COMPARISON OF OXIDATIVE STRESS LEVELS AMONG PATIENTS WITH PRIMARY OPEN ANGLE GLAUCOMA (POAG) AND PRIMARY ANGLE CLOSURE GLAUCOMA (PACG)

by

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I hereby certify that the work in this my own except for the quotations and summaries which have been duly acknowledged.

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ABBREVIATIONS

POAG	Primary open angle glaucoma
PAC	Primary angle closure glaucoma
PACS	Primary angle closure suspect
PACG	Primary angle closure glaucoma
PXG	Pseudoexfoliation glaucoma
IOP	Intraocular pressure
VCDR	Vertical cup- disc- ratio
RGC	Retinal ganglion cells
ONH	Optic nerve head
RNFL	Retinal nerve fibre layer
DM	Diabetes mellitus
HPT	Hypertension
HPL	Hyperlipidemia
IHD	Ischemia heart disease
ROS	Reactive oxygen species
GPx	Glutathione peroxidise
NO	Nitric oxide
NOS	Nitric oxide synthase
TAS	Total antioxidant status
BDNF	Brain-derived neurotrophine factor
SOD	Superoxide dismutase
MDA	Malondialdehyde
TNF-α	Tumour necrosis factor alpha

TNF-A	Tumour necrosis factor-A
NMDA	N-methyl-D-asparate
oxo8dG	Oxo-2,7-dihydro-2'-deoxyguanosine
8-OH-dG	8-hydroxydeoxyguanosine
8-OHG	8-hydroxyguanosine
8-oxoG	8-*oxoguanine*
TBA	Thiobarbituric acid
DNA	Deoxyribonucleic acid
H_2O_2	Hydrogen peroxide
O^{2-}	Superoxide
PUFA	Polyunsaturated fatty acids
HO•	Hydroxyl radical
LOO•	Lipid peroxyl radicals
LOOH	Lipid hydoperoxide
dH_2O	Distilled water
FRAP	Ferric reducing ability of plasma
GC-MS	Gas chromatographic and mass spectrometry
MMP	Matrix metalloproteinase
ECM	Extracellular matrix
TGF-B2	Transforming growth factor
ET-1	Endothelin-1
MYOC	Myocillin
OPTN	Optineurin
SD	Standard deviation
LCOS II	Lens Opacities Classification System II

SIMES	Singapore Malay Eye Study

ABSTRAK

Pengenalan :

Stres oksidatif dipercayai menyebabkan kemusnahan sel ganglion retina, degenerasi jaringan trabekular dan meningkatkan rintangan pada pengaliran keluar akueus. Keadaan ini dipercayai meningkatkan tekanan intraokular dan seterusnya menyebabkan kerosakan saraf mata yang merupakan penyebab utama penyakit glaukoma. Memahami kesan stres oksidatif adalah penting dalam perawatan pesakit glaukoma. Paras stress oksidatif selalunya diambil dari cecair di dalam mata, akues dan vitreus. Kaedah ini adalah sukar dan invasif. Manakala paras stress oksidatif di dalam darah dipengaruhi olef pelbagai faktor dan tidak spesifik kepada penyakit ocular.

Objekif:

Kajian ini bertujuan untuk membuat perbandingan antara aras SOD, catalase dan MDA dalam air mata di kalangan pesakit glaukoma sudut terbuka, pesakit glaukoma sudut tertutup dan kumpulan kawalan. Selain itu, perbandingan aras SOD, catalase dan MDA antara glaukoma sudut terbuka dan glaucoma sudut tertutup turut dikaji.

Metodologi:

Kajian keratan rintas ini telah dijalankan di antara Mei 2014 and November 2015. Pesakit yang menghidapi penyakit glaukoma sudut terbuka primer dan glaukoma sudut tertutup primer yang menghadiri klinik mata Hospital Raja Permaisuri Bainun dan Hospital Universiti Sains Malaysia telah dipilih sebagai kumpulan kajian. Manakala, kumpulan kawalan dipilih melalui padanan umur di kalangan pesakit mata yang bukan berpenyakit glaukoma. Soal selidik mengenail sejarah penyakit, sejarah mata dan sejarah ubat seperti antioksidan dijalankan ke atas kedua-dua kumpulan. Pemeriksaan mata yang lengkap termasuk analisis

medan penglihatan Humprey telah dilaksanakan. Sampel air mata diambil menggunakan kertas khas Whatmann mengikut teknik ujian Schirmmer. Analsis makmal dijalankan untuk menguji tahap SOD, catalase dan MDA air mata pesakit glaukoma dan kumpulan pengawal. Analisis statistik dijalankan dengan menggunakan Statistical Package untuk Social Science (SPSS Inc Versi 20). Independent t-test digunakan untuk membandingkan aras SOD,catalase dan MDA dalam air mata antaran kumpulan glaukoma dan kawalan.

Keputusan :

Seramai 62 pesakit glaukoma sudut terbuka primer, 57 pesakit glaukoma sudut tertutup primer dan 72 pesakit kawalan terlibat di dalam kajian ini. Pesakit glaukoma didapati lebih tua daripada pesakit kumpulan kawalan. Purata tahap SOD dan catalase pesakit glaukoma didapati lebih tinggi daripada kumpulan kawalan. Manakala, purata aras MDA pesakit glaukoma didapati lebih rendah daripada kumpulan kawalan. Tetapi tiada perbezaan signifikan antara tahap SOD (p=0.191), catalase(p=0.259) dan MDA (0.309) dari air mata antara pesakit glaukoma dan kumpulan kawalan. Selain itu, tiada perbezaan signifikan antara tahap SOD, aras catalase dan MDA dari air mata di antara pesakit glaukoma sudut terbuka primer dan glaukoma sudut tertutup primer. Walaubagaimanapun, purata tahap SOD didapati lebih tingi bagi pesakit glaukoma sudut terbuka primer jika dibandingkan dengan kumpulan kawalan selepas dianalisis menggunakan MANCOVA (351.67 U/ml vs 315.12 U/ml, p=0.006) dengan mengambil kira penyebab yang lain.

