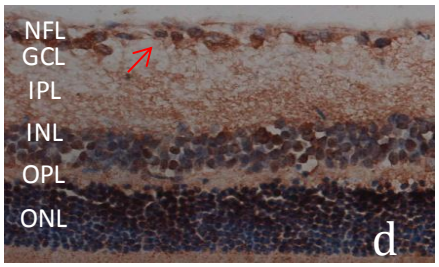
TNF-R1 stained OHT retina at 1 week



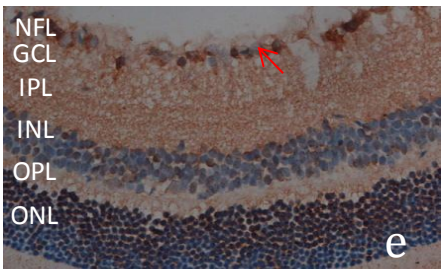
TNF-R1 stained control retina at 1 week



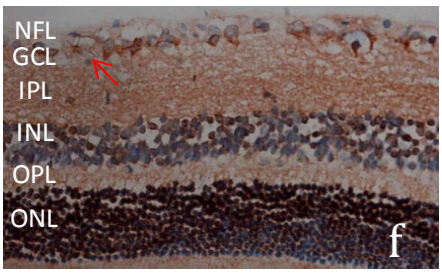
TNF-R1 stained OHT retina at 3 weeks



TNF-R1 stained control retina at 3 weeks



TNF-R1 stained OHT retina at 12week



TNF-R1 stained control retina at 12 week

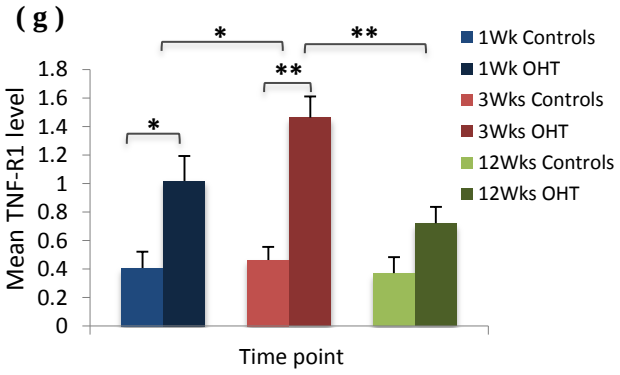


Figure 3.3 TNF-R1 Immunohistochemistry

(Figure a-f, brown) shows TNF R1 immunoreactivity on paraffin-embedded cross sections in the OHT retina and age-matched controls at 3 time points. Note labeling of the RGCs and inner plexiform layer. (Figure 3-3, g) illustrate comparison of TNF-R1 in OHT and age-matched controls, n=18 slide/time point (All treatment groups were compared to each other and the control by one-way ANOVA followed by *post hoc* comparisons * $P < 0.05$, * * $P < 0.01$).

3.2 Investigation of Aß in OHT

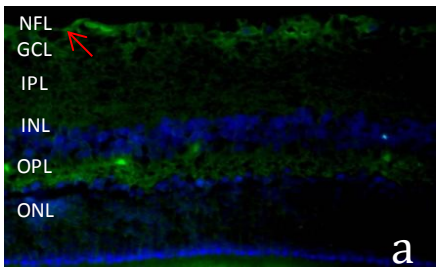
To examine the potential role of Aß in RGCs apoptosis in glaucoma, APP and Aß expression in the same experimental glaucoma (OHT) model was examined in this study.

3.2.1 Immunohistochemistry for APP and A*ß*

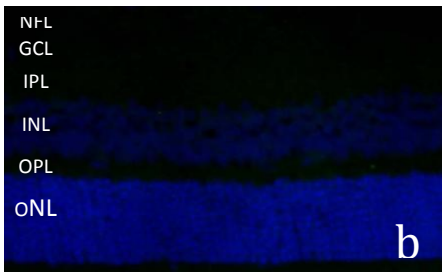
Leica image capture of APP in OHT eyes and age–matched controls are shown in (figure 3.4 a-f, green), enhanced expression of APP (arrow head) was found in the inner retinal layers (RGCs and nerve fiber layer) of OHT retinal sections while minimal immunoreactivity were observed in age-matched control; masked grading of the retinal sections (figure 3.4, g) illustrate significant reduction in APP expression in OHT rats over time ($P < 0.05$ or $P < 0.01$) to reach the lowest level at 12 weeks of OHT elevation. There was no apparent difference in APP labeling in the control sections.

We have observed enhanced Aß immunohistochemical expression in RGCs of all OHT rats in comparison with the control, as shown in (figure 3.5 a-f) (arrow head). Aß accumulation in the OHT retinal sections increased gradually during the observed time points, and the maximum deposition was evident at 12 weeks after OHT surgery. The graph displayed in (figures 3.5, g) shows the masked grading of this retinal sections and the statistically significant Aß accumulation at 3 and 12 weeks time points in comparison with the control ($p < 0.01$).

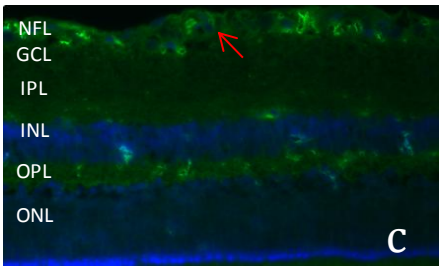
Leica Image Capture of APP in OHT model and age –matched controls



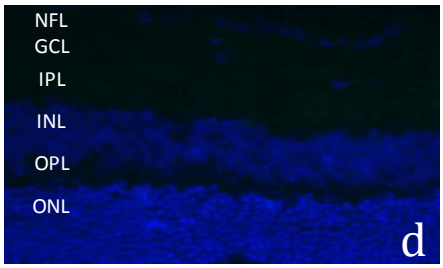
APP stained OHT retina at 1 week



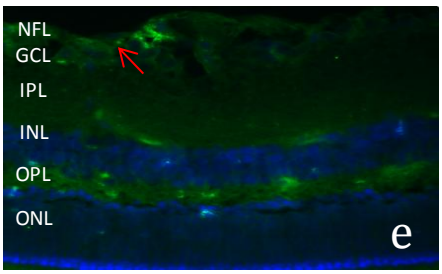
APP stained control retina at 1 week



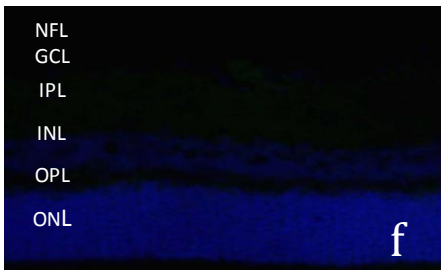
APP stained OHT retina at 3 weeks



APP stained control retina at 3 weeks



APP stained OHT retina at 12 weeks



APP stained control retina at 12 weeks

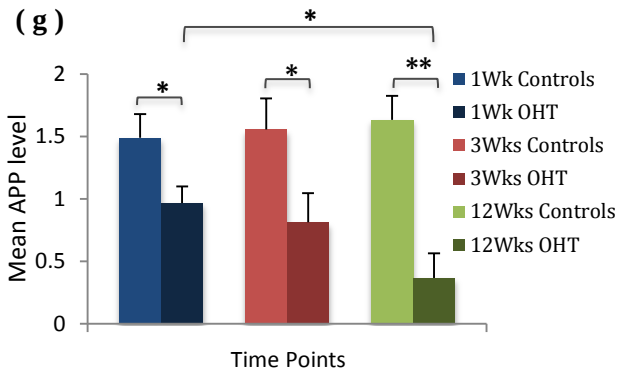
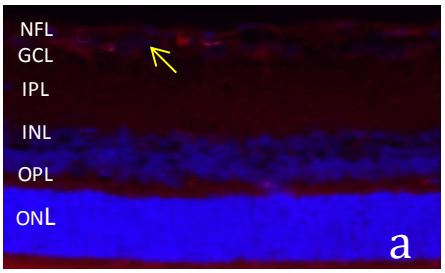


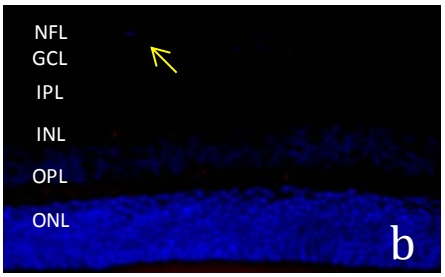
Figure 3.4 APP Immunohistochemistry

(Figure 3-4 a-f, green) shows apparent reduction in APP immunoreactivity in RGC layer on paraffin-embedded cross sections of the OHT retina compared to age-matched controls at 3 time points. Notice intense labelling of the RGCs and NFL in the OHT whereas no intensity observed in the age-matched controls, (figure 3-4 g) statistically significant reduction in APP level of OHT and age-matched controls at 3 time points, n=30 slide/time point. (All treatment groups were compared to each other and the control by one-way ANOVA followed by *post hoc* comparisons * $P < 0.05$, * * $P < 0.01$).

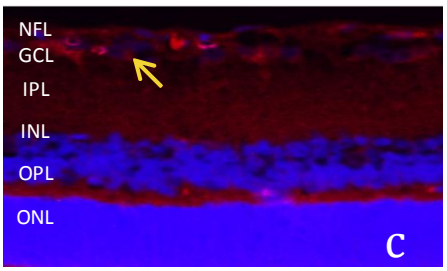
Leica Image Capture of Aß in OHT model and age–matched controls



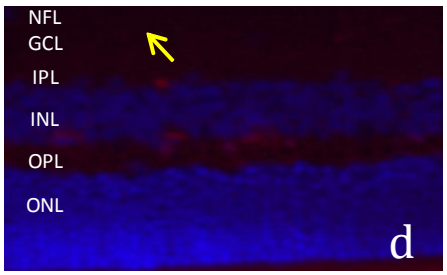
Aß stained OHT retina at 1 week



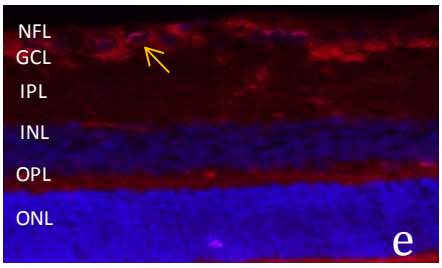
Aß stained control retina at 1 week



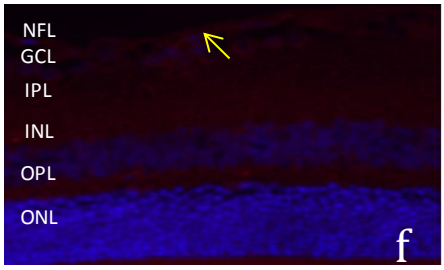
Aß stained OHT retina at 3 weeks



Aß stained control retina at 3 weeks



Aß stained OHT retina at 12 weeks



Aß stained control retina at 12 weeks

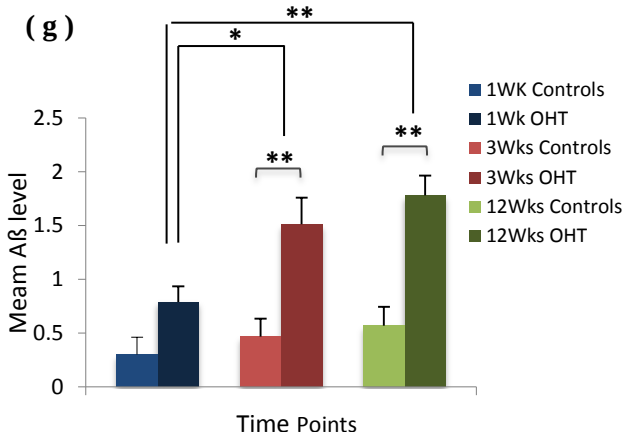


Figure 3.5 Aß Immunohistochemistry

(Figure 3-5 a-f, red) shows enhanced Aß immunoreactivity (red) in RGC and NFL on paraffin-embedded cross sections in the OHT retina and age-matched controls at 3 time points. Notice increased immunofluorescence intensity of Aß over time in OHT eyes, whereas minimum increase observed in the age-matched controls, (figure 3-5, g) comparison of Aß level among glaucoma rats and age matched controls at 3 time points, n=30 slide/time point. (All treatment groups were compared to each other and the control by one-way ANOVA followed by *post hoc* comparisons * $P < 0.05$, * * $P < 0.01$).

3.3 Investigation of RGC apoptosis in vitro

To mimic RGCs mitochondrial injury occurring in glaucoma, RGC-5 were exposed to the apoptotic inducers: DMSO, UVC and Na N3, and their effect on RGC-5 viability were examined using the MTT assay.

3.3.1 Effect of DMSO on RGC-5 survival

RGC-5 were treated with 1%, 2.5%, 5% and 10% DMSO, DMEM and 0.5% Triton and an MTT assay was performed 24 hours later to assess cell survival. Results showed all concentrations of DMSO significantly reduced RGC-5 viability compared to control (figure 3.6). DMSO treatment resulted in a dose dependent reduction in cell survival (MTT reduction) in comparison with the control (DMEM), 1% DMSO resulted in 27% reduction in RGC-5 viability, 57% cell survival reduction observed with 2.5% DMSO, 69% with 5% DMSO and 86% with 10% DMSO. Statistical analysis showed significant effects between different concentrations of DMSO and in comparison with the control (DMEM) ($P < 0.01$).

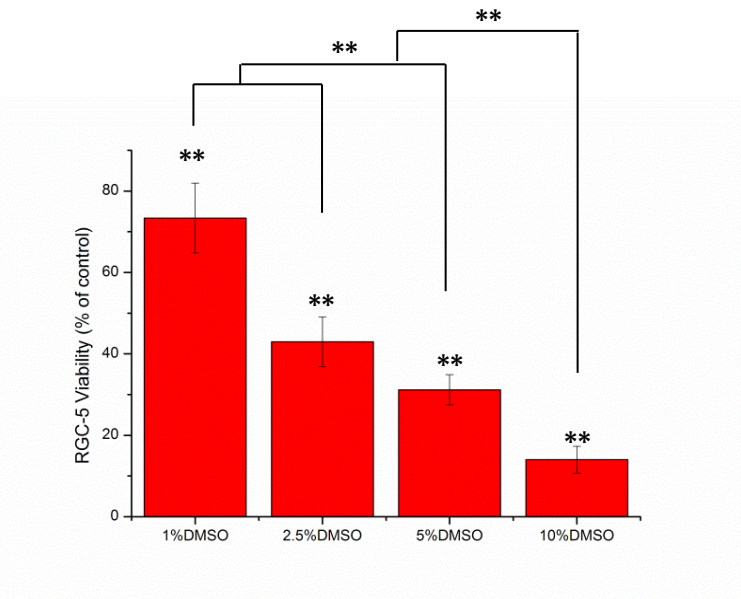


Figure 3.6 DMSO-induced RGC-5 death

Treating RGC-5 with a concentration range of 1-10% DMSO were associated with a dose dependent reduction in cell survival as confirmed by MTT assay, in comparison with base line DMEM the indicated concentration of DMSO has resulted in 27%, 57%, 69% and 86% reduction in RGC-5 viability, data are expressed as mean ± 95% CI, n=8. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * $P < 0.01$).

3.3.2 Effect of UVC on RGC-5 survival

Figure 3.7 shows the influence of UVC exposure on the viability of cultured RGC-5. There was a dose dependent reduction in RGC-5 viability following exposure to 40, 60 and 80 mj/cm^2, compared with cells incubated without UV treatment; RGC-5 viability has been reduced by 20% following UV40 and 47% after increasing the dose to 60 mj/cm^2, while doubling the radiation to 80 mj/cm^2 has resulted in 67% reduction in RGC-s viability by MTT assay. Compared with the control (DMEM), the effect of all UVC exposure on RGC-5 viability was statistically significant ($P < 0.01$).