Kesimpulan :

Pengesahan tahap stres oksidatif dari air mata secara kuantitatif adalah mudah dan tidak invasif. Melalui kajian ini dapat disimpulkan bahawa SOD bermungkinan menjadi penanda tekanan oksidatif yang berpotensi bagi mengenal pasti glaukoma sudut terbuka primer.

Walaubagaimanapun, tahap stress oksidatif dari air mata mungkin dipengaruhi oleh pelbagai faktor lain yang mengurangkan kemampuan air mata sebagai penanda tahap stress oksidatif yang lebih spesifik.

ABSTRACT

Introduction:

Oxidative stress has been postulated to cause retinal ganglion cells death, trabecular meshwork degeneration leading resistance of aqueous outflow. Subsequently, it may lead to IOP elevation and glaucomatous optic neuropathy. Thus, it is worthwhile to look for the oxidative stress level in glaucoma patients. Detection of oxidative stress level in ocular tissue may perhaps provide insight into a role of oxidative stress in pathogenesis of glaucoma.

Objective:

Our objective was to compare the oxidative stress level in tears between glaucoma and age-matched controls. The comparison of SOD, catalase and MDA level between POAG and PACG patients were also conducted.

Methods:

A cross sectional study was conducted between May 2014 and November 2015 involving patients with confirmed diagnosis of POAG and PACG attending eye clinic of two tertiary hospitals in Malaysia; Hospital Universiti Sains Malaysia and Hospital Raja Permaisuri Bainun. Age-match non glaucoma patients were recruited as controls. The detail medical history, ocular history and drug history such as antioxidant supplement were obtained through direct questioning from patients and medical record. Complete ophthalmic evaluations were conducted including Humphrey visual field analysis. Tear samples collected by using schirmer paper. Laboratory analysis was performed to test on SOD, catalase and MDA level of tears using commercially available immunological kits. Statistical analysis was done using Statistical Package for the Social Science (SPSS Inc Version 20). Independent t-

test was used to compare the SOD, catalase and MDA level in tears between glaucoma and controls.

Results:

A total of 62 POAG patients, 57 PACG patients and 72 controls were recruited. Patients with glaucoma were older. Mean SOD and catalase level were slightly higher in glaucoma patients as compared to controls. Meanwhile mean MDA level was slightly lower in glaucoma patients compared with controls. However, there was no significant difference of SOD level (p=0.191), catalase level (p=0.259) and MDA level (p=0.309) between glaucoma and controls using multivariate analysis. There was no significant difference of SOD, catalase and MDA level between POAG and PACG patients. However, mean SOD activity was statistically significant higher in patients with POAG compared to controls after adjusting confounding factors (351.67 U/ml vs 315.12 U/ml), p=0.006) .

Conclusions

Quantification of oxidative stress level in tears is non invasive and easy. Catalase and MDA may not a play role in oxidative stress in glaucoma. SOD is a potential oxidative stress marker for POAG.

Chapter 1
Introduction

1.0 INTRODUCTION

1.1 Glaucoma

Glaucoma is a leading cause of irreversible world blindness, which is second among the visual disorders (Quigley and Broman, 2006). Glaucoma is a progressive optic neuropathy characterised by loss of retinal ganglion cells, with characteristic appearance of structural optic nerve damage and functional visual field defect (Quigley and Green, 1979).

Glaucoma can be divided into 2 main groups, which are open or closed angle glaucoma. It can be further divided into primary or secondary. Primary open angle glaucoma (POAG) is the most common form of glaucoma. Primary Angle closure glaucoma (PACG) is known to cause more blindness than POAG in Asians (He *et al.*, 2006).

1.1.1 Types of Glaucoma

1.1.1.1 POAG

POAG is the most common form of glaucoma (Quigley and Broman, 2006). POAG has no identifiable secondary cause, normally it affects both eyes. In open glaucoma, the anterior chamber is deep with features of open angle on gonioscopy. POAG is optic nerve damage with structural and functional evidence. It can be with elevated or without elevated intraocular pressure. Thus, the diagnosis of POAG is made if any of three categories of evidence without feature of angle closure on gonioscopy and no identifiable secondary cause (Foster *et al.*, 2002).

1.1.1.2 PACG

Angle closure glaucoma is where the trabecular drainage system is closed without identifiable secondary cause. The old concept of PACG classification is defined as people presents with

sudden onset of IOP elevation resulting from total angle closure associated with symptoms, an episode of sudden IOP elevation that is spontaneously aborted, chronic IOP elevation due to presence of peripheral anterior synechiae that close the anterior chamber angle permanently or latent evidenced that an open angle but narrow angle under certain circumstances (Lowe, 1988). The lack of standardization and frequent overlap in clinical presentation make it difficult for comparison in epidemiologic studies. In addition, old form of classification does not offer any insight as to the natural history of disease, presence or absence of glaucomatous optic neuropathy. Therefore, it is not useful for visual prognostication. This classification is differs from new concept. The new classification takes into consideration of the difference between the mechanism of elevation of IOP and the resultant insult caused by it. There are three subcategories: Primary angle closure suspect (PACS), primary angle closure (PAC) and primary angle closure glaucoma (PACG). PACS is an eye which appositional contact between peripheral iris and posterior trabecular meshwork. PAC is when occludable drainage angle and presence of features trabecular meshwork obstruction such as elevated of intraocular pressure, peripheral anterior synechiae, iris whirling and glaucomflecken lens opacities. PACG is progression of PAC together with evidence of glaucoma (Foster *et al*., 2002).

1.1.2 Prevalence of Glaucoma

It has been estimated 285 million peoples will visually impaired and 39 million blind in 2010 (Pascolini and Mariotti, 2011). The most common cause of visual impairment is uncorrected refractive error (43%). Glaucoma (2%) is rank as the third common cause of visual impairment (Pascolini and Mariotti, 2011). Cataract remains the most common cause of blindness (51%) followed by glaucoma (8%).

It was estimated that nearly 64.3 million people (aged 40-80 years old) were affected by glaucoma in 2013, estimated to increase to 76.0 million by 2020 and may further escalate to 111.8 million in 2040 (Tham *et al.*, 2014). Africa is a region with the highest of prevalence POAG (4.20%) while PACG is more prevalence in Asia (1.09%). It is projected Asia will be the largest people affected by POAG and PACG by 2020 (Tham *et al*, .2014).

Baltimore Eye Survey reported that African Americans have four to five times higher risk to develop POAG compared to Caucasian in the United State (Tielsch *et al.*, 1991). Another study reported that 2.71 million people diagnosed to have POAG in United States in 2011 with 31% of were 70 to 79 years old, 53 % of female and 44 % of white population but is non- Hispanic ethnicity (Vajaranant *et al.*, 2012). POAG was found disproportionately affect those of African descendent while 80 % of those with PACG was found in Asia (Cedrone *et al.*, 2008). However, POAG was more common (2.1%) than PACG (1.5%) in Southern Chinese population (He *et al.*, 2006).

PACG affects approximately 1.09% of Asian adults (Tham *et al.*, 2014). The prevalence of PACG (1.4%) was approximately two folds higher than POAG (0.7%) among Chinese (Cheng *et al.*, 2013). In China, the prevalence of glaucoma among those aged more than 40 years old was reported to be 3.6% in Beijing Eye Study, 2.6% with POAG and 1.0% with PACG (Wang *et al.*, 2010).

The Singapore Malay Eye Study (SIMES) had reported the prevalence of glaucoma in ethnic Malay of more than forty years old was 3.4% which is comparable to ethic Chinese people (Shen *et al.*, 2008). Currently, there is no actual statistic on prevalence of glaucoma in Malaysia.

1.1.3 Pathogenesis of Glaucoma

The pathogenesis of glaucoma remains unknown. Currently, the most popular theory is based on pressure dependent and pressure-independent theory (Caprioli, 2007). Elevation of IOP is believed to cause direct mechanical injury nerve fibre later responsible for glaucomatous damage (Quigley *et al.*, 1980). Thus, elevated IOP induces physical changes at the ONH visualized clinically as optic disc cupping, which cause optic nerve axonal compression at the lamina cribrosa, reduce retrograde axoplasmic flow, interference in retrograde neurotrophine transport to retinal ganglion cells which can stress retinal ganglion cells (RGC) (Guo *et al.*, 2005). Elevated IOP is the only modifiable risk factor for POAG (Le *et al.*, 2003).

Pressure-independent theory is based on neurodegenerative nerve fibre layer of retinal and ONH with normal IOP (Gupta and Yucel, 2007). The potential triggers are genetics, inadequate ocular blood flow, vascular degeneration and oxidative stress. Strong family nature of POAG has been recognized as important risk factor, suggesting the potential of genetic predisposition to glaucoma (McNaught *et al.*, 2000). Based on single gene studies, myocillin(MYOC)/TIGR (GLC1A), optineurin (OPTN) (GLC1E) and WDR 36 (GLC1G) (Wiggs, 2007). As POAG is a complex disease, it is unlikely only a single gene is responsible for POAG development. Currently with the advancement of genetic analysis, more genetic variations and mutations were identified (Fingert, 2011; Fujiwara *et al.*, 2003; Unal *et al.*, 2007). Impairment of insufficient autoregulation of optic nerve blood flow may lead to optic nerve ischemia (Flammer and Mozaffarieh, 2008). On the other hand, low systemic blood pressure for long duration may lead to reduction of ocular perfusion pressure chronically (Caprioli and Coleman, 2010). Therefore, reduce ocular perfusion to optic nerve head resulting to optic nerve ischemia, which commonly seen in POAG and NTG patients. Reduction of diastolic perfusion pressure is an important risk factor for POAG (Bonomi *et al.*,

2000). Vasospasm may lead to inadequate blood flow to the surrounding tissue and consequent ischemia of optic nerve due to vasoconstriction or insufficient dilatation of microcirculation (Flammer and Orgul, 1998). It can be seen in the condition of such as migraine and raynaud phenomenon. In additions, vascular degenerative contributing for progressive decline in ocular perfusion has been observed with increasing age (Agarwal *et al.*, 2009). Such condition can be seen in the patients with arteriosclesorosis which accelerated if associated with hypertension, diabetes mellitus and hyperlipidemia. At the molecular mechanism leading to RGC death due to vascular dysregulation are still not clear. There are few postulations reported before, they postulated that vascular insufficiency can cause RGC apoptosis directly. Or the other hand, they believed that insufficiency of blood flow to optic nerve head may lead to response of upregulation of MMP-9 expression in circulating leucocytes. The increased of MMP expression leading RGC death in the absence of IOP elevation (Golubnitschaja-Labudova *et al.*, 2000; Golubnitschaja *et al.*, 2004). Different studies provide cumulating evidence which supports the association of reactive oxygen species (ROS) with different aspect. Tezel (2006) reported that oxidative stress direct effect damage to macromolecules such as DNA, protein and lipids ensues of RGC, consequences leading to RGC death. ROS are involved in signalling RGC death by acting as second messenger and modulating protein function by redox modifications of downstream effectors through enzymatic oxidation of specific amino acid residuals. For the indirect damage to RGC death through activation of apoptosis factors such as glutamate, tumor necrosis factor-alpha (TNF-α), mitogen activated protein kinases, nuclear factor-kappa B or extracellular signal-regulated kinases (Tezel, 2006).