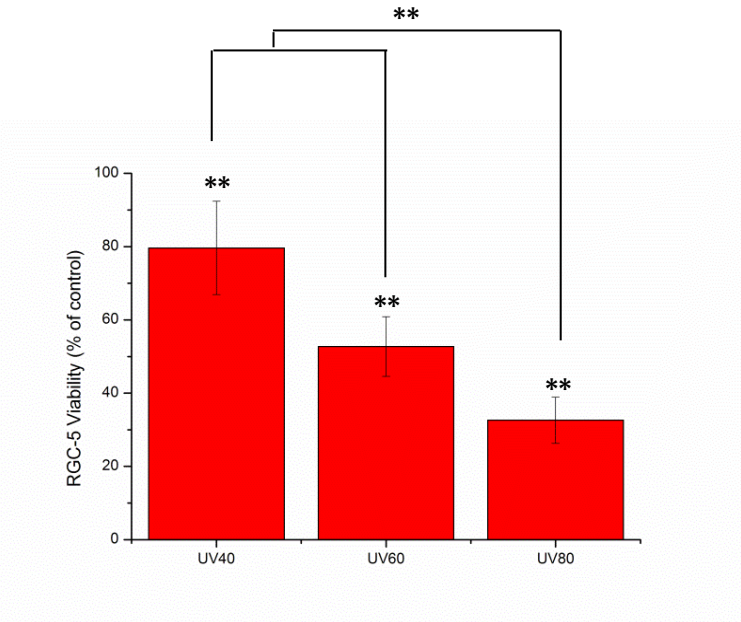


Figure 3.7 UV-induced RGC-5 toxicity

RGC-5 exposed to 40, 60 and 80 mj/cm^2 UV, were associated with a dose dependent reduction in cell survival as confirmed by MTT assay, in comparison with base line DMEM the indicated exposure of UV, has resulted in 20%, 47% and 67% reduction in cell survival, data are expressed as mean ± 95% CI, n=8. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * P < 0.01).

3.3.3 Effect of NaN_3 on RGC-5 survival

As shown in figure 3.8, 24 hours exposure of RGC-5 to 1, 2.5, 5 and 10mM NaN_3 resulted in a concentration dependent decline in cell survival. Exposing RGC-5 to the indicated concentration of NaN_3 had the effect of reducing viability on MTT assay to 35%, 49%, 51% and 62% of control level respectively. In comparison with the control (DMEM) this effect was statistically significant at all used concentrations of NaN_3 ($P < 0.01$ or $P < 0.05$).

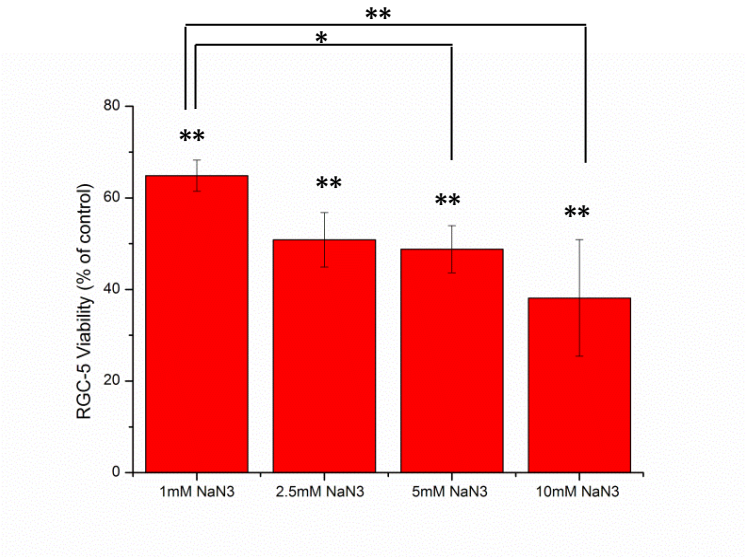


Figure 3.8 NaN_3-induced RGC-5 toxicity

RGC-5 treated with 1, 2.5, 5 and 10mM NaN_3, were associated with a dose dependent reduction in cell survival as confirmed by MTT assay, in comparison with base line DMEM RGC-5 exposure to the indicated concentration of NaN_3 has resulted in 35%, 49%, 51% and 62% reduction in cell survival, data are expressed as mean ± 95% CI, n=8. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * P < 0.05, * * P < 0.01).

3.4 Effect of Ginkgolide A, Ginkgolide B and Bilobalide on RGC-5 viability

From the previous section, it has been shown that all 3 pro-apoptotic insults decreased RGC survival, and before carrying on these investigations, it was necessary to examine and compare effects of Gingko biloba constituents alone on RGC-5 viability. RGC-5 were incubated with different concentrations (0.5-25µg/ml) of GA, GB and Bil alone for 24 hours to access any possible cytotoxicity using these agents alone might have on RGC-5 viability. As shown in (figure 3.9), toxicity was observed with higher doses of GA (5, 10, and 25 µg/ml). These doses has largely reduced RGC-5 viability leading to 78%, 65% and 55% reduction in cell viability respectively in comparison with the control (DMEM), whereas lower doses (0.5, 1 and 2.5µg/ml) of GA appear to have no significant differences in comparison to control (DMEM)

(P < 0.01), and were associated with 99%, 92% and 88% reduction in cell viability, suggesting that GA is not cytotoxic at lower doses.

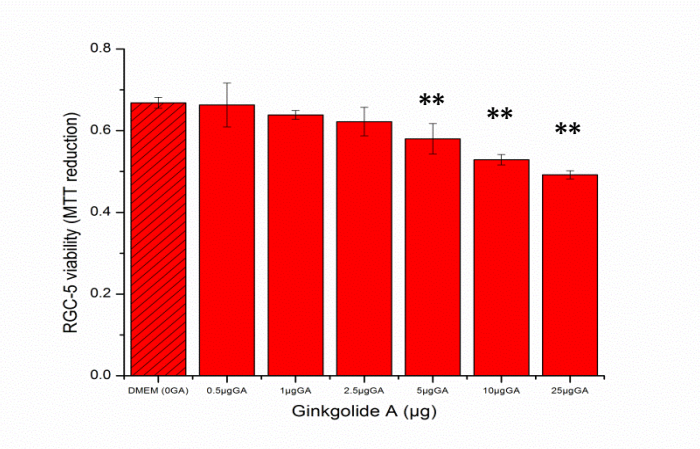


Figure 3.9 Effects of Ginkgolide A on RGC-5

RGC-5 treated with a concentration range (0.5-25µg) Ginkgolide A, and MTT assay to access cell viability was performed after 24 hours, as in (figure 3.9) in comparison with baseline DMEM the indicated concentration of GA were associated with dose dependent reduction in the mitochondrial activity of living RGCs with no apparent toxicity at the lower doses in comparison with control (DMEM), data are expressed as mean ± 95% CI, n=5, Y axis= Abs $_{570nm}$. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * P < 0.01).

Looking at the effect of Ginkgolide B (Figure 3.10), it is visible that higher RGC-5 viability than the control (DMEM) were observed with (0.5, 1 and 2.5µg/ml) GB leading to 134%, 115% and 107% higher RGC-5 survival in comparison with the control while (5, 10, and 25 µg/ml) GB were found to be associated with 100%, 91% and 85% reduction in RGC-5 survival. No toxicity was evident with (0.5-10 µg/ml) GB. Statistical analysis revealed significant difference between the control and the lowest and the highest used concentration (P < 0.01).

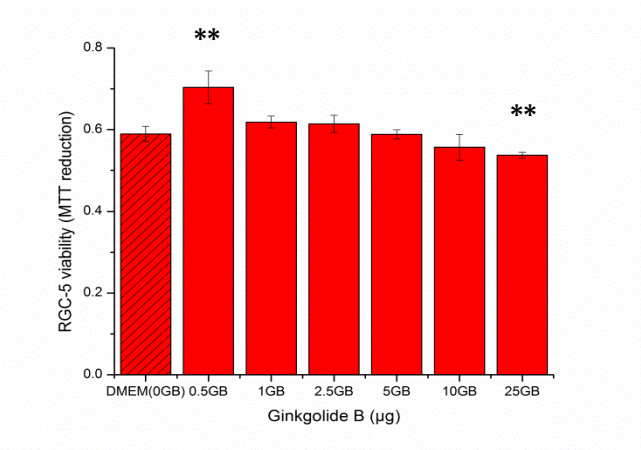


Figure 3.10 Effect of Ginkgolide B on RGC-5

A concentration range (0.5-25µg) Ginkgolide B was used to treat RGC-5 and MTT assay was performed 24 hours later. (Figure 3.10) displays RGC-5 viability in comparison with baseline DMEM, higher cell viability than the control (DMEM) were observed with (0.5, 1 and 2.5µg/ml) GB with no apparent toxicity at the doses (0.5-10µg/ml), data are expressed as mean ± 95% CI, n=5, Y axis= Abs $_{570nm}$. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * $P < 0.01$).

The comparison of the effect of Bilobalied and the control (DMEM) are illustrated in (figure 3.11). Bil application has resulted in enhancement of cell survival at the lower dose and then dose dependent reduction in RGC-5 viability in comparison with the control, 0.5 µg/ml Bil were associated with 104% higher viability than the control while Bil at (1, 2.5, 5, 10 and 25 µg/ml) were associated with about 99%, 89%, 86%, 82% and 58% reduction in cell viability respectively, no significant difference ($P < 0.01$) was observed with the control at (0.5, 1, 2.5 and 5 µg/ml) Bil suggesting lack of cytotoxicity at these concentrations.

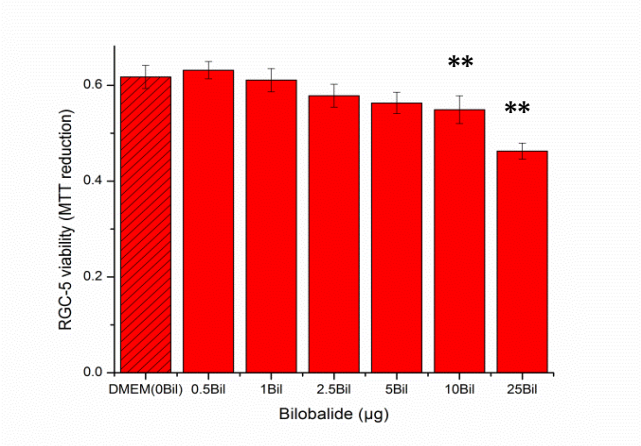


Figure 3.11 Effect of Bilobalide on RGC-5

Treating RGC-5 with a concentration range of (0.5-25µg) Bilobalide has resulted in enhancement of cell survival at the lower dose and then dose dependent reduction in RGC-5 viability in comparison with the control. No significant difference was observed at (0.5, 1, 2.5 and 5 µg/ml) Bil and the control suggesting lack of cytotoxicity at these concentrations, data are expressed as mean ± 95% CI, n=5, Y axis= Abs $_{570nm}$. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * P < 0.01).

3.5 Neuroprotective effect of Ginkgolide A, Ginkgolide B and Bilobalide against DMSO, UV40 and NaN_3 toxicity

To screen neuroprotective potential of Ginkgolide A, Ginkgolide B and Bilobalide on RGC-5 exposed to those apoptotic inducers discussed in section 3.3, the following studies were then performed at doses where RGC-5 viability was 20-35%. Hence, for DMSO, 1% was identified as UV40 and 1mM NaN_3 were chosen as the average reduction in RGC-5 viability using these protocols was between 20-35% and using higher concentrations of those insults would result in massive reduction in RGC-5 viability where cell damage may not be prevented by GA, GB and Bil.

3.5.1 Effects of Ginkgolide A, B and Bilobalide on 1% DMSO-induced RGC-5 death

3.5.1.1 Effects of Ginkgolide A, B and Bilobalide on DMSO-induced toxicity using MTT assay

From the results illustrated in the previous section 3.4, no cytotoxicity was observed with (0.5, 1 and 2.5 µg/ml) GA, (0.5, 1, 2.5, 5 and 10 µg/ml) GB and (0.5, 1, 2.5 and 5 µg/ml) Bil. However, all the following experiments were conducted using the full range of (0.5-25 µg/ml) of these compounds to examine their EC_{50} on dose response curve. In these investigations, the effect of GA was dose dependent, and it appeared that the lowest doses of GA (0.5 and 1µg/ml) were the most effective in enhancing cell viability, however, no statistically significant difference was observed in comparison with baseline 1% DMSO (figure 3.12). In comparison with baseline 1%DMSO, 0.5 and 1µg/ml GA had resulted in 10% and 9% enhancement in cell viability compared to baseline 1% DMSO respectively. The reduction in RGCs survival at higher doses is most probably related to the toxicity of the drug by itself as shown earlier in figure 3.9 in which toxic effect was observed at 5, 10 and 25µg/ml GA.

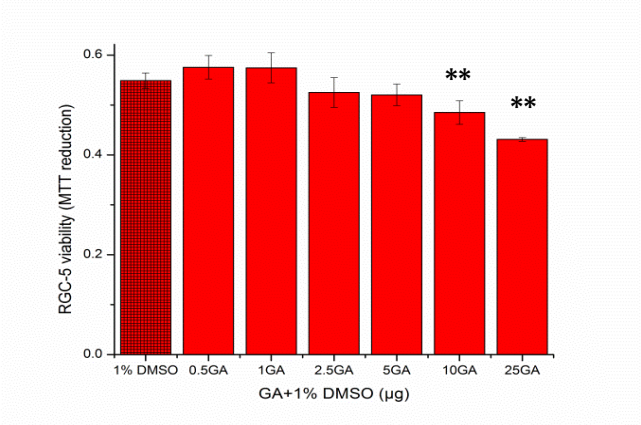
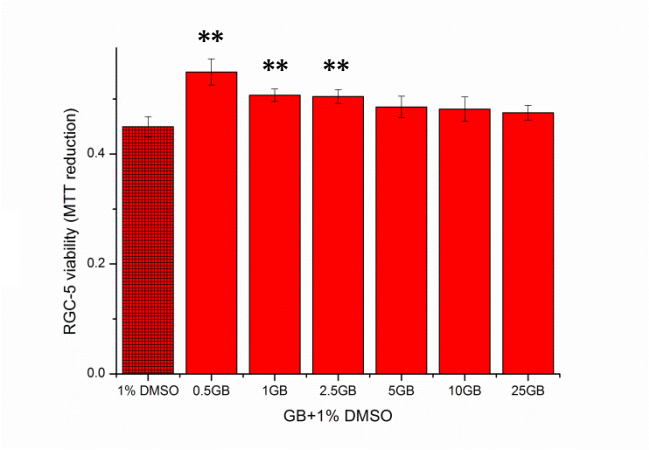


Figure 3.12 Effects of Ginkgolide A on 1% DMSO-induced RGC-5 death

0.5-25 µg/ml Ginkgolide A was applied to RGC-5 for 2 hours before DMSO treatment. MTT absorbance at 570nm was performed 24 hours later, as seen in (figure 3-12). There is a trend for 0.5-1 to induce RGC survival, this was statistically not significant, the effects of 1% DMSO on RGC-5 was dose dependent, and in comparison with baseline1% DMSO, 0.5 and 1 µg/ml GA were associated with 10% and 9% enhancement in RGC-5 viability, each experiment was performed five times and the result is expressed as mean ± 95%CI, Y axis= Abs_{570nm}. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * $P < 0.01$).

Ginkgolide B treatment in presence of 1%DMSO was associated with neuroprotective effect at 0.5-2.5 µg/ml ($P < 0.01$), and the best protection was seen at the lowest concentration tested (0.5 µg/ml) as displayed in (figure 3.13 a). The range of used concentration of GB of 0.5, 1, 2.5, 5, 10 and 25 µg/ml was associated with about 52%, 30%, 29%, 19%, 17% and 14% higher RGC-5 viability respectively in comparison with baseline 1% DMSO. GB effect on RGC-5 was dose dependent with EC_{50} of 2.21µM as shown in (figure 3.13, b).