1.1.3.1 Pressure dependent mechanism

Intraocular pressure (IOP) is a well-known modifiable risk factor for development of POAG. Elevated IOP is result from diminished aqueous outflow rather than over production of aqueous (Kwon *et al.*, 2009). It was found that IOP more than 25mmHg increased the risk of POAG by 19% in predominantly African origin population based on Barbados Eye Study (Nemesure *et al.*, 2007).

Elevation of IOP is believed to cause direct mechanical damage to RGC at the optic nerve head. Damage of RGC enhances the secretion of matrix metalloproteinase (MMP) that cause changes extracellular matrix and apoptosis of RGC (Agarwal *et al.*, 2009). High IOP is also believed to activate glial cells such as microglial and astrocytes. Glial cells then release tumour necrosis factor alpha (TNF-α) that further damages the RGC accelerating the apoptosis of RGC. The molecular mechanism causing apoptosis of RGC has been uncertain. Neurotransmitter such as glutamate is also activated by IOP elevation. Glutamate has been act as a neurotoxin which exerts its toxic effect on RGC predominantly through the N-methyl-D-asparate (NMDA) subtype of glutamate receptor (Sucher *et al.*, 1997). Elevation of IOP is believed to initiate cascade of events that accelerate apoptosis of RGC.

It is known that growth factors play an important role in the regulation of cellular function, cytoskeletal organization and components of extracellular matrix (ECM). Growth factor such as neurotrophine factor and transforming growth factor (TGF-B2) are found in astrocytes of optic nerve head, lamina cribosa and trabecular meshwork. Thus, elevated IOP reduce retrograde axoplasmic flow can stress the RGC. Deprivation of neurotrophic factor such as brain-deprived neurotrophic factor to supply retinal ganglion cells may further leads to

progression of apoptosis. Oxidative stress will induce apoptosis by stimuli the production of TNF-α (Kwon *et al.*, 2009).

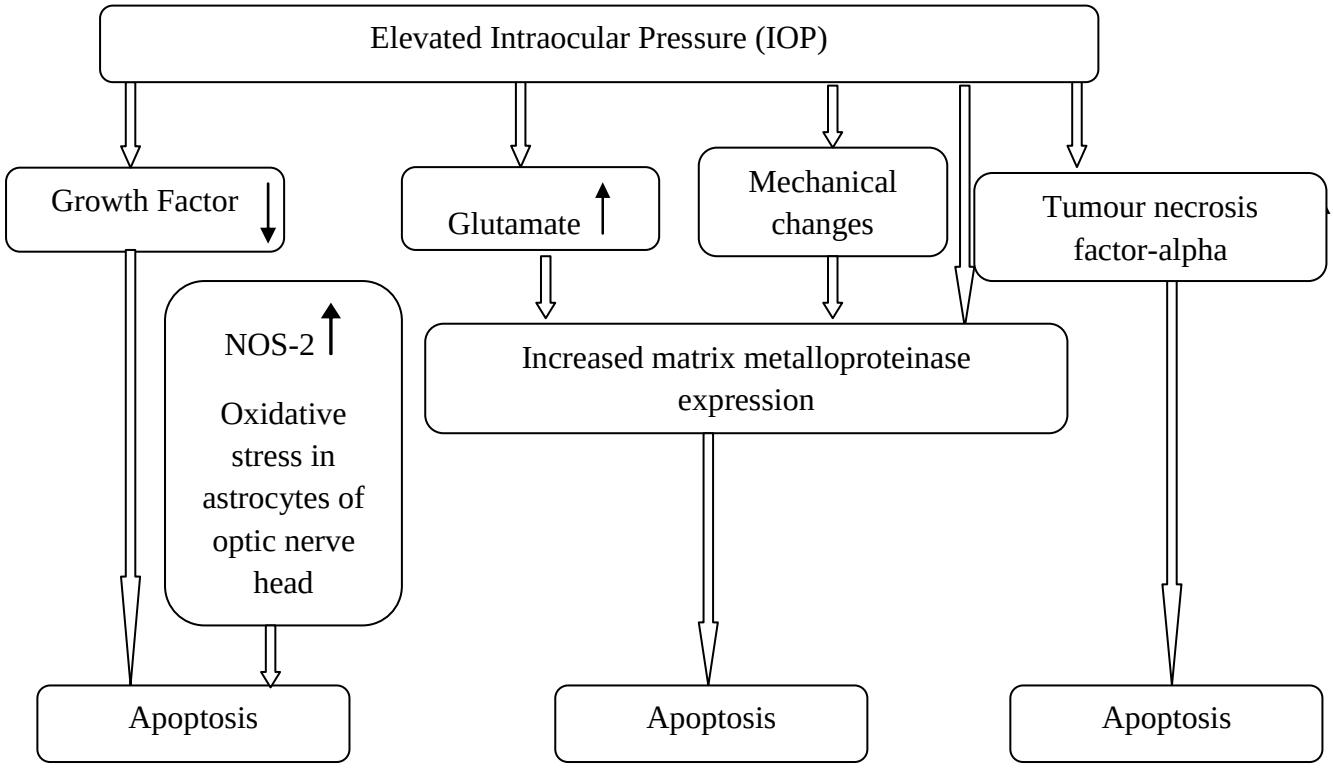

Figure 1.1: Factors causing retinal ganglion cells apoptosis in glaucoma (Agarwal *et al.*, 2009)