(a)



(b)

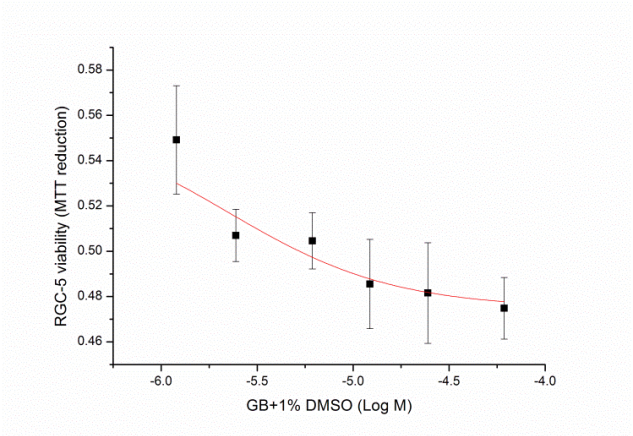


Figure 3.13 Protective effect of Ginkgolide B on 1% DMSO-induced RGC-5 toxicity

Ginkgolide B 0.5-25 µg/ml was applied to RGC-5 2 hours before 1% DMSO treatment. 24 hour later cell viability was assessed using MTT assay. In (figure 3-13 a) there was a significant inhibition in RGC-5 viability by 1%DMSO, which was blocked by 0.5-2.5 µg/ml GB. GB enhancement of cell viability ranged between 52% with 0.5 µg/ml and 14% with 25µg/ml GB. (Figure 3-13 b) illustrate dose response curve of GB+1%DMSO showing dose dependent effect with an EC_{50} of 2.21µM. Each experiment was performed five times and the result is expressed as mean ± 95%CI, Y axis= Abs $_{570nm}$. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * $P < 0.01$).

The profile of protection observed with Bilobalide was similar to GA as displayed in (figure 3.14) 0.5 and 1µg/ml Bil had the most pronounced effect on 1%DMSO neurotoxicity in cultured RGC-5, however, the effect was statistically not significant ($P < 0.01$). In comparison with baseline 1%DMSO, those doses enhanced RGC-5 survival by 23% and 18%.

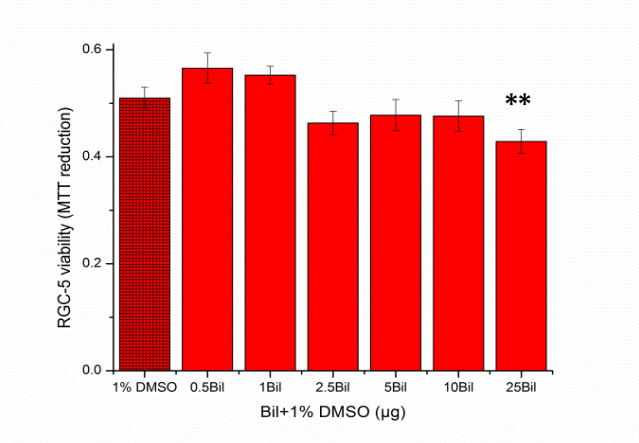


Figure 3.14 Effects of Bilobalide on 1% DMSO-induced RGC-5 death

0.5-25 µg/ml Bilobalide were applied to RGC-5 for 2 hours before1% DMSO treatment. MTT absorbance was performed 24 hour later. As seen in (figure 3-14) the DMSO induced reduction in cell viability was attenuated by 0.5 and 1 µg/ml Bil. Each experiment was performed five times and result is expressed as mean ± 95%CI, Y axis= Abs $_{570nm}$. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * $P < 0.01$).

3.5.1.2 Effects of Ginkgolide A, B and Bilobalide on DMSO-induced toxicity using Hoechst 33342 staining

To examine the apoptotic potential of DMSO in cultured RGC-5 and to further assess the neuroprotective effect of Ginkglide A, Ginkgolide B and Bilobalide on 1% DMSO induced RGC-5 death; cells were stained by Hoechst 33342. This viable dye allows condensation of DNA, a feature of apoptosis, to be detected. After 24 hours of 1% DMSO, GA, GB and Bil treatment, cells were fixed and stained with Hoechst 33342 dye at a concentration of 5µl/ml for 5 minutes. On observing the 12 well plates under the Leica Image Capture Microscope, as seen in figures 3.15-3.17, the majority of

RGC-5 in the control culture (DMEM) had normal cell density, whereas the density of RGC-5 reduced markedly in the 1% DMSO treated culture with higher percentage of apoptotic nuclei. The apoptotic cells in all wells with condensed chromatin and fragmented DNA take up the Hoechst 33342 stain and fluorescence typical for apoptotic nuclei (arrows head). Apoptotic cells were counted from 5 field per well and their proportion was calculated and the results are shown as apoptotic cells as a percentage of the total number of cells (Massieu et al., 2004;Ban et al., 2006b;Santiago et al., 2007).

In figure 3.15 the effect of GA on the 1% DMSO treated cells was illustrated, when RGC-5 was exposed to DMEM only (figure 3.15 a) this resulted in 3.9% apoptosis whereas 1% DMSO (figure 3.15 e) increased the number of apoptotic cells to 5%, among the used concentration of GA 0.5, 1 and 2.5 µg/ml showed neuroprotective effect as measured by reduction in RGC apoptosis (figure 3.15 b, f, c). These doses had the lowest proportion of apoptosis of 3.4%, 3.7% and 4% respectively and reversed the DMSO-induced cytotoxicity, on the other hand 5, 10 and 25 µg/ml (figure 3.15 d, g, h) were associated with 5.5%, 5.9%, and 6.8% apoptotic nuclei respectively.

It was clearly evident on examining those plates that 0.5, 1 and 2.5 µg/ml GA had remarkably reduced the number of condensed and apoptotic nuclei; quantitative data of apoptotic cells as a percentage of the total number of cells are displayed in (figure 3.15, i). At 0.5, 1 and 2.5 µg/ml GA was found to be neuroprotective ($P < 0.01$). This result using the Hoechst 33342 staining to measure reduction in RGC apoptosis, was different from the previous MTT assay results of the effect of 0.5, 1 and 2.5µg/ml GA

against 1% DMSO-induced cytotoxicity, because each assay has different implication for cell death.

Figure 3.16 illustrates the neuroprotective effects of GB on 1% DMSO-induced toxicity. From this data it is apparent that cell density of the GB treated RGC-5 was maintained at all concentration range 0.5-25 µg/ml used in the study. The control culture (DMEM) in (figures 3.16, a) shows the majority of cells with apparently normal nuclear morphology resulting in only 2.9% apoptosis, while 1% DMSO treated RGC-5 (figures 3.16, e) led to 4.6% apoptotic cells. Pretreatment with 0.5, 1, 2.5, 5, 10 and 25 µg/ml GB two hours before DMSO exposure (figures 3.16 b, c, d, f, g, h) reduced nuclear condensation (arrow head) and the percentage of apoptotic nuclei to 1.8%, 1.9%, 2%, 2.5%, 2.8%, and 2.6% respectively. Figures 3.16, i illustrate that, at 0.5 and 1 µg/ml GB was found to be neuroprotective $P < 0.01$ as measured by a reduction in RGC apoptosis.

Figure 3.17 shows the Hoechst 33342 staining of Bil and 1% DMSO treatment. In (figures 3.17, a) the control culture with 2.9% apoptosis and maintained cellular density was displayed. Figures 3.17, e shows 1% DMSO treatment, which resulting in 4.4% apoptosis with visible reduction in cell density. In (figures 3.17 b, f, c) the effect of 0.5, 1, 2.5 µg/ml of Bil on DMSO treated RGC-5 was shown, which has inhibited the percentage of apoptotic RGC-5 to 2.3%, 3.4%, 3.9%, while Hoechst staining of 5, 10, 25 µg/ml Bil treated cultures failed to reduce the apoptosis and increased the apoptotic cells to 4.7%, 5.5 and 5.5% respectively. Nevertheless, at all concentrations Bil effect on RGC apoptosis was statistically not significant.

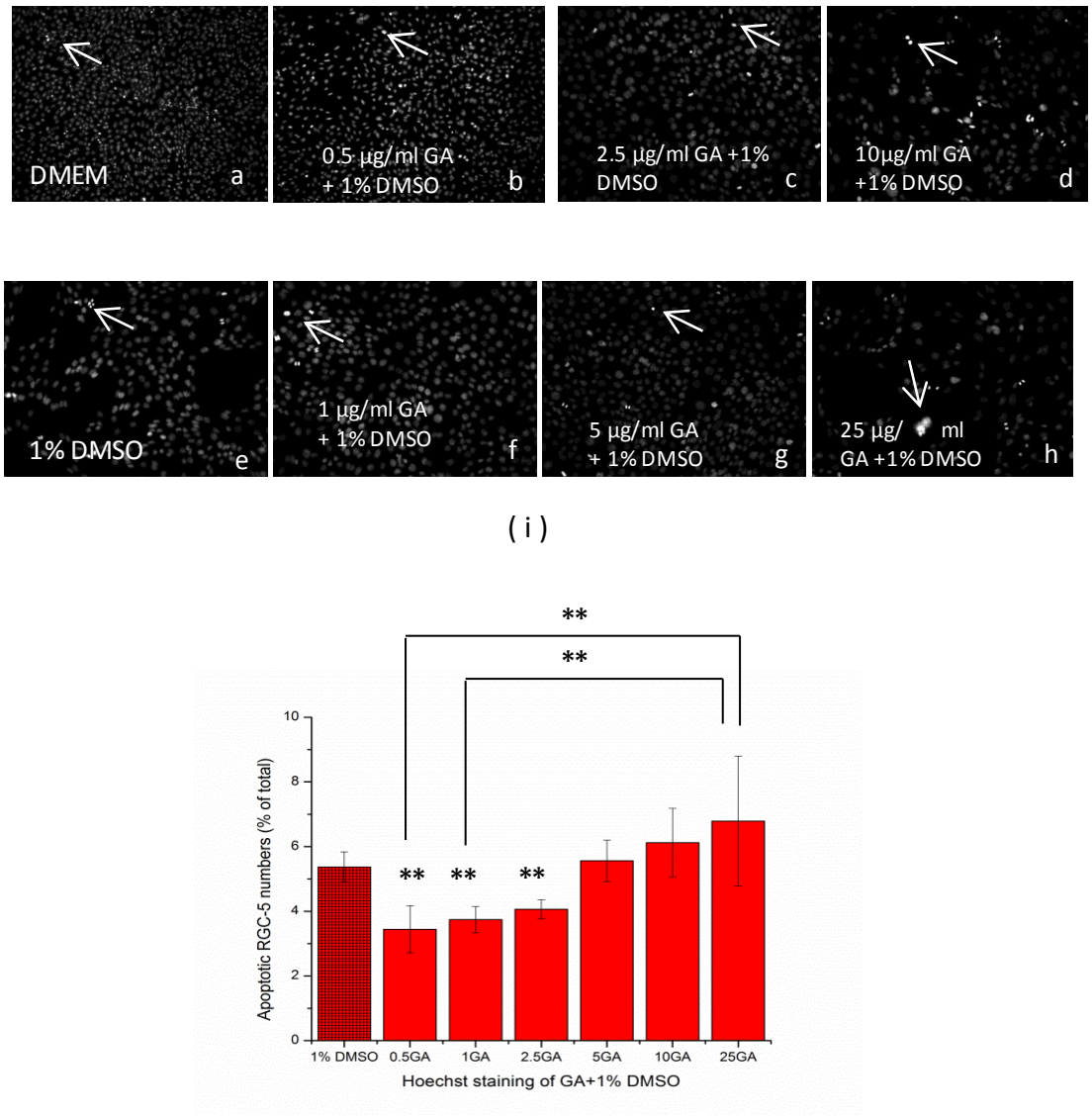
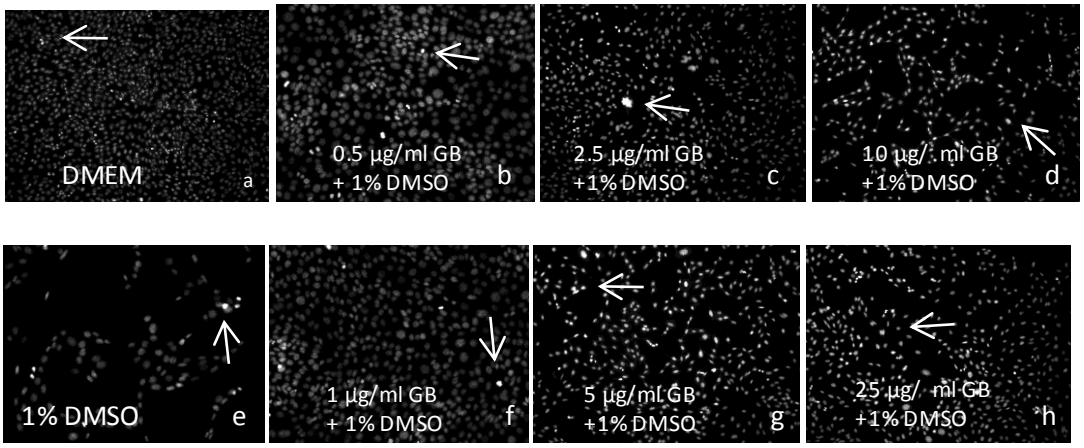


Figure 3.15 Fluorescent microscopy images of Hoechst 33342 staining of GA and 1% DMSO treated RGC-5

RGC-5 were pre-treated with the indicated concentration of GA, 2 hours later 1% DMSO was added and cells were incubated for 24 hours, after that RGC-5 stained with Hoechst 33342. Five fields for each indicated concentration were counted and tabulated using Microsoft excel software, the brightly stained RGCs with condensed nuclei were considered to be apoptotic whereas the lightly stained smooth RGCs were considered as normal non apoptotic cells. The apoptotic degree was calculated by dividing the number of counted apoptotic cells over the total number of visible cells in the field and displayed as percentage. (Figure 3.15, a) shows normal RGC-5 density and fewer apoptotic cells in the control culture, (figure 3.15, e) RGC-5 loss with higher number of apoptotic nuclei in 1% DMSO treated cells, (figures 3.15, b, c, f) reduced nuclear condensation with 0.5, 1 and 2.5 µg/ml GA treatment, (figures 3.15, d, g, h) RGC-5 loss with higher percentage of apoptotic nuclei with 5, 10 and 25 µg/ml, quantitative data are displayed in (figure 3.15, i) as shown in the graph At 0.5, 1 and 2.5 µg/ml GA was found to be neuroprotective. Each column represents mean ± 95%CI. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * $P < 0.01$).