1.1.3.1 Pressure –independent mechanism: Vascular Cause

Imbalance or inadequate ocular perfusion to the optic nerve head is also believed responsible for glaucomatous optic nerve damage. Elevated IOP and vascular dysfunction is believed to cause impairment of optic nerve head (ONH) perfusion such as autoregulation deficit or vasospasm. In addition, fluctuating blood pressure, a resultant decrease in ocular perfusion pressure and a rise in local tissue metabolic demand will impair blood supply or nutrient supply or to ONH. Therefore failure of stable flow regulation resulting increase sensitivity of Endothelin-1 (ET-1) mediated vasoconstriction (Moore *et al.*, 2008). At a lower homestatic state, there will be a tendency for some of glutamate leak out from RGC or astroglial cells into extracellular space. Over the time, accumulation of extracellular glutamate becomes toxic to RGC directly and leading to RGC apoptosis (Osborne *et al.*, 1999). In addition, surrounding cells such as amacrine cells or muller glia bear the brunt of accumulation of glutamate, leading to RGC death as secondary effect. Whether glutamate is directly or indirectly toxic to RGC, blocking excessive glutamate activation remains an area for investigation (Pang *et al.*, 2005). Growth factor such as neurotrophine factor and transforming growth factor-β2 (TGF-β2) may play an important role by affecting the normal development and cellular functions in trabecular meshwork as well as retina (Agarwal *et al*., 2009). Elevated IOP resulting in blocked of retrograde axoplasmic transport in retina. This will lead to reduce the supply of brain-derived neurotrophine factor (BDNF) to RGC. BDNF is important for regulating RGC metabolism and cell survival. Deficiency of BDNF to RGC can further lead to progression of RGC apoptosis (Nickells, 1996). These effects seem to be further modulated by increased release of TGF-β2 b activated astrocytes in response of elevated IOP (Pena *et al.*, 1999). An up regulating of tumour necrosis factor-A (TNF-A) in the astrocytes was detected in glaucomatous optic nerve. Yuan and Neufeld (2000)

documented that TNF-α stimulation may contribute to neuronal damage by both a direct effect on the axons of the RGC and by inducing nitric oxide synthase (NOS)-2 in astrocytes.

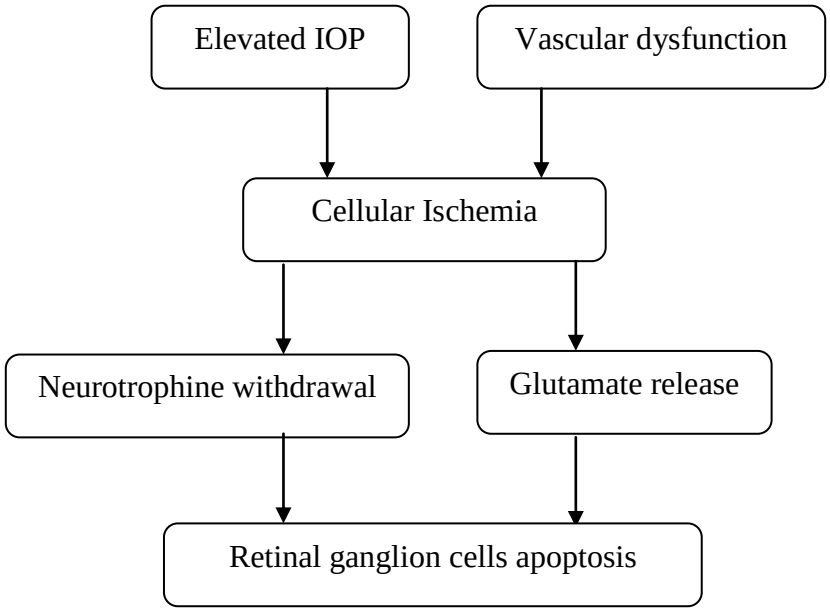

Figure 1.2: Potential stimuli leading to apoptotic retinal ganglion cells death (adapted from (Chung *et al.*, 1999).

In addition, age related changes to the vessel especially arteriosclerosis may also alter perfusion to RNFL and ONH (Harris *et al.*, 2000). Perhaps the ageing process and derangement of ocular blood flow may responsible for pathogenesis of glaucoma (Chung *et al.*, 1999; Flammer *et al.*, 1999; Hayreh, 2001).

1.1.3.3 Genetic Mutation

Family history of glaucoma has been identified as an important risk factor in development of POAG (Leske *et al.*, 2007). Genetic analyses has been identified three potential genes; myocillin(MYOC)/TIGR (GLC1A), optineurin (OPTN) (GLC1E) and WDR 36 (GLC1G) (Wiggs, 2007).

MYOC gene is expressed almost every ocular tissue of human such as cornea, iris, sclera, trabecular meshwork, ciliary body and sclera spur. However, MYOC gene is found abundant in trabecular meshwork, ciliary body and sclera in normal human eye (Karali *et al.*, 2000). Mutations of the MYOC gene play a critical role in manifestation of glaucoma. Misfolded mutant mycolin secretion forms secretion-incompetent aggregates. The block of mycolin secretion was proposed to alter the extracellular matrix environment of trabecular meshwork. Subsequently, resistant of aqueous humor outflow is increased and leading elevation of IOP (Challa, 2008; Kwon *et al.*, 2009). However, the molecular pathogenesis of mycolin-caused glaucoma is still poorly defined. There were studies reported that heteromeric mycolin were retained in the rough endoplasmic reticulum and aggregating to form inclusion bodies of Russell bodies. The presence of mycolin aggregates activate capases 12 and 3 and expression of C/EBP homologous protein (CHOP)/GADD 153 resulting apoptosis of trabecular meshwork cells. As a consequent, phagocytotic capacity of trabecular meshwork cells will be insufficiency for effective cleaning aqueous humor and leading elevation of IOP in POAG (Yam *et al.*, 2007).