(i)

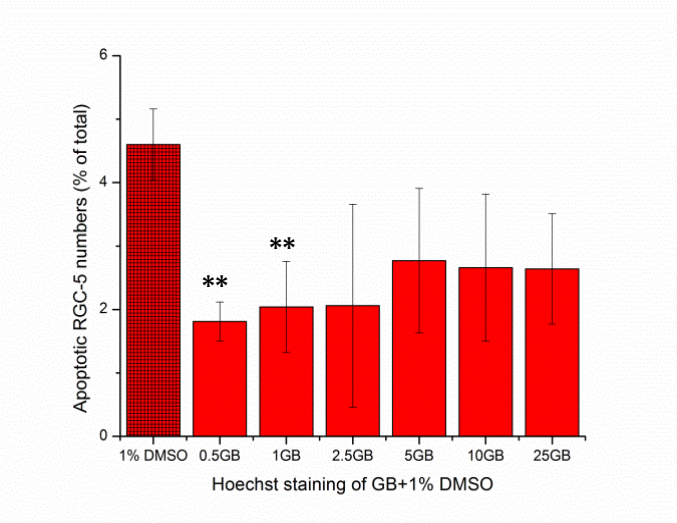
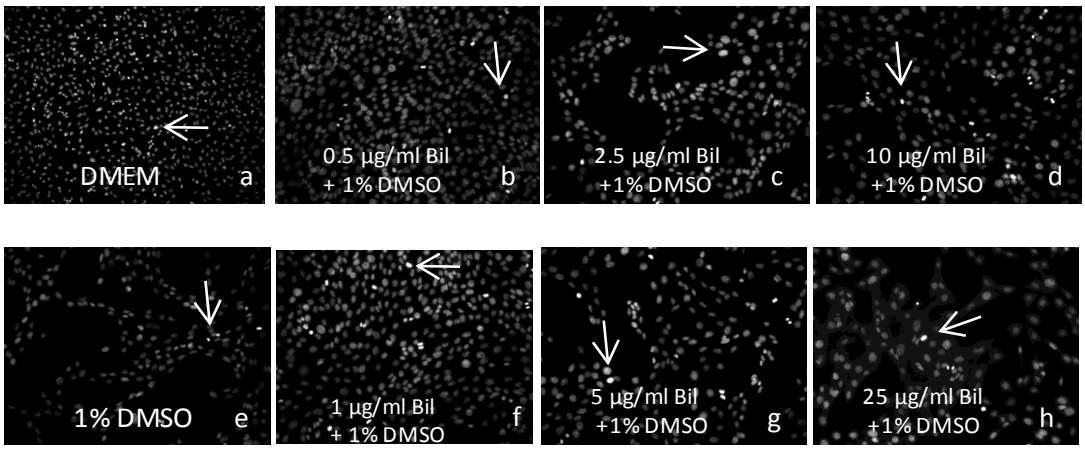


Figure 3.16 Fluorescent microscopy images of Hoechst 33342 staining of GB and 1% DMSO treated RGC-5

Representitive fluorescence microscopy of Hoechst 33342 after 1% DMSO and Ginkgolide B treatment. RGC-5 were pretreated with the indicated concentration of GB and two hours later 1% DMSO was added, after 24 hours incubation, RGC-5 stained with Hoechst 33342. Five fields for each indicated concentration were counted and tabulated using Microsoft excel software, fluorescent cells were counted and expressed as a percentage of the number of Hoechst positive cells. (figure 3.16, a) shows maintained cell density and fewer apoptosis in the control culture, (figure 3.16, e) RGC-s loss and higher apoptosis percentage, (figures 3.16 b, c, d, f, g, h) relatively maintained density and reduction in apoptosis with all the used concentration of GB. Quantitative data are displayed in (figures 3.16, i), at 0.5 and 1µg/ml GB was found to be neuroprotective, each column represents mean ± 95%CI. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * $P < 0.01$).



(i)

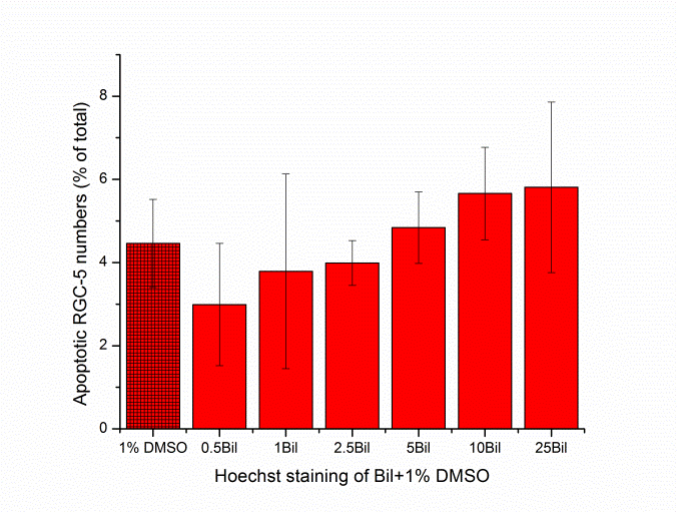


Figure 3.17 Fluorescent microscopy images of Hoechst staining of Bil and 1% DMSO treated RGC-5

Hoechst 33342 after 1% DMSO and Bilobalide treatment. RGC-5 were pre-treated with the indicated concentration of Bil and 2 hours later 1% DMSO was added and cells were incubated for 24 hours, after that RGC-5 stained with Hoechst 33342. Five fields for each indicated concentration were counted and tabulated using Microsoft excel software, cells were counted and expressed as percentage of the number of Hoechst positive cells. In (figure 3.17, a) the control culture displays normal cells density with 2.9% apoptosis, (figure 3.17, e) 1% DMSO treatment with resultant reduction in RGC-5 density and increasing the percentage of apoptotic cells to 4.4%, applying 0.5, 1 and 2.5 µg/ml Bil as in (figures 3.17 b, c, f) has inhibited the percentages of apoptosis. No protection was observed with the higher doses of Bil which failed to reduce the apoptosis as in (figures 3.17 d, g, h). Quantitative data are displayed in (figures 3.17, i) at all concentrations Bil effect on RGC apoptosis was statistically not significant, the apoptotic degree was calculated by dividing the number of counted apoptotic cells over the total number of visible cells in the field and displayed as percentage. Each column represents mean ± 95% (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons).

To detect DNA fragmentation and identify the apoptotic features of DMSO toxicity in RGC-5 line, the TUNEL assay was performed on cells treated with the indicated concentration of DMSO for 24 hours using the protocol described previously in section 2.2.4. Micrographs of DMSO treated RGC-5 were taken using Leica Image Capture microscope and the number of The TUNEL-positive cells were counted. No quantitative evidence of apoptosis in the DMSO treated RGC-5 was detected with the TUNEL assay (data not shown).

3.5.2 Effects of Ginkgolide A, B and Bilobalide on UV40-induced RGC-5 death

UVC light is a known apoptotic inducer in several cell lines and has been associated with oxidative cellular damage. In these experiments RGC-5 survival fell significantly following two minutes exposure to 40 mj/cm^2 of UVC light (254nm) and Ginkgolide B and Bilobaliode were able to significantly restore cell viability. As displayed in (figure 3.18), UV40 has reduced RGC-5 viability, Ginkgolide A at concentration (0.5-2.5 µg/ml) was respectively associated with 10%, 17 % and 14% enhancement in RGC-5 survival in comparison with baseline UV40. However, this enhancement was not significant using one way-ANOVA.

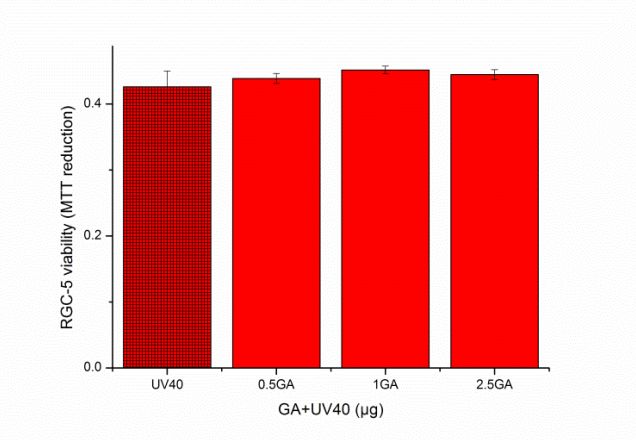


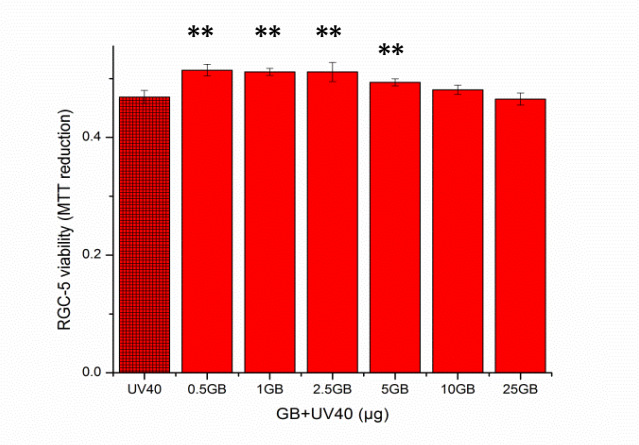
Figure 3.18 Effect of Ginkgolide A on UV40-induced RGC-5 death

Pre-exposing RGC-5 cells to 0.5, 1 and 2.5 µg/ml GA for 2 hours before UV40 treatment has increased RGC-5 survival in comparison with baseline UV40, n=6 and the result is expressed as mean ± 95%CI, Y axis= Abs $_{570nm}$. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons).

Figure 3.19 shows the MTT assay result of incubating RGC-5 with Ginkgolide B prior to UV40 mj/cm2 exposure. As illustrated in figure 3.19, a the decline in RGC-5 viability was significantly blocked by (0.5-5µg/ml) GB with maximal effect at 0.5 µg/ml ($P < 0.01$), which has resulted in 22%, 21%, 21% and 12% enhancement in cell

viability respectively. The dose response curve of GB protective effect is illustrated in (figure 3.19, b) with an EC_{50} of 15µM.

(a)



(b)

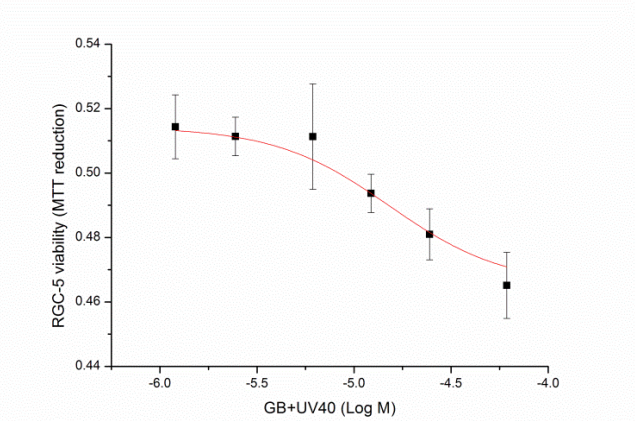


Figure 3.19 Effect of Ginkgolide B on UV40-induced RGC-5 toxicity

0.5-25 µg Ginkgolide B were applied to RGC-5 for 2 hours before exposure to UV40. MTT absorbance was performed 24 hours later; there was a decline in RGC-5 viability after UV-C exposure which was dose dependently blocked by (0.5-10 µg/ml) GB (figure 3.19, a). Figure 3.19, b displays dose response curve of GB with an EC_{50} of 1.53 µM. n=6 and result is expressed as mean ± 95% CI, Y axis= Abs_{570nm}. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * $P < 0.01$).

The effect of Bilobalide on UV40 treated RGC-5 is displayed in (figure 3.20), and as seen UV40-induced cell death was significantly ($P < 0.01$) reversed by 0.5 and 1µg/ml Bil, with the best protection observed at 1µg/ml Bil. The illustrated concentration range of 0.5-5 µg/ml Bil in (figure 3.20), has resulted in 18%, 23%, 17% and 12% enhancement of cell viability as compared with baseline UV40.

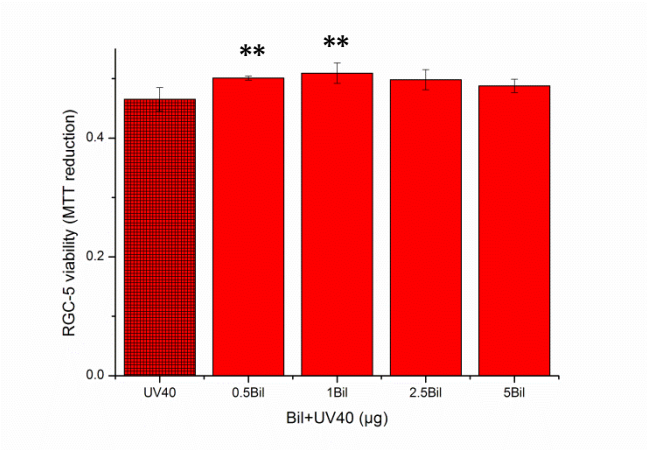


Figure 3.20 Protective effect of Bilobalide on UV40-induced RGC-5 death

0.5-5 µg Bilobalide were applied to RGC-5 for 2 hours before exposing RGC-5 to UV40, MTT absorbance was performed 24 hours later; (figure 3.20) shows significant reduction in RGC-5 survival which was totally and dose dependently blocked by the applied Bil, the percentage of RGC-5 protection by (0.5-5 1µg/ml) Bil in comparison with baseline UV40 was 18%, 23%, 17% and 12% respectively, n=6 and result is expressed as mean ± 95% CI, Y axis= Abs $_{570nm}$. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * $P < 0.01$).

3.5.3 Effects of Ginkgolide A, B and Bilobalide on 1Mm NaN_3-induced RGC-5 death

Applying 1mM NaN_3 to RGC-5 has resulted in a significant fall in cell viability as estimated by MTT assay and Ginkgolide A, B and Bilobalide pretreatment failed to demonstrate a protective effect. Interestingly, the reduction in RGC-5 viability following NaN_3 treatment was attenuated by GA, GB and Bil at 0.5µg/ml concentration. However, the result of ANOVA revealed that this effect was not significant ($P < 0.01$).

As shown in (figure 3. 21) pre-incubating RGC-5 with the lowest concentration used 0.5µg/ml GA led to 24% enhancement in RGC-5 viability as compared with baseline 1 mM NaN_3, GA at 1 and 2.5 µg/ml GA were associated with dose dependent reduction in RGC-5 viability.

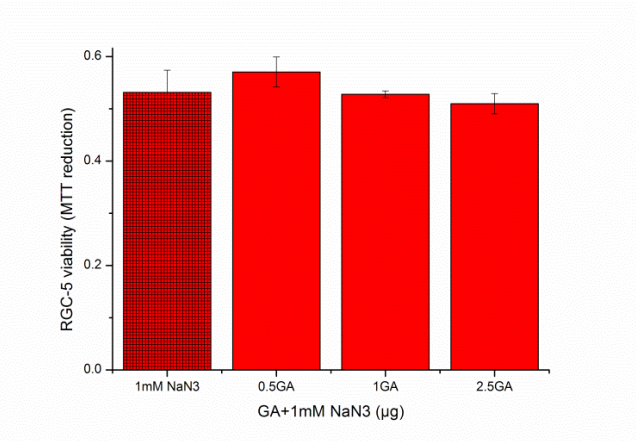


Figure 3.21 Effect of Ginkgolide A on NaN_3-induced toxicity

Ginkgolide A 0.5-2.5 µg/ml was applied to RGC-5 for 2 hours before 1Mm NaN_3 treatment. MTT assay was performed 24 hours later; as shown in (figure 3.21) there was a reduction in RGC-5 survival with 1 mM NaN_3 that is attenuated by 0.5 µg/ml GA. In comparison with baseline 1Mm NaN_3, 0.5 µg/ml GA were associated with 24% increase in RGC-5 viability, whereas, GA at 1 and 2.5 µg/ml GA were associated with dose dependent reduction in RGC-5 viability, n=5, result is expressed as mean ± 95% CI, Y axis= Abs_{570nm}. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons).

The profile of Ginkgolide B on NaN_3 treated RGC-5 was similar to GA as displayed in (figure 3.22), GB at 0.5µg/ml was associated with statistically not significant enhancement in RGC-5 viability of 1% compared to baseline NaN_3, whereas (1-25µg/ml) were associated with dose dependent reduction in RGC-5 viability.

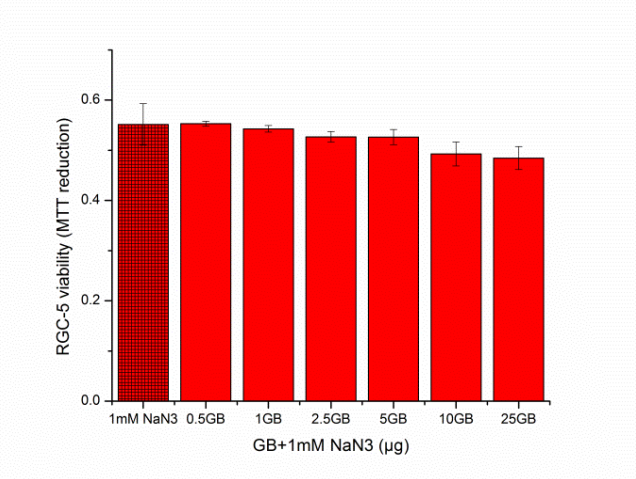


Figure 3.22 Effect of Ginkgolide B on NaN_3-induced RGC-5 toxicity

0.5-25 µg/ml Ginkgolide B was applied to RGC-5 for 2 hours before 1 mM NaN_3 exposure. MTT absorbance was performed 24 hours later; as seen in (figure 3.22) there is a decline in RGC-5 survival with 1 mM NaN_3, this was attenuated by 0.5 µg/ml GB leading to 1% enhancement in cell viability in comparison with baseline1 mM NaN_3. Other doses 1-25µg/ml GB failed to improve RGC-5 survival, the effect of GB on 1Mm NaN_3-induced cytotoxicity was dose dependent and statistically not significant ($P < 0.01$), n=5 and result is expressed as mean ± 95% CI, Y axis= Abs $_{570nm}$. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons).