Optineurin (OPTN) is located at GLC1E locus, chromosome 10p14 (Rezaie *et al.*, 2002). It is commonly found at trabecular meshwork, non pigmented ciliary epithelium and retina. The process of pathogenesis of glaucoma is still unknown (Leung *et al.*, 2003; Willoughby *et al.*,

2004). OPTN may play a neuroprotective role by reducing retinal ganglion cells susceptibility to apoptosis in response to trabecular stress or oxidative stress (Challa, 2008; Fingert, 2011; Kwon *et al.*, 2009). In the other hand, OPTN has been postulated in playing a role in TNF-α signalling pathway. Mutation of OPTN will increase the level of TNF-α in POAG. Subsequently, TNF-α will induce retinal ganglion cells damage and apoptosis. WDR 36 is located in GLG1G locus, chromosome 15q22.1. It encodes a protein which may involve in T cells activation and proliferation (Kwon *et al*., 2009). In addition, it seems to be associated with severity of glaucoma. The exact role of WDR 36 in pathogenesis need to be further studied (Challa, 2008). Chi *et al.* (2010) postulated that WDR 36 play an important role in haemostasis retinal ganglion cells in animal study. Mutations of WDR 36 altered axon growth of RGCs, lost of RGCs and leading RGC death in mice. Apoptotic cell deaths are one of the hallmarks of glaucoma pathogenesis. However, despite of many replicative studies, only 4% of POAG patients reported mutation of MYOC gene (Fingert *et al.*, 1999).

1.1.3.4 Oxidative Stress

The endogenous production of free radicals and neutralization by antioxidant defense mechanism is in a state of equilibrium under normal physiological condition (Uttara *et al.*, 2009). Oxidative stress is a condition whereby the production of radicals exceeded the neutralizing capacity (Wochenschrift, 1991). Cells damage can occur when defense mechanisms are inadequate. Oxidative damage causes loss of normal structural and functional integrity of cells (Pamplona, 2008).

Nitric oxide (NO) has been found to play a role in the regulation of IOP, ocular blood flow and apoptosis retinal ganglion cells (Aslan *et al.*, 2008; Haefliger *et al.*, 1999). NO is an enzymes that was found in cornea, conjunctiva, lens, trabecular meshwork, ciliary muscle and retinal (Becquet *et al.*, 1997). The presence of NO in aqueous humour was found to lead to formation of a powerful oxidant peroxynitrite which increase the aqueous outflow resistance (Liton and Gonzalez, 2008). It was found that NO in aqueous humour is higher in POAG compared to those without glaucoma (Chang *et al.*, 2000). However, there was no difference of NO levels in plasma between glaucoma and cataract patients (Altintas *et al.*, 2005; Chang *et al.*, 2000). This further illustrates that serum level is non-specific compared to aqueous or any ocular tissue.

A series of studies found that impairment in antioxidant defense mechanism is an important contribution for pathogenesis of POAG (Bagnis *et al.*, 2012; Ferreira *et al.*, 2004; Ferreira *et al.*, 2009; Zanon-Moreno *et al.*, 2009). Ferraira *et al* (2009) reported that significant increase of 67% in superoxide dismutase (SOD) activity was observed in aqueous humour of POAG and glaucoma associated with exfoliation syndrome (EFG) compared with cataract patients. Moreover, glutathione peroxidise (GPx) activity was found higher in aqueous humour in both

POAG and EFG as compared with cataract patients. Ascorbic acid in both POAG and EFG was significant lower than cataract patients. On the other hand, Leite *et al.* (2009) found ascorbic acid concentration in aqueous humour was two folds lower in comparison of POAG and cataract patients. It believed that oxidative stress plays a role in glaucoma pathogenesis, affecting on trabecular meshwork cells, ganglion cells on retinal and optic nerve head.

Oxidative stress triggers trabecular meshwork degeneration and alternation of aqueous pathway, which subsequently lead to elevation IOP (Sacc à*et al.*, 2005). Decline of trabecular meshwork cellularity and mitochondria function are linearity related to age. In normal condition, about 1-5% of oxygen consumed by mitochondria is converted to ROS including hydrogen peroxide (H_2O_2) and superoxide anions (Trounce *et al.*, 1989). Thus, production of ROS is increased by age. Oxidative stress reduces the effect on adhesion of trabecular meshwork cells to extracellular matrix protein results in rearrangement of cytoskeletal structure of trabecular meshwork. It may lead to disruption of trabecular meshwork cells and compromise the trabecular meshwork integrity. Subsequently, increase the resistance of aqueous humour outflow (Zhou *et al.*, 1999). Schachtschabel and Binninger (1993) reported that ascorbic acid stimulate hyaluronic acid synthesis in trabecular meshwork. The function of hyaluronic acid is to maintain the patency of aqueous humour outflow. Oxidative stress impairs the synthesis of hyaluronic acid, resulting down regulation of maxtrix metalloproteinases (MMP) in trabecular meshwork. Therefore, aqueous humour outflow was impaired due to accumulation of extracellular matrix (ECM) (Guo *et al.*, 2012). Oxidative DNA damage was found in the trabecular meshwork may lead to IOP elevation and visual field defect (Izzotti *et al.*, 2011). A recent report has demonstrated that lipid peroxidation, DNA damage and protein damage were demonstrated in patients with PACG (Chang *et al.*, 2011). The increase in 8-OH-dG levels in glaucoma patients reflected an oxidative stress-

dependent accumulation of DNA damage in the trabecular meshwork. DNA damage in trabecular meshwork cells is expected to evolve into degeneration of trabecular by inducing protein dysfunction and alter cellular response. Thus, decrease in the number of trabecular meshwork cells and increase the resistant of aqueous humour outflow.