The effect of Bilobalide on 1mM NaN_3-induced cytotoxicity in RGC-5 line was interestingly similar to GA and GB as displayed in (figure 3.23). Only the smallest used concentration of 0.5 µg/ml Bil was able to rescue RGC-5 increasing the cellular viability by 10% in comparison to baseline 1mM NaN_3. Other doses (1-5 µg/ml Bil) were associated with 6%, 22% and 24% reduction in RGC-5 viability respectively.

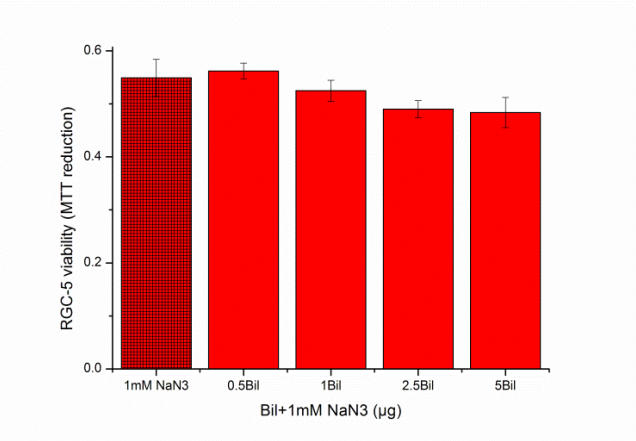


Figure 3.23 Effect of Bilobalide on NaN_3-induced RGC-5 toxicity

0.5-5 µg/ml Bilobalide was applied to RGC-5 for 2 hours before applying1 mM NaN_3. MTT absorbance was performed 24 hours later; as displayed in (figure 3.23) there is a significant fall in RGC-5 survival with 1 mM NaN_3 which is attenuated by 0.5 Bil. (figure 3.23) in comparison with baseline1 mM NaN_3, 0.5 µg/ml Bil were associated with 2% enhancement in RGC-5 survival while other doses failed to protect the cell resulting in 4%, 11%, 12%, 14% and 4% reduction in RGC-5 survival. Each experiment was performed five times and result is expressed as mean ± 95% CI, Y axis= Abs $_{570nm}$. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * P < 0.01).

3.6 Aβ toxicity in RGC-5 line

Since Aβ expression in the retinal sections from the OHT model established in the group has been demonstrated, using immunohistochemistry (section 3.2). The effect of the full length peptide $A\beta_{1-42}$ and $A\beta_{25-35}$ on RGC-5 survival *in vitro* was next examined in the following experiments.

3.6.1 Effect of $A\beta_{1-42}$ on RGC-5 survival

To investigate the ability of the full length peptide $A\beta_{1-42}$ to induced RGC-5 death, RGC-5 was incubated with 10, 25, 50 and 100 µM $A\beta_{1-42}$, cell viability assay which was performed 24 hours after the insult failed to show significant reduction in RGC-5 survival, as shown in figure 3.24. Interestingly, in comparison with the control (DMEM) addition of 10 µM Aβ1-42 led to 6% increase in RGC-5 survival, other

doses were associated with 6%, 5% and 4% reduction in RGC-5 viability respectively.

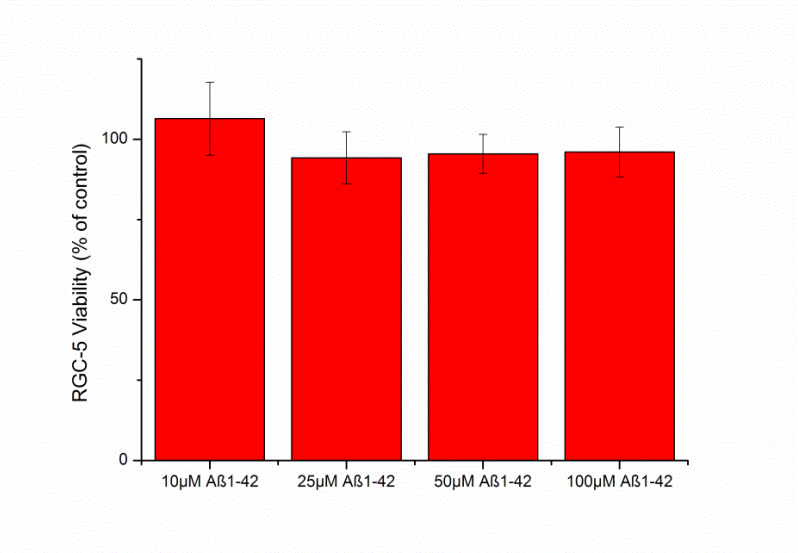


Figure 3.24 Effect of $A\beta_{1-42}$ on RGC-5 survival

The effect of 10, 25, 50 and 100µM $A\beta_{1-42}$ on RGC-5 viability was measured after 24 hours. $A\beta_{1-42}$ 25-100µM $A\beta_{1-42}$ resulted in mild insignificant reduction in cell viability as determined by MTT assay, absorbance was measured at 570nm. There has been 6%, 5% and 4% reductions in cell survival compared with baseline DMEM. Result is expressed as mean ± 95% CI, n=8, Y axis= Abs_{570nm}. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons).

3.6.2 $A\beta_{25-35}$ leads to RGC-5 death

The apoptotic effect of $A\beta_{25-35}$ has been demonstrated *in vitro* in many neuronal cell lines. Thus in this experiment RGC-5 was challenged with a concentration range of $A\beta_{25-35}$ up to 100µM that has been used by other researchers to stress PC12 cell line (Zhou et al., 2000) as displayed in figure 3.25. The colorimetric MTT assay was performed to investigate the effect of 24 hours exposure to concentration range of 5-100µM $A\beta_{25-35}$ on RGC-5. A concentration dependent decrease in RGC-5 survival was observed on treating RGC-5 with 5µM, 10µM, 25µM, 50µM and 100µM $A\beta_{25-35}$, which led to a 16%, 17%, 18%, 27% and 60% reduction in survival respectively, in comparison with the control (DMEM). One way-ANOVA showed significant reduction in RGC-5 viability ($P < 0.01$) compared with the control (DMEM). The

50µM $A\beta_{25\text{-}35}$ was associated with nearly 30% reduction in cell viability and was used in the next experiment for the determination of neuroprotective effect of Ginkgolide A, Ginkgolide B and Bilobalide on $A\beta_{25\text{-}35}$-induced RGC-5 cytotoxicity.

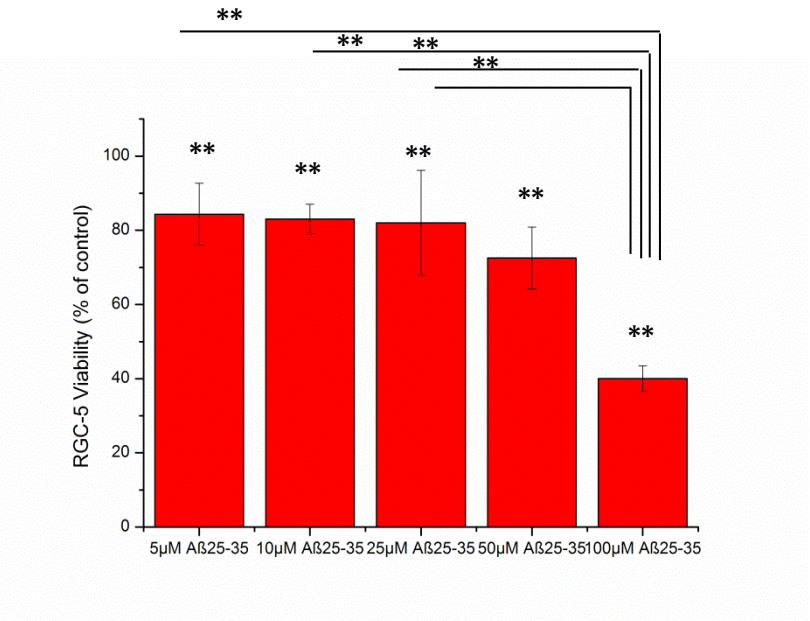


Figure 3.25 Effect of $AB_{25\text{-}35}$ on RGC-5 survival

RGC-5 was exposed to concentration range of 5-100µM Aβ25-35 and MTT assay done 24 hours later. There was a concentration dependent reduction in RGC-5 viability which was statistically significant at all used concentration; absorbance was measured at 570nm. Results are expressed as mean ± 95% CI, Y axis= Abs_{570nm}. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * $P < 0.01$).

3.7 Effects of Ginkgolide A, Ginkgolide B and Bilobalide aganist $A\beta_{25\text{-}35}$-induced toxicity in RGC-5

To examine the effect of Ginkgolide A, Ginkgolide B and Bilobalide on RGC-5 death induced by 50µM $A\beta_{25\text{-}35}$, RGCs were pretreated with various doses (0.5-25µg) GA, GB and Bil for 2 hours, followed by simultaneous administration of the indicated concentration of $A\beta_{25\text{-}35}$, cells were then incubated for 24 hours. As estimated by MTT assay, the result of these experiments showed that 1 µg/ml GA and 0.5µg/ml

GB had a protective effect on RGCs and were able to significantly reverse the neuronal toxicity of 50µM $A\beta_{25-35}$.

(Figure 3.26) shows that, Ginkgolide A at 1µg/ml had significantly ($P < 0.01$) prevented 50µM $A\beta_{25-35}$-induced RGC-5 death. Applying 0.5, 1 and 2.5µg/ml GA to RGC-5 resulting in 21%, 28% and 11% enhancement in cell viability in comparison with the baseline $A\beta_{25-35,}$ higher doses of GA (5, 10 and 25 µg/ml) were associated with reduction in cell viability (data not shown).

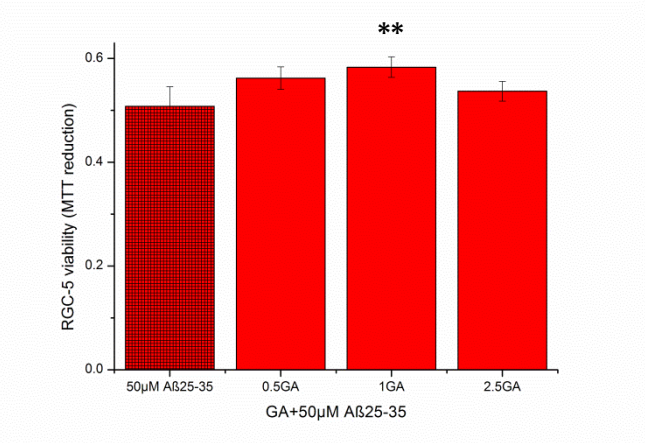


Figure 3.26 Protective effect of Ginkgolide A on AB_{25-35}-induced RGC-5 toxicity

RGC-5 were pre-treated with 0.5-2.5 µg/ml Ginkgolide A for 2 hours and then exposed to 50µM $A\beta_{25-35}$. MTT absorbance was performed after 24 hours to access cell viability, in (figure 3.26) as displayed the reduction in cell viability after exposure to 50µM $A\beta_{25-35}$ was reversed by 1 µg/ml GA. At 0.5, 1, 2.5 and 5 µg/ml GA there was 11%, 15% and 6% enhancement in cell survival in comparison with the control. Each experiment was performed five times and the result is expressed as mean ± 95%CI, Y axis= Abs $_{570nm}$. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * $P < 0.01$).

Figure 3.27 illustrates the dose dependent effect with 0.5-25µg/ml GB on Aβ-induced toxicity. In comparison with baseline, 50µM Aβ $_{25-35}$, pre-treating RGC-5 with these concentrations of GB was associated with 27%, 21%, 13% and 11% enhancement of cell viability. However, one-way ANOVA showed significant protective effect only at 0.5 µg/ml concentration ($P < 0.01$).

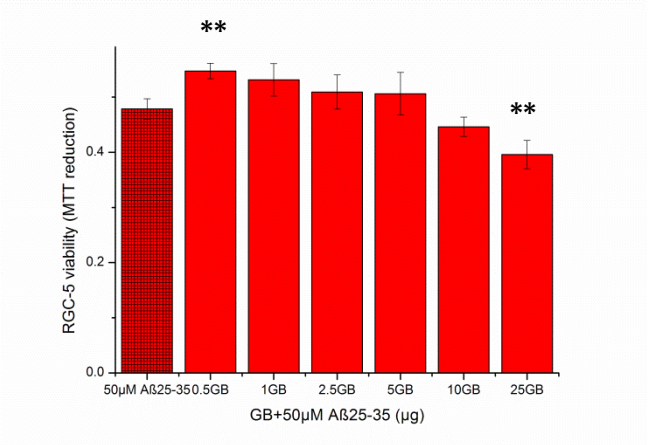


Figure 3.27 Protective effect of Ginkgolide B on Aβ$_{25-35}$-induced Rgc-5 toxicity

0.5-25µg/ml Ginkgolide B were applied to RGC-5 for 2 hours before insulting the cells with 50µM Aβ$_{25-35}$. MTT absorbance was performed after 24 hours and seen in (figure 3.27) GB enhanced RGC-5 survival resulting in 27%, 21%, 13%, 11% increase in cell viability at 0.5, 1, 2.5 and 5 µg/ml concentration whereas 10 and 25 failed to protect RGC-5. However, the protective effect was observed at 5µg/ml. Each experiment was performed five times and the result is expressed as mean ± 95% CI, Y axis= Abs $_{570nm}$. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * $P < 0.01$).

The effect of Bilobalide on $A\beta_{25\text{-}35}$-induced toxicity in RGC-5 is displayed in (figure 3.28), among the used concentrations only 1 µg/ml Bil was able to enhance cell viability by 16%, as compared with baseline $A\beta_{25\text{-}35}$, Nevertheless, this enhancement was statistically not significant ($P < 0.01$).

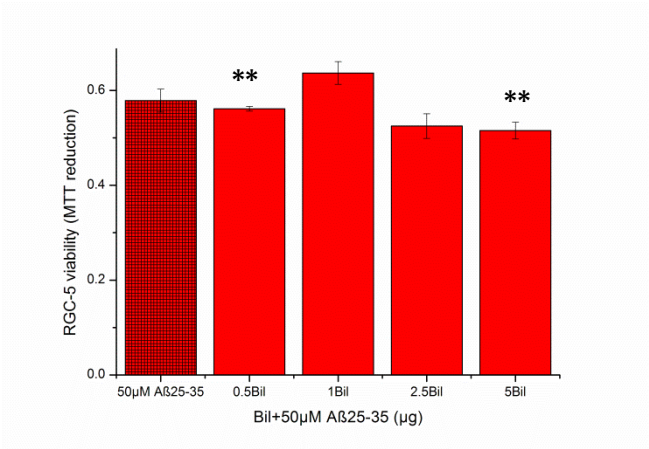


Figure 3.28 Protective effects of Bilobalide on $AB_{25\text{-}35}$-induced RGC-5 toxicity

RGC-5 was incubated with 0.5-25 µg/ml Bilobalide for 2 hours before 50µM $A\beta_{25\text{-}35}$ exposure. MTT absorbance was performed after 24 hours and as displayed in (figure 3.28, a) 1 µg/ml Bil reversed Aβ toxicity resulting in 16%, reduction in RGC-5 apoptosis as compared with 50µM $A\beta_{25\text{-}35}$. Each experiment was performed five times and the result is expressed as mean ± 95% CI, Y axis= Abs_{570nm}. (All treatment groups were compared to each other and the control by one-way ANOVA followed by post hoc comparisons * * $P < 0.01$).