Moreover, oxidative stress alters the equilibrium between NO and endothelins. In the presence of free radicals, NO reacts with anion superoxide to form peroxynitrite (ONOO-) which can lead to neurotoxicity of ONH or RGC (Lipton, 1999). Thus apoptosis of ONH and RGC occurred. In the other hand, oxidative stress can increase the synthesis of endothelin-1 in smooth muscle cells of vessel (Kahler *et al.*, 2001). Therefore, it may lead to vasoconstriction of vessels. Reduction of optic nerve blood flow can result in RGC death. In addition, Yorio *et al.* (2002) had found that the activation of endothelin results in changes in axonal transport of optic nerve head, proliferation of astrocytes in ONH, ischaemic and increased production of neurotoxin compounds. These may result in apoptosis of RGC and subsequently development of glaucoma.

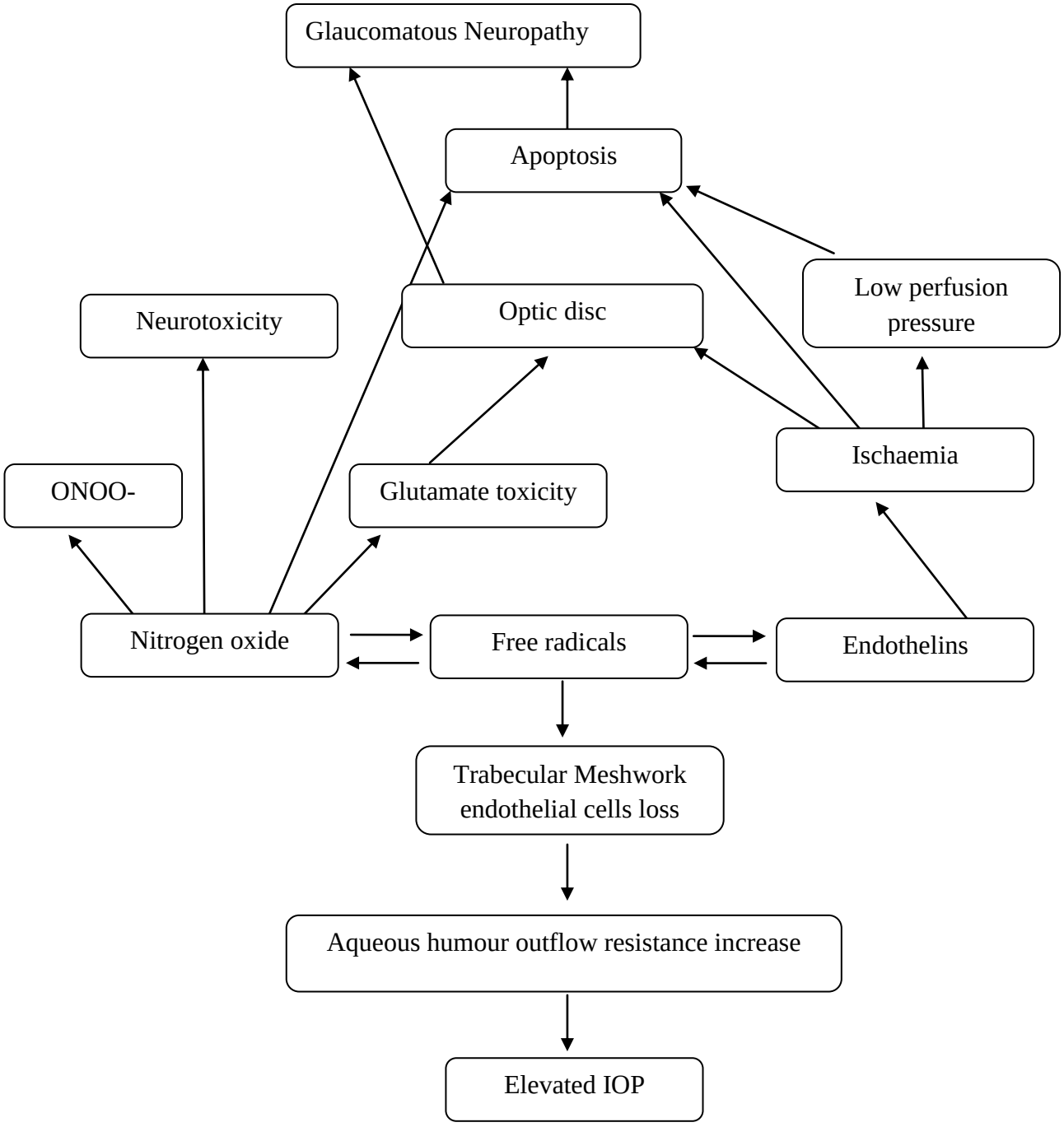

Figure 1.3: Elevation of IOP in glaucoma may probably related to oxidative- related related degenerative processes which affecting endothelial cells of trabecular meshwork. Vascular damage and neuronal cell death associated with glaucoma may relate to oxidative stress. (Adapted from Sacca et al., 2007).