Chapter Four

4 Discussion

In glaucoma, the major cause of global irreversible blindness (Quigley and Broman, 2006), there is an urgent need for treatment modalities that directly target RGCs (Cheung et al., 2008a;Baltmr et al., 2010). The discovery of an alternative therapeutic approach, independent of IOP reduction, is highly sought after, due to the indirect nature and limited effectiveness of IOP lowering therapy in preventing RGC loss (Oliver et al., 2002;Leske et al., 2007). As discussed in the introduction (section 1.1.4), several mechanisms have been implicated in initiating the apoptotic cascade in glaucomatous retinopathy, and numerous drugs have been shown to be neuroprotective in animal models of glaucoma. Ginkgo biloba, a naturally occurring herb, has been advocated as a neuroprotective agent for several years in progressive glaucoma, especially when IOP-lowering strategies are ineffective (Ritch, 2000). However, the mechanism by which Ginkgo biloba exerts its action is not well established, although several pathways have been implicated (Diamond et al., 2000;Ahlemeyer and Krieglstein, 2003). These include reduction of oxidative stress via Aβ (Yao et al., 2001;Bastianetto and Quirion, 2002;Luo et al., 2002;Shi et al., 2009), mitochondrial dysfunction (Tendi et al., 2002;Abdel-Kader et al., 2007), and regulation of apoptosis-related caspases (Luo et al., 2002;Massieu et al., 2004;Gong et al., 2005). All of these mechanisms ultimately lead to programmed cell death with loss of RGCs (Osborne et al., 1999;Tatton et al., 2001;McKinnon et al., 2002a;Tezel, 2006).

4.1 Summary of immunohistochemical findings

The aim of the immunohistochemical work in this thesis was to examine several of the potential molecular targets of Ginkgo biloba related to RGC-5 apoptosis in OHT retinal sections and their correlation with IOP elevation. Immunohistochemistry showed cytochrome *c* and TNF-R1 expression peaking at 3 weeks (Fig 3.1 and 3.3), and active caspase 3 activity at 12 weeks after IOP elevation (Fig 3.2). Furthermore, the results have revealed a strong inverse correlation between Aß and APP in OHT animals, with APP and Aß accumulation peaking at 1 and 12 weeks after IOP elevation respectively (Fig 3.4 and 3.5).

4.2 Interpretation

4.2.1 Mitochondrial dysfunction and RGC apoptosis in OHT

Mitochondrial dysfunction has previously been described as one of the key intracellular lesions associated with the pathogenesis of glaucoma (Mittag et al., 2000;Tatton et al., 2001;Tezel and Yang, 2004), and many experimental studies have demonstrated the protective effect of Ginkgo biloba on mitochondrial function (Tendi et al., 2002;Eckert et al., 2003;Abdel-Kader et al., 2007). Literature does not show a direct link between Ginkgo biloba treatment and cytochrome *c*, nevertheless, Cheung et al (2003) who correlated mitochondrial failure with cytochrome *c* release and subsequent activation of the executor caspase, caspase-3, have observed cytochrome *c* release in RGCs post axotomy (Cheung et al., 2003). The activation of the death receptors TNF-R1 can also lead to caspase-3 activation in RGCs (Tezel and Yang, 2004). Therefore, in these experiments, the expression of those molecular targets has been investigated in retinal sections of OHT and age-matched controls at different time points following IOP elevation.

4.2.1.1 Cytochrome c release in RGCs

Work in this study provides evidence that cytochrome *c*, a water soluble protein located in the mitochondrial intermembrane space (Cheung et al., 2003), is detected in the cytoplasm of RGCs in OHT eyes. The results displayed in section 3.1.1, showed marked up-regulation of cytochrome *c* immunoreactivity 1 week post OHT surgery to reach a peak at 3 weeks' time point, which coincides with the development of peak RGC apoptosis in the same model as previously described (Cordeiro et al., 2004;Guo et al., 2005a). The diffuse cytoplasmic staining pattern of cytochrome *c* in RGCs post IOP elevation and the absence of obvious nuclear chromatin condensation in this study was similar to those described in RGCs post axotomy (Cheung et al., 2003;He et al., 2004). Relocation of cytochrome *c* from the inner mitochondrial membrane to the cytoplasm has also been reported in neuronal cells post transient focal cerebral ischemia (Fujimura et al., 1998), post cold injury induced brain trauma (Morita-Fujimura et al., 1999) and in traumatic axonal brain injury (Buki et al., 2000).

Cytochrome *c* expression in the normal control retinas was nearly undetectable in substantial agreement with Cheung et al (2003), who looked at cytochrome *c* in normal and axotomized RGCs (Cheung et al., 2003) and in the control cortex of post cold injury induced brain trauma (Morita-Fujimura et al., 1999). The decline in cytochrome *c* immunoreactivity in the OHT retinal sections after 3 weeks may be explained by considering the fact that mitochondrial damage is an early event in the apoptotic process in glaucoma, and will lead to cytochrome *c* release, which then potentially contributes to RGCs apoptosis by activating caspases (Tezel and Yang, 2004). Additionally cytochrome *c* expression appears to be related to the type and duration of insult. While work in this thesis has shown cytochrome *c* expression to be

inconstant at different time points of IOP elevation (Fig 3.1), Cheung et al (2003) documented localized cytochrome *c* immunoreactivity increasing in one day to reach a peak at three days post axotomy (Cheung et al., 2003). Furthermore, in a study, which was conducted on adult hamsters, He et al 2004, who also looked at caspase-3 as will be shown later, have observed more RGCs death and a significantly higher number of RGCs releasing cytochrome-*c* when optic nerve transection was performed closer to the optic disc (He et al., 2004). Released cytochrome-*c* has been shown to activate Apaf-1 and procapse-9 resulting in caspase-3 activation, ending in nuclear DNA fragmentation in an experimental model of traumatic brain injury (Morita-Fujimura et al., 1999). In addition, the balance between Bcl-2/Bax also appears to be essential in the activation of cytochrome *c* (Ow et al., 2008).

4.2.1.2 Active caspase-3 level in RGCs

In addition to cytochrome *c*, activation of caspases has also been shown to be involved in the death of cultured RGCs exposed to different apoptotic stimuli (Tezel and Wax, 1999, 2000), in optic nerve axotomy induced RGC death (Chaudhary et al., 1999;Kermer et al., 1999), and in rat models of experimental glaucoma (McKinnon et al., 2002a). Moreover, gene therapy conveying a potent caspase inhibitor, in a rat glaucoma model has been found to promote optic nerve axon survival (McKinnon et al., 2002b).

Caspases, cysteine-aspartic proteases, are naturally occurring as proenzymes, which are activated in response to apoptotic insult (Tezel and Wax, 1999). Among them, activated caspase-3 is believed to be the primary effecter enzyme in neuronal apoptosis (Cheng et al., 1998;Thornberry and Lazebnik, 1998;Wang et al., 2007). In

this study, as shown in section 3.1.2, there was gradual enhancement in active caspase-3 expression over time in the retinal sections of OHT animals peaking at 12 weeks of IOP elevation compared to age-matched controls. The intense labeling of active caspase-3 in RGC layer after IOP elevation was similar to that observed in RGCs after axotomy, additionally, the level of caspase-3 in the control retina was similar, indicating the presence of baseline level of caspase-3 in control retinas (Kermer et al., 1999). This result was also consistent with previous research conducted by McKinnon et al (2002), who examined the involvement of active caspase-3 in experimental glaucoma model, using several assays including immunohistochemistry. In that experiment, an active caspse-3 antibody was detected in the RGC layer of OHT retinas more extensively than the control retinas. However, unlike work presented in this thesis, the animals were observed for a period of 4 weeks only after IOP elevation (McKinnon et al., 2002a).

The peak of caspase-3 expression at 12 weeks of IOP elevation in this study was rather delayed. This is in agreement with previous groups who suggested activation of caspase-3 may be a delayed event in traumatic and ischemic brain injury (Buki et al., 2000) and in RGCs after optic nerve transection (He et al., 2004). This delay is probably due to the presence of other effectors downstream of cytochrome *c* such as caspase-9 which might affect the activation of caspase-3 (Tezel and Yang, 2004).

Caspase-3 activation has also been considered as the underlying mechanism of Ginkgo biloba's neuroprotective effect in age related hearing loss (Nevado et al., 2010), and in Aß treated neuroblastoma cell line (Luo et al., 2002;Nevado et al., 2010), which could be via either the mitochondrial or the death receptor pathway.

A similar mechanism could therefore be involved in RGCs apoptosis in the OHT model established by the group, caspase-3, similar to other caspases is produced as proenzyme and is usually sliced after its activation to smaller subunits of 12 kDa and 17 kDa (Liu et al., 1999). A higher level of the 12 kDa subunit of caspase-3 was observed using immunoblotting analysis in the retina of transgenic rats with Rhodopsin mutation than in age-matched controls (ibid).

Activation of apoptotic pathways in AD brains has been widely reported, where caspase activation was implicated in neurofibrillary tangle formation (Rohn et al., 2001b;Rohn et al., 2002b). Furthermore, the brain section from Down Syndrome patients showed extensive Aß deposition and neurofibrillary tangle formation which was associated with accumulation of caspase-3 cleavage products of fodrin (Head et al., 2002). Gervais et al (1999) have previously shown APP cleavage by caspases during apoptosis, resulting in elevated Aß production in hippocampal neurons (Gervais et al., 1999).

There appears to be more than one way of involving activated caspase-3 in apoptosis: one is through APP cleavage which is supported by substantial elevation of caspase-3 and its implication in APP cleavage in Alzheimer's disease (Gervais et al., 1999) and the other is as a key executor of the mitochondrial pathway (Tezel and Yang, 2004).

4.2.1.3 TNF-R1 in RGCs

Up-regulation of pro-inflammatory cytokines and excessive expression of TNF-α has been documented in RGCs incubated in ischemic conditions (Fuchs et al., 2005), as well as in coculture of RGCs and glial cells exposed to elevated hydrostatic pressure

(Tezel and Wax, 2000). Further evidence to support this hypothesis was provided by Kitaoka et al (2006) who observed substantial RGC loss following intravitreal injection of TNF-α in rat model of optic nerve axonal degeneration (Kitaoka et al., 2006). Moreover, TNF-R1 was also found to be localized to RGCs in glaucomatous eyes (Tezel et al., 2001;Tezel, 2008) and TNF-α and its subsequent binding to the death receptor, TNF-R1, in RGCs has been proposed to trigger a caspase-dependent and a caspase-independent component of the mitochondrial death pathways in glaucoma (de Kozak et al., 1997;Tezel, 2008).

Immunohistochemical staining of TNF-R1 (section 3.1.3), documented enhanced TNF-RI receptor expression on the RGC's surface and in the cytoplasm, that peaked at 3 weeks after IOP elevation. This appears to follow a similar profile of developing RGC apoptosis in the OHT model, leading to the assumption that blockade of TNF-R1 signaling could be an effective strategy to protect RGCs in glaucoma.

Ginkgo biloba is known to suppress expression of TNF-α in the brain of atherosclerotic rats (Jiao et al., 2005). It also inhibits TNF-α and H_2O_2 activated primary human T lymphocytes through down-regulation of activator protein-1 signal transduction (Tsao et al., 2008). As TNF-α pathway has been implicated in initiating RGCS apoptosis (Tezel, 2008), this result highlights its neuroprotective potential in glaucoma.

In this study, positive immunoreactivity for TNF-R1 was observed in the cytoplasm in addition to the RGCs surface, and was more intense in OHT eyes in comparison to control (Fig 3.3). This observation further indicates that binding of TNF-α to the

death receptor is essential for signaling the apoptotic pathway (Tezel et al., 2001). The result of this study was in agreement with previous observations on cultured RGCs exposed to ischemia or elevated hydrostatic pressure (Tezel and Wax, 2000) and on retinal section from 20 eyes of glaucoma patients, in which excessive expression of TNF-α and TNF-R1 was observed in glaucoma eyes compared to age-matched normal subjects (Tezel et al., 2001).

The role of the TNF-R1 and TNF-R2 has been investigated, with substantial evidence that TNF-R1 promotes neurodegeneration (Tezel et al., 2001), whereas TNF-R2 is neuroprotective (Fontaine et al., 2002). In mice deficient for TNF, TNF-R1 and TNF-R2, TNF has been observed to aggravate cell death, and absence of TNF-R1 was associated with reduction of neurodegenerative process, furthermore, selectively activating TNF-R2 was found to promote neuroprotection, in the ischemia–reperfusion model. This was associated with the presence of activated Akt/protein kinase B (Fontaine et al., 2002).

Receptor mediated death of RGCs through TNF-R1 has been observed and it is believed to involve a caspase-dependent and caspase–independent component of the mitochondrial cell death pathway (Tezel et al., 2001). In this process caspase-8 cleaves a proapoptotic member of the Bcl-2 family, Bid, and the activated Bid consequently participates in the activation of the mitochondrial cell death pathway (Li et al., 1998;Luo et al., 1998).

Rohn et al (1999) examined the role of TNF-R1 activation in Alzheimer's disease and found that receptor activation can initiate the apoptotic cascade by recruiting adaptor

proteins that activate the proximal effector caspase, caspase-8 (Rohn et al., 2001a), and caspase-8 in turn activates the final executor caspase, caspase-3 (Stadelmann et al., 1999). Inhibition of caspase-8 in RGCs exposed to TNF-α has resulted in a relatively greater protection than in RGCs exposed to hypoxia. This enhancement in RGC survival was, however, temporary and caspase inhibition was not sufficient to block RGC death if the mitochondrial membrane potential was depleted and mitochondrial mediators including cytochrome *c* and AIF were released (Tezel and Yang, 2004).

TNF- R1 and c-Jun N-terminal protein kinase (JNK) signaling have been implicated in the secondary degeneration of RGCs following optic nerve injury, where RGCs loss was remarkably lower in TNF-R1 deficient mice compared to controls (Tezel et al., 2004). In addition, this RGC loss was more prominent two weeks after optic nerve crush. This time period was correlated with the period of glial activation and increased glial immunoreactivity for TNF-α in these eyes (ibid).

4.2.2 Aß and RGC apoptosis in OHT

Apoptosis of RGCs has been demonstrated in experimental animal models of glaucoma as well as in human glaucoma. Many mechanisms have been implicated in initiating RGCs apoptosis in literature as in section 1.1.4, and it is believed that more than one mechanism might be involved in different glaucoma patients at different stages of disease progression (Yin et al., 2008).

Colocalization of Aß and RGC apoptosis in experimental glaucoma has been shown earlier by the group *in vivo*, where a dose and time dependent apoptosis has been

confirmed with Aß administration (Guo et al., 2007a). Aß, polypeptide of 39-43 amino acids that is generated by proteolytic cleavage of APP, is believed to play a significant role in RGC apoptosis, and blocking Aß cytotoxicity may help to prevent the occurrence or progression of glaucoma (McKinnon et al., 2002a). This work showed a strong inverse correlation between Aß and APP immunohistochemistry in retinal cross sections of OHT animals, and in the *in vitro* section of this study as shown in the next section, Aß $_{25\text{-}35}$-induced toxicity in RGC-5 was confirmed, which was blocked by Ginkgolide A and Ginkgolide B treatment. This converse association between Aß and APP is believed to be due to an increase in APP proteolysis and Aß formation that accompanies neuronal apoptosis in glaucoma. This is consistent with Aß colocalization with apoptotic RGCs, that was previously investigated by the group *in vivo* in experimental glaucoma (Guo et al., 2007a).

In section 3.2.1, the presence of APP in the inner retinal layers (RGCs and nerve fiber layer) of OHT rats and age-matched control was demonstrated, however, more intense labeling was observed in the OHT eyes. This observation is in agreement with previous studies that examined APP and Aß in the OHT model (McKinnon et al., 2002a;Guo et al., 2007a). McKinnon et al (2002), using immunoblots, confirmed significant reduction in the level of APP and elevation in Aß in hypertensive eyes in comparison with the controls (McKinnon et al., 2002a). In partial disagreement with this result, however, is the work of, Goldblum et al 2007 who documented higher intensity of both APP and Aß labeling in retinal sections of old DBA/2J glaucomatous mice than young and old controls (Goldblum et al., 2007). This study showed a reduction in APP immunoreactivity in OHT rats over time, which was highest at 12 weeks of OHT elevation. This is postulated to be due to cleavage of APP into Aß by

caspase-3 during apoptosis (Gervais et al., 1999;McKinnon et al., 2002a). The predominant site of caspase-mediated proteolysis of APP in hippocampal cells was found to be within its cytoplasmic tail, and two mechanisms have been proposed to be responsible for caspases mediated processing of APP: the cleavage of APP at endogenous caspase sites and, probably interfering with the normal intracellular processing of APP that would otherwise prevent it from Aß formation (Gervais et al., 1999). The result in this study has also showed minor increases in APP immunohistochemistry in all age-matched controls over time, which could be attributed to the physiological aging changes (Loffler et al., 1995).

Abnormal metabolism of APP, a neuronal transmembrane precursor of Aß, has been implicated in the pathogenesis of several neurodegenerative diseases such as Alzheimer's disease (Anandatheerthavarada and Devi, 2007). Accumulation of APP and Aβ in the mitochondria has been proposed as a causative role in impairing mitochondrial functions (ibid). Furthermore, knocking down of APP inhibited Aß-induced RGCs death (Tsuruma et al., 2010). APP which is believed to play a critical role in growth (Saitoh et al., 1989) and synaptogenesis (Moya et al., 1994) is hugely synthesized in RGCs and then transported into the optic nerve in small transport vesicles, from where it is then transferred to the axonal plasma membrane, as well as to the nerve terminals and metabolized (Morin et al., 1993). A reduction in APP level on chronic administration of EGb 761 have been reported by Augustin and associates (2009) in the cortex of transgenic mouse model of AD (Augustin et al., 2009) which indicated that APP is an important molecular target of Ginkgo biloba. Furthermore, Colciaghi et al (2004) have reported that EGb 761 was capable of inducing the metabolism of APP toward the non amyloidogenic pathway in hippocampal slices

therefore blocking the formation of Aß (Colciaghi et al., 2004).

Using immunohistochemical techniques Gong and colleagues (2005) have observed that EGb 761 treatment reduced the content of APP and caspase-3 in the hippocampus of aluminum-treated rats in a dose dependent manner (Gong et al., 2005). Conversely, Luo et al (2003) using immunohistochemistry have shown that EGb 761 raises the levels of caspase-3 and APP in the hippocampus of a normal rat (Luo et al., 2003), and this could be due to the controversial effect of Ginkgo biloba on the normal and degenerated tissues.

Atypical processing of APP has been implicated in the pathogenesis of glaucoma as well (McKinnon et al., 2002a;Goldblum et al., 2007), additionally, elevated levels of Aß either due to intracellular accumulation or extracellular administration possibly via interaction with the death receptor, has been suggested to act as a stimulus to initiate the apoptotic pathway within neurons (Du and Yan, 2010). This triggers the activation of caspases that subsequently cleave fodrin, a neuronal cytoskeletal protein and known molecular target for caspase-3, APP tau and protein (Head et al., 2002), which is supported by the strong association between Aß deposition and caspase-3 activation that has been observed in neuronal section from patients with Down syndrome, where neuronal cells found to be positive for caspase-3 cleavage product of fodrin (Head et al., 2002), and in patients with Alzheimer's disease (Stadelmann et al., 1999).

This study has observed enhanced Aß immunohistochemical expression in RGCs of all OHT rats, with maximum deposition at 12 weeks after OHT surgery. A similar

pattern of Aß accumulation was found in the rat ocular hypertensive model by others (McKinnon et al., 2002a). The strong inverse correlation between Aß and APP immunohistochemistry observed in this work is believed to be due to an increase in APP proteolysis and Aß formation that accompanies neuronal apoptosis in glaucoma. This is consistent with Aß colocalization with apoptotic RGCs, that was previously investigated *in vivo* in experimental glaucoma (Guo et al., 2007a).

4.3 Summary of in vitro experimental findings

Table 4.1 Neuroprotective effects of Ginkgolide A, B and Bilobalide on RGC-5

Apoptotic Inducers	Assay	Ginkgolide A Effect/Dose	Ginkgolide B Effect/Dose	Bilobalide Effect/Dose
1% DMSO	MTT assay	-	+/ 0.5,1 and 2.5µg/ml	-
	Hoechst 33342	+/ 0.5,1 and 2.5µg/ml	+/ 0.5 and 1µg/ml	-
UV40	MTT assay	-	+/ 0.5,1, 2.5 and 5µg/ml	+/ 0.5 and 1µg/ml
1mM NaN_3	MTT assay	-	-	-
50µM Aβ25-35	MTT assay	+/ 1µg/ml	+/ 0.5µg/ml	-

In the *in vitro* section of this study, RGC-5 was exposed to different concentrations of DMSO, UVC, NaN_3 and Aβ25-35. This has led to a dose dependent reduction in cell viability at 24 hours. To screen the neuroprotective potential of Ginkgolide A, Ginkgolide B and Bilobalide on RGC-5, 1% DMSO, UV40, 1mM NaN_3 and 50µM Aβ25-35 were used as apoptotic inducers, as the average reduction in RGC-5 viability using these concentrations was between 20-35% and using higher concentrations of

those insults would result in a loss of RGC-5 viability that may not be recovered by Ginkgo biloba treatment.

To prove the apoptotic potential of 1% DMSO, Hoechst 33342, which is capable of penetrating plasma membranes and staining the DNA of apoptotic RGCs has been used on DMSO treated RGCs. While all three compounds showed a dose-related response on RGC-5, some intrinsic toxicity was observed with GA. As shown in table 4.1 using the MTT assay, the significant protective effect of Ginkgolide A was observed only against 50μM Aβ25-35 at 1μg/ml concentration. Although GA failed to rescue RGCs from DMSO toxicity on the MTT assay, Hoechst 33342 staining showed that 0.5,1 and 2.5μg/ml of this compound was able to reduce the percentage of apoptotic RGCs after DMSO treatment.

Work in this thesis shows that Ginkgolide B had the most profound neuroprotective effects, against different apoptotic inducers in the majority of assays at a concentration range of 0.5-5μg/ml. Using the MTT assay, 0.5- 2.5μg/ml GB treatment was found to significantly enhance RGC viability after 1% DMSO insult. In addition, on Hoechst staining, the lower two doses 0.5-1μg/ml were able to reduce DMSO-induced apoptosis in RGCs. The protective potential of GB was also observed against UV40 at 0.5-5μg/ml and against 50μM Aβ25-35 at 0.5μg/ml, whereas Bilobalide was effective only against UV40 at a concentration of 0.5-1μg/ml. Interestingly, none of the Ginkgo biloba constituents tested in this work were able to rescue RGC-5 from the toxic effect of sodium azide in both assays.

4.4 Interpretation

4.4.1 1% DMSO and the effects of Ginkgolide A, Ginkgolide B and Bilobalide

DMSO, a widely used solvent, has been reported to have a multitude of actions both *in vivo* and *in vitro* (Santos et al., 2003) and it has been implicated in apoptotic death in several cell lines (Trubiani et al., 1996;Marthyn et al., 1998;Liu et al., 2001;Koiri and Trigun, 2011). In section 3.3.1 of this study, applying ascending concentrations of 1-10% DMSO to RGC-5, resulted in a dose dependent decline in cell survival as accessed by the MTT assay. In this study DMSO was found to cause significant toxicity to RGCs even with the smallest used concentration of 1%. This is consistent with an earlier finding by Cao et al 2007, who found ≥ 10% reduction in cell viability and increased cellular apoptosis in human lens epithelial cell line (HLECs) line treated with 1% DMSO in addition to up regulation of Bax expression in HLECs (Cao et al., 2007). In another study, which was conducted in the EL-4 murine lymphoma cell line, Liu et al (2001) found no apoptotic changes with 0.5% and 1.5% DMSO treatment but with 2.5% DMSO (Liu et al., 2001). This could be due to variable sensitivity of different cell lines to DMSO. Furthermore, the previous research has confirmed that, DMSO-induced apoptosis in the EL-4 murine lymphoma cell line is mediated via caspase cascade of the mitochondrial death pathways (ibid).

DMSO has been observed to promote death receptor mediated apoptosis in human myeloid leukemia cell line via enhancement of mitochondrial membrane depolarization (Vondracek et al., 2006). Involvement of the mitochondrial pathway in DMSO-induced toxicity through stimulation of TNFα-p53 *in vitro* has also been investigated (Brown et al., 2007) and more recently in Dalton lymphoma cells *in vivo*,

DMSO-induced apoptosis have been found to be mediated via inducing TNFα-p53 mitochondrial apoptotic pathway and caspase-9 activation (Koiri and Trigun, 2011),

To evaluate the neuroprotective potential of Ginkgo biloba constituents against 1% DMSO, cells were pre-treated with GA, GB and Bil for two hours before DMSO treatment at a dose range chosen based on previous descriptions in various cell lines and disease models as will be shown in section 4.4.5. Using the MTT assay, GA failed to demonstrate significant protective effects on RGCs viability at all used concentrations, which was expected at higher doses as this cannot be attributed only to DMSO toxicity, but as displayed in section 3.4 at 5, 10 and 25 µg/ml GA was also toxic to RGC. However, lower doses of GA were also insufficient to rescue RGCs. GB treatments on the other hand were associated with significant neuroprotective effects on RGCs, observed at 0.5,1 and 2.5 µg/ml with the greatest protection seen at the smallest used concentration. This protection was dose dependent with an EC_{50} of 2.21µM.

As the MTT assay relies on activity of the mitochondrial succinate dehydrogenase enzyme of living cells, any drug that affects cellular mitochondrial function could unduly influence the result. This possibility could be ruled out using a more sensitive mitochondrial test such as direct evaluation of the mitochondrial membrane potential (Tezel and Yang, 2004;Abdel-Kader et al., 2007) and the mitochondrial ROS level using fluorescence dyes, bioluminescent measurement of ATP level and measurement of caspase-9 activity (Abdel-Kader et al., 2007).

To demonstrate that apoptosis occurred after 1% DMSO treatment and to assess the anti-apoptotic potential of GA, GB and Bil on these cells, Hoechst 33342 staining was used as displayed in section 3.5.1.2. The morphological changes of apoptotic RGCs on Hoechst 33342 staining, such as chromatin condensation seen in this study was the same confirmed by others in different cell lines (Massieu et al., 2004;Griffin et al., 2007;Santiago et al., 2007). In this study, Hoechst 33342 staining of 1% DMSO treated RGC-5 was associated with higher level of apoptosis and contrasting the MTT results 0.5, 1 and 2.5 µg/ml GA treatment significantly reduced the percentage of apoptotic RGCs. A significant reduction of apoptotic RGCs number was also observed on treatment with 0.5 and 1 µg/ml GB. However, surprisingly no significant protective effect was observed with Bil using both MTT and Hoechst assays.

To confirm the apoptosis inducing potential of DMSO on RGC-5, the TUNEL assay was carried out. However, no quantitative data of apoptotic RGCs were obtained. This failure to obtain positive results with the TUNEL assay in this study might be attributed to cell line resistance or the used protocol.

4.4.2 UV40 and the effects of Ginkgolide A, Ginkgolide B and Bilobalide

UV light induced apoptosis has been linked to several cytoplasmic and membrane bound molecular targets (Schwarz, 1998). Several other research studies have investigated whether UV light-induced DNA damage was associated with apoptotic or necrotic process. In a study which looked at DNA repair deficient and proficient isogenic cells, UVC (200-280nm) induced DNA damage has been found to be mainly due to apoptotic pathway (Dunkern et al., 2001).

UVB (280-320nm) has also been found to induce apoptosis in RGC-5. In the RGCs, UVB induced a dose dependent reduction in cell viability, which was also associated with enhanced expression of cytochrome *c*, Bax and absence of bcl2 (Balaiya et al., 2010). A similar observation was reported by another group who documented caspase-3 and Bax activation after exposing RGC-5 culture to visible light (more than 400nm) for 48 hours (Wood et al., 2008).

The duration of UVC exposure in this experiment was 2 minutes in all treated RGCs, but another group (Balaiya et al., 2010) have exposed RGC-5 to a different time period of UVB light, and they have observed a dose dependant reduction in cell viability corresponding with increased cytochrome *c* expression in cultured RGC-5. In 1994 Sachsenmaier et al investigated the involvement of growth factor receptors in UVC induced apoptosis in HeLa cells. This group found that UVC and growth factor have common signal transduction pathway to the nucleus, which involves Ras, Raf, Src and MAP kinases (Sachsenmaier et al., 1994).

In this study, the MTT assay indicated that the viability of RGC-5 culture was significantly and dose dependently declined after exposure to 40, 60 and 80 mj/cm^2 UVC light, which is likely due to chronic oxidative damage in those cells. The protective effect of Ginkgolide A, Ginkgolide B and Bilobalide against UV40 was further accessed. As shown in table 4.1, no significant effect was observed with GA, whereas GB at 0.5, 1. 2.5, and 5 µg/ml, and Bil at 0.5 and 1 µg/ml were associated with significant enhancement in cell viability, which is probably mediated via their antioxidant potential (Ahlemeyer et al., 1999;Zhou and Zhu, 2000). This result is in

line with a recent observation by Liu and Yu, 2008, who used UVC as an apoptotic inducer in a primary culture of rat cortical neurons. Here; significant increase in MTT absorbance and neuroprotective effect was observed with green tea polyphenols (Liu and Yu, 2008).

4.4.3 NaN_3 and the effects of Ginkgolide A, Ginkgolide B and Bilobalide

Examining the effects of 24 hours exposure of RGC-5 to 1, 2.5, 5 and 10mM of the mitochondrial complex inhibitor NaN_3, it has been observed that concentration dependent reduction in RGC-5 viability was produced, which is in agreement with another group who had dose dependent reduction in cellular viability and mitochondrial membrane potential of primary cortical neuron, using 1, 3 and 10 mM NaN_3 (Selvatici et al., 2009). In that study, Selvatici and coworkers confirmed that MK801, trolex, N-acetyl-L-carnitine, and the nitric oxide synthase inhibitor, L-NAME were able to prevent the cytotoxicity of 10 minutes exposure to NaN_3, which disagree with the result of this experiment in which no neuroprotection was achieved with Ginkgolide A, B and Bilobalide. However, the protective effect of EGb 761 on mitochondrial respiratory chain function in PC 12 cells using complex inhibitors including NaN_3 has been documented (Abdel-Kader et al., 2007). Possible explanations for this effect is NaN_3, which is known to cause irreversible loss of cytochrome *c* oxidase activity (Leary et al., 2002), and is also known to cause mitochondrial failure leading to both apoptotic and necrotic effects depending on dose and duration of exposure to NaN_3 as well as on the type of cultured cells (Selvatici et al., 2009). As mentioned in section 3.3.3 in this thesis RGCs were exposed to NaN_3 for 24 hours.

It is conceivable to assume that RGCs death after NaN_3 treatment in this experiment was via non-apoptotic pathway. That is why on using the same assay (MTT), RGC-5 viability was reduced after NaN_3 exposure and GA, GB and Bil failed to reuse them. This is actually supported by the absence of a significant increase in annexin V-FITC labeling of phosphatidyl serine (PS) which is believed to signal early apoptosis on the external surface of cortical neurons after NaN_3 (Selvatici et al., 2009). Additionally, as mentioned earlier our assessment of neuroprotective potential of Ginkgo biloba constituents was through the MTT assay which is a measure of mitochondrial function, so its validity may be criticized.

4.4.4 Aß and the effects of Ginkgolide A, Ginkgolide B and Bilobalide

In these experiments RGC-5 were resistant to 10-100 µM Aß $_{1-42}$ treatment, unlike human neuroblastoma SH-SY5Y cells which showed significant apoptosis and its viability dropped to around 50% after exposure to 100µg/ml Aß $_{1-42}$ (Shi et al., 2009). One possible explanation is that RGC-5 are much more resistant cell line with ability of survival in the presence of high level of ROS (Maher and Hanneken, 2005). The result of these experiments was also partly inconsistent with Bastianetto et al 2000 who observed reduction in hippocampal primary cells survival on MTT assay after exposure to 5 µM Aß $_{1-40}$, 25 µM Aß $_{1-42}$ and 25 µM Aß $_{25-35}$ (Bastianetto et al., 2000a). The authors also found that the whole extract EGb 761 and the flavonoid fraction of the extract were able to rescue rat hippocampal primary cells from the indicated concentrations of Aß toxicity, interestingly; with Aß $_{25-35}$. This protection was significant even if EGb 761 was applied 8 hours after Aß $_{25-35}$ treatment. On the

other hand, in the same research Ginkgolide B and Bilobalide failed to protect neuronal cells from Aß-induced neurodegenerative process (Bastianetto et al., 2000a).

In the present *in vitro* study Aß $_{25-35}$ caused significant reduction in RGC-5 viability in agreement with previous observation showing similar effect at similar concentrations: at 5-20 µM Aß$_{25-35}$ in RGC-5 (Tsuruma et al., 2010), at 10 µM Aß $_{25-35}$ in cultured rat cerebral cortical neurons (Ban et al., 2006a) and finally at 50 µM Aß $_{25-35}$ (Zhang et al., 2010) and at 100 µM Aß $_{25-35}$ (Zhou et al., 2000) in PC 12 cell line.

In this work, the protective effect of 1µg/ml GA and 0.5µg/ml GB against 50 µM Aß $_{25-35}$-induced death in RGC-5 was confirmed, while Bilobalide failed to confer any protection. Krieglstein et al (1995) hypothesised that Ginkgolides exert their action on neurons possibly via PAF receptors, whereas bilobalide could act on both neurons and astrocyte (Krieglstein et al., 1995). Despite this, the exact mechanism of protection by the Ginkgolides against Aß $_{25-35}$ toxicity was not addressed in this thesis and remains to be elucidated. Ginkgolides especially Ginkgolide B have demonstrated protective effects against apoptotic inducers known to target mitochondrial pathway such as DMSO, given the fact that the mitochondrial permeability transition pore (mPTP) is believed to be involved in Aß-induced toxicity (Du and Yan, 2010). It is possible that this Ginkgolid's neuroprotection was mediated via this pathway.

In literature, there are several proposed mechanisms through which Aß exert its apoptotic effect including: oxidative stress and Ca^{2+} influx (Abramov et al., 2011), mitochondrial dysfunction (Du and Yan, 2010) and modulation of apoptosis related caspases (Troy et al., 2000;Rohn et al., 2002a). Moreover, Tsuruma and coworkers

2010 strongly suggested that Aß $_{25-35}$ up-regulation of APP is the leading cause for RGC-5 death (Tsuruma et al., 2010). A recent research on mixed culture of hippocampal neurons and astrocytes has implicated astrocyte membrane cholesterol content in mediating Aß neurotoxicity (Abramov et al., 2011). This recent work might provide an explanation for an earlier finding by Yao and colleagues 2004 in PC12 cells where they observed that EGb 761 inhibited overproduction of APP and Aß induction by free cholesterol (Yao et al., 2004).

Ginkgo biloba has exerted its protective effect on Aß treated cells via several mechanisms including a direct inhibitory effect against Aß aggregation and Aß induced apoptosis in neuronal cell culture (Yao et al., 2001;Shi et al., 2009). This property of EGb 761 is believed to be mediated via direct interaction with Aß in neuroblastoma cell line expressing AD-associated double mutation (Luo et al., 2002). In that experiment, EGb 761 and Bilobalide were more powerful than GA and GB in inhibiting Aß aggregation. Furthermore, EGb 761 significantly attenuates mitochondrial initiated apoptotic pathway and decreased the activity of caspase-3 in neuroblastoma cells (Luo et al., 2002).

4.4.5 Comparison of *in vitro* effects of Ginkgolide A, Ginkgolide B and Bilobalide

Despite having certain structural and chemical properties in common, especially three lactone groups and a tertiary-butyl group (Krieglstein et al., 1995;Ivic et al., 2003), the pharmacological activity of Ginkgolide A, Ginkgolide B and Bilobalide is different as shown in section 1.2.3, and the effective neuroprotective concentration of

GA, GB and Bil on RGC-5 in these experiments was also variable in different experimental conditions as displayed in table 4.1

In this work, overall the most profound neuroprotective effects were observed with Ginkgolide B against 1% DMSO, UV40, and 50μM Aβ25-35 at a concentration range of 0.5-5 μg/ml, with the best protection against all apoptotic inducers observed at 0.5 μg/ml. This raises a question of whether doses lower that 0.5μg/ml would confer greater protection, however, the dose range used in these experiments was based on previous work by (Ahlemeyer et al., 1999;Bastianetto et al., 2000b;Shi et al., 2009) in different neuronal cells. Similar neuroprotective effect by GB was observed at 0.25-1 μg/ml, in iNOS producing THP-1 macrophages which were treated with lipopolysaccharide and TNF-α (Cheung et al., 2001) which is believed to be mediated via the anti-oxidant property of GB. Conversely, Bastianetto et al (2000) have observed a negative effect with 1–5 μg/ml GB in hippocampal cells, treated with the NO donor SNP (Bastianetto et al., 2000b).

GB has also demonstrated neuroprotective anti-oxidant effects against serum deprivation and staurosporine-induced ROS production and apoptosis in chick embryonic neurons and in mixed cultures of neurons and astrocytes from neonatal rat hippocampus, however, higher concentrations were used to achieve anti-apoptotic effects (Ahlemeyer et al., 1999). In those experiments, GB at 10μM was required to rescue serum deprived and staurosporine treated chick neurons whereas in the mixed neuronal culture 100 μM GB was found to be effective against staurosporine-induced apoptosis (Ahlemeyer et al., 1999). GB at 100 μM has also been found to protect primary culture of hippocampal neurons against glutamate-induced excitotoxicity (Xu

et al., 2010). The neuroprotective effect of GB on glutamate and NMDA-induced excitotoxicity has been investigated by another group, Zhu et al (1997), who have observed enhancement in neuronal viability with reduction of Ca^{2+} level in primary culture of cerebral cortical neurons with 2mg/L GB (Zhu et al., 1997).

In contradiction with the results of this study, both Ginkgolide A and B treatment enhanced cellular viability and were associated with enhancement of the mitochondrial membrane potential after SNP treatment in PC12 cells at a concentration of 0.01 mg/ml and in dissociated brain cells at concentrations of 0.05 mg/ml (Abdel-Kader et al., 2007).

In this study, no significant effect was observed with Ginkgolide A after 1% DMSO, UV40 and NaN_3 treatment. However, GA at 0.5, 1 and 2.5µg/ml reduced the number of apoptotic RGCs after DMSO treatment as revealed by Hoechst staining. In line with these findings, no protection was observed with Ginkgolide A in either serum-deprived or staurosporine-treated neuronal cells (Ahlemeyer et al., 1999). However, dose dependent protective effect was observed with GA at 0.25-1 µg/ml with the best protection at the lower dose in THP-1 macrophages (Cheung et al., 2001).

This discrepancy in the potency between GA and GB has been described by Ivic et al (2003) when they were examining the effect of terpenoid fraction of Ginkgo biloba on the glycine and GABA, type A receptors. These authors noticed that GB was significantly more potent than GA, and they have explained this by the fact that GB have a 1-OH group whereas GA does not, and the higher potency of GB is due to the direct interaction of the 1-OH group with the receptor. Furthermore, the position of

the lactone group in GA and GB was found to be different which has an implication on receptor binding (Ivic et al., 2003).

In this work, the protective effect of 1µg/ml GA and 0.5µg/ml GB on 50 µM Aß $_{25-35}$-induced death in RGC-5 was confirmed, which is partially consistent with an earlier finding in SH-SY5Y cells were both GA and GB were able to block neurotoxicity of Aß $_{1-42}$ (Bate et al., 2004). However, it seems that the effective concentration of different Ginkgo biloba constituents is variable in different cell lines, and in different models of inducing cell death. GB has previously demonstrated its best protection against 100µg/ml Aß $_{1-42}$-induced toxicity in SH-SY5Y cells at 10µg/ml whereas 5 and 20µg/ml GB could not display any protection (Shi et al., 2009). Another experiment highlighted the lower potency of GA when they found that GB but not GA was able to suppress the K^+-evoked Ach release in rat hippocampal slices treated with Aß $_{25-35}$ (Lee et al., 2004).

In these experiments only 0.5 and 1 µg/ml Bil were able to protect RGC-5 from UV40-induced cell loss with higher neuroprotection at 1 µg/ml. No other used concentration of Bil was effective in enhancing cell survival with other apoptotic induces: 1% DMSO, NaN_3 and 50mM $A\beta_{25-35}$ treated RGC-5. This discrepancy in the neuroprotective potential of Bilobalide was observed by other researchers, where the prominent protective effect of Bil was observed at 10 µM against hypoxic damage of chick neurons, while poor effect was seen against excitotoxic damage of hippocampal neurons (Krieglstein et al., 1995). The same group also found that Bil demonstrated neuroprotective effects in the rat model of focal cerebral ischemia but no effect was seen in the rat model of global ischemia (ibid).

The effective concentration of Bilobalide in our experiments were 0.5 and 1 µg/ml, which is equal to 1.53, 3.06 µM. Similar protection by Bil was observed in macrophages derived from a human monocytic cell line at a concentration range of 0.25-1 µg/ml, via selective inhibitory effect on iNOS mRNA expression (Cheung et al., 2001). In agreement with Cheung et al (2001), Bil (25-100µM) demonstrated a dose dependent reduction in apoptosis which was induced in PC12 cells with the ROS producer, xanthine, in addition to the anti-oxidant property of Bil, in the same experiment the authors found reduction in ROS-induced elevation of Bax and active caspase-3 (Zhou and Zhu, 2000). The anti-oxidant effect of Bil was also observed in chick embryonic neurons exposed to serum deprivation and staurosporine (Ahlemeyer et al., 1999), Bil protection was observed at 1 µM in chick embryonic neurons exposed to serum deprivation and at 10 µM in those exposed to staurosporine (ibid). Furthermore, Bil at 100 µM was found to rescue mixed culture of hippocampal neurons and astrocytes from serum deprivation induced apoptosis (ibid). Conversely, no protective effect with Bilobalide was observed in work by other groups in the same cell line. Rapin et al 1998 found that addition of 0.1-1 µg/ml (0.3-3 µM) Bil failed to protect hippocampal neurons from peroyl radical-induced apoptosis (Rapin et al., 1998) and Bastianetto et al (2000) have also documented that treatment with 1–5 µg/ml Bil did not protect hippocampal cells from the SNP-induced toxicity. In this research the authors believed that the documented neuroprotective potential of EGb 761 against NO-induced toxicity was attributed to its flavonoid fraction (Bastianetto et al., 2000b).

The neuroprotective effects of Bilobalide have been attributed to its positive effects on mitochondrial membrane potential (Abdel-Kader et al., 2007), and to enhancement

of the mitochondrial respiratory chain (Tendi et al., 2002). The protective effects of Bil which were observed in, PC 12 cells, at 10 µg/ml, a higher concentration than our study, is believed to be mediated via up regulation of mitochondrial gene expression of NADH dehydrogenase (ibid).

Glycine and GABA receptors antagonism are also believed to mediate the neuroprotective effects of Bil (Ivic et al., 2003). This was recently investigated by another group where Bilobalide at concentration of 10 µM was able to decrease NMDA-induced choline release by more than 90% (Kiewert et al., 2008), and exert antagonistic effect on GABA receptors (Kiewert et al., 2007).

In this work Bilobalide failed to confer any protection against 50 µM $A\beta_{25-35}$ induced toxicity. This result was partially consistent with another group where they found that both Bil and GB failed to confer any protection against Aß toxicity in primary culture of hippocampal cells (Bastianetto and Quirion, 2002). Conversely in PC12 cells 25-100 µM Bil, dose dependently blocked the toxicity of 100 µM $A\beta_{25-35}$ (Zhou et al., 2000).

Beside cell line variations and the differences in apoptotic inducers used, another reason that might explain the discrepancies in the neuroprotective potential of GA, GB and Bil in this experiments and work conducted by other groups is the solvent used to dissolve Ginkgo biloba constituents, while most other groups have used DMSO and ethanol to dissolve GA, GB and Bil, in this work GA was dissolved in DMF, GB in DMSO and Bil in Acetone according to the manufacturer's instruction.

Chapter Five

5. Conclusion and summary

Managing glaucoma patients can be clinically challenging to ophthalmologist due to the fact that most glaucoma patients are asymptomatic until a considerable amount of visual field loss occurs. However, during the last years, much progress has been made in understanding pathogenic mechanisms involved in glaucoma and mechanism of action of neuroprotective agents. Even though, there has historically been a problem in translating preclinical and experimental drugs to patients for many reasons in particular: the lack of good experimental models of disease; the narrow therapeutic index of the neuroprotective drugs due to undesirable side effects on the patients (Levin and Peeples, 2008), as well as the lack of good clinical end points. It is now believed that better clinical end points are necessary to access the new therapeutic agents. Unfortunately a failure of phase III clinical trial of memantine in glaucoma patients has reduced enthusiasm for those drugs as neuroprotective (Osborne, 2008), however, recent advance in imaging technology should provide clinician and researchers with more reliable tools to access the efficacy neuroprotective agents (Cordeiro et al., 2004;Cordeiro et al., 2010;Cordeiro et al., 2011). The results of a recently published, well-designed glaucoma clinical trial, the Low Pressure Glaucoma Treatment Study (LoGTS), showing evidence of Brimonidine neuroprotection (Krupin et al., 2011) is highly encouraging for using the non IOP lowering agents in glaucoma patients (Cordeiro and Levin, 2011).

Ginkgo biloba has been shown to have neuroprotective effect in several neurodegenerative diseases via modulation of APP, Aß, inflammatory mediators and apoptosis related caspases, in the this study we investigated the immunoreactivity of cytochrome *c*, caspase-3,TNF RI, APP and Aß, in the normal and OHT rat retina as potential pharmacological target for Ginkgo biloba. We can report that RGCs in the

OHT model established by the group, express these molecular targets and that could be used to slow the progression of RGCs loss in glaucoma.

We can conclude with certainty that individual component of Ginkgo biloba terpene fraction: Ginkgolide A, Ginkgolide B and Bilobalide have some neuroprotective effect on RGC-5 death whether it was produced by 1% DMSO, UV40, 1mM NaN_3 or 50 µM $Aß_{25-35}$ toxicity with Ginkgolide B having the profound neuroprotection. However, the neuroprotective potential of GA, GB and Bil was different depending on the apoptotic stimuli used. More studies are needed to understand the precise mechanism by which DMSO, UV40, $Aß_{25-35}$ induced RGC-5 death and to clarify the mechanism of neuroprotection by Ginkgo biloba constituents in blocking RGC-5 apoptosis, which will help to provide the pharmacological basis of its use in preventing or retarding RGCs loss in glaucoma patients.

5.1 *Future perspective*

Work in this study support the role of neuroprotection in glaucoma management by using non-IOP treatment modalities that directly target RGCs. in the *in vitro* experiments Ginkgolide B showed the most profound nuroprotective effects on RGC-5 against apoptotic inducers which simulate glaucomatous stress. Therefore, it could be the most active ingredient of the commonly used herb Ginkgo biloba in glaucoma and it would be very interesting to do a clinical trial to access the nuroprotective potential of Ginkgolide B in glaucoma patients.

Chapter Six

6. References

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