1.2 Detection of Oxidative Stress in Ocular Tissue

Metabolism in the eye is of increasing interest because the organ is highly susceptible damage by sunlight, oxygen, various chemicals and pollutants. Oxidative stress mechanism in ocular tissues has been associated with a wide variety of disease such as ocular surface disease, cataract, glaucoma, uveitis, retinopathies and age related macular degeneration. Atalla *et al.* (1988) found that antioxidant (glutathione peroxidise) presence in ocular tissue predominantly in the cornea epithelium and endothelium, choroid, inner segment of photoreceptor and retinal pigmented epithelium. Besides, SOD also was found in ocular tissue which predominantly in the corneal epithelium, endothelium, non pigmented inner ciliary epithelium, apical regions of the posterior epithelium of iris, inner segments of photoreceptor of retina and retina pigment epithelium (Roo *et al.*, 1985). There was a study reported catalase activity was six-fold greater in retinal pigment epithelium (Liles *et al.*, 1991). Recent information is included about antioxidants not previously known to present in the eye, but it also present in the vitreous humour and tear fluid. They found that hydrogen peroxide and ascorbic acid is normally present in aqueous humour (Rose *et al.*, 2014). Ascorbic acid is found to be a primary antioxidant in ocular protection because of it high concentration in the eye. The function of ascorbic acid in eye is to protect membrane lipids from peroxidation(May, 1999). It also acts as a filter-like function against UV radiation in corneal epithelium and aqueous humour from radiation damage. Concentration of ascorbic acid in cornea is almost same as concentration in aqueous humour (Ringvold *et al.*, 2000). It also presents at high concentration in tear film (Dreyer and Rose, 1993).

1.2.1 Tears Film

Ocular surface of eye is as an inert barrier against infection. Tear film is a complex mixture consisting of protein, enzymes, lipids, electrolytes and metabolites. Oxygen radical formation has been measured before in human tears by using reagent of xanthine, xanthine oxidase, human milk lactoferrin, bovin serum albumin, SOD and catalase (Kuizenga *et al.*, 1987). Kuizenga *et al.* (1987) did not detect scanvergers of superoxide (O^{2-}) and H_2O_2 in human tears. They postulated that human tears played an important role in protecting the outer parts of the eye against the OH^- induce tissue damage. Besides, Frei (1991) proved that other type of antioxidants such as cysteine, glutathione, urate and ascorbic acid presence in human tears (Frei, 1991). However, ascorbic acid was proved to be a good antioxidant by completely against peroxidative damage by lipid peroxidation (Choy *et al.*, 2011). The ascorbic acid concentration and total antioxidant capacity (FRAP) were 3-4 folds higher in plasma than tears (Choy *et al.*, 2003). There were limited literature reviews on the normal level of antioxidants in aqueous humour and tears.

1.2.2 Schirmer's Test

Schirmer's test is an assessment of tear production. There was a cross-sectional study done on 383 adult patients seeking for primary health care to evaluate whether tears could be used as tool for health screening. Tear collection was done using schirmer strips and pain score of the schirmer tear collection was elicited as well as pain score of previous experience of antecubital venous puncture and finger prick test. They found that the pain score for schirmer tear collection was significant lower than antecubital venous puncture. In addition, 70% agreed for their tears being collected to screen for eye problems (Quah *et al.*, 2014) .Therefore, to collect the human tear by using schirmer strip is less invasive and less painful as compared to plasma collection.

Glass capillary tube is another method for tears collection. The capillary tube is rested in the lateral tears meniscus and minimizes contact with bulbar conjunctiva (Lam *et al.*, 2014).Tears are immediately collected by capillary tube. The average protein concentration obtained by the microcapillary tube showed the values was lower than the tears collection by schirmer strip (Farias *et al.*, 2013). However, Choy *et al* (2011) suggested that capillary tube for human tears collection was less invasive as compared with schirmer strip (Choy *et al.*, 2011) .Human tears are relatively easier to be collected simultaneously form eyes by using schimmer' strip and it's convenient as well.

1.3 Free Radicals

Free radical is any molecular species that contains of unpaired electron (Cheeseman and Slater, 1993).It can be divided into reactive oxygen species (ROS) and reactive nitrogen species. These radical can be generated by our body by various endogenous systems, physiological state or exposure to different physiochemical condition (Fialkowa *et al.*, 2007; Lobo *et al.*, 2010). Many radicals are unstable and highly reactive (Lobo *et al.*, 2010). They can either donate an electron or accept electron from other. Free radicals are able to cause damage to biological molecules in the nucleus and in the membranes of cells. Free radicals damage to base form of DNA yielding products such as 8-OH-dG, thymine glycol, damage to deoxyribose sugar and DNA protein cross-links. This damage can result in mutations that are heritable change in DNA.

Polyunsaturated fatty acids (PUFA) that contain 2 or more double bonds are particularly susceptible to oxidation by free radicals and other highly reactive species (Yin *et al.*, 2011). In brief, an allylic hydrogen is abstracted by a reactive species, such as the hydroxyl radical (HO•), resulting in the formation of lipid peroxyl radicals (LOO•). This radical can then react with a second PUFA, forming a lipid hydoperoxide (LOOH) and a second LOO•, resulting in the propagation of the lipid oxidation (Esterbauer *et al.*, 1991). Alternatively, LOO• can attack an intramolecular double bond and form a cyclic endoperoxide which decomposes to malondialdehyde (Yin *et al.*, 2011). Malondialdehyde (MDA) is one of many low molecular weight end-products of lipid hydoperoxide decomposition and is the most often measured as an index of lipid peroxidation. An increase of free radicals results in over production of MDA.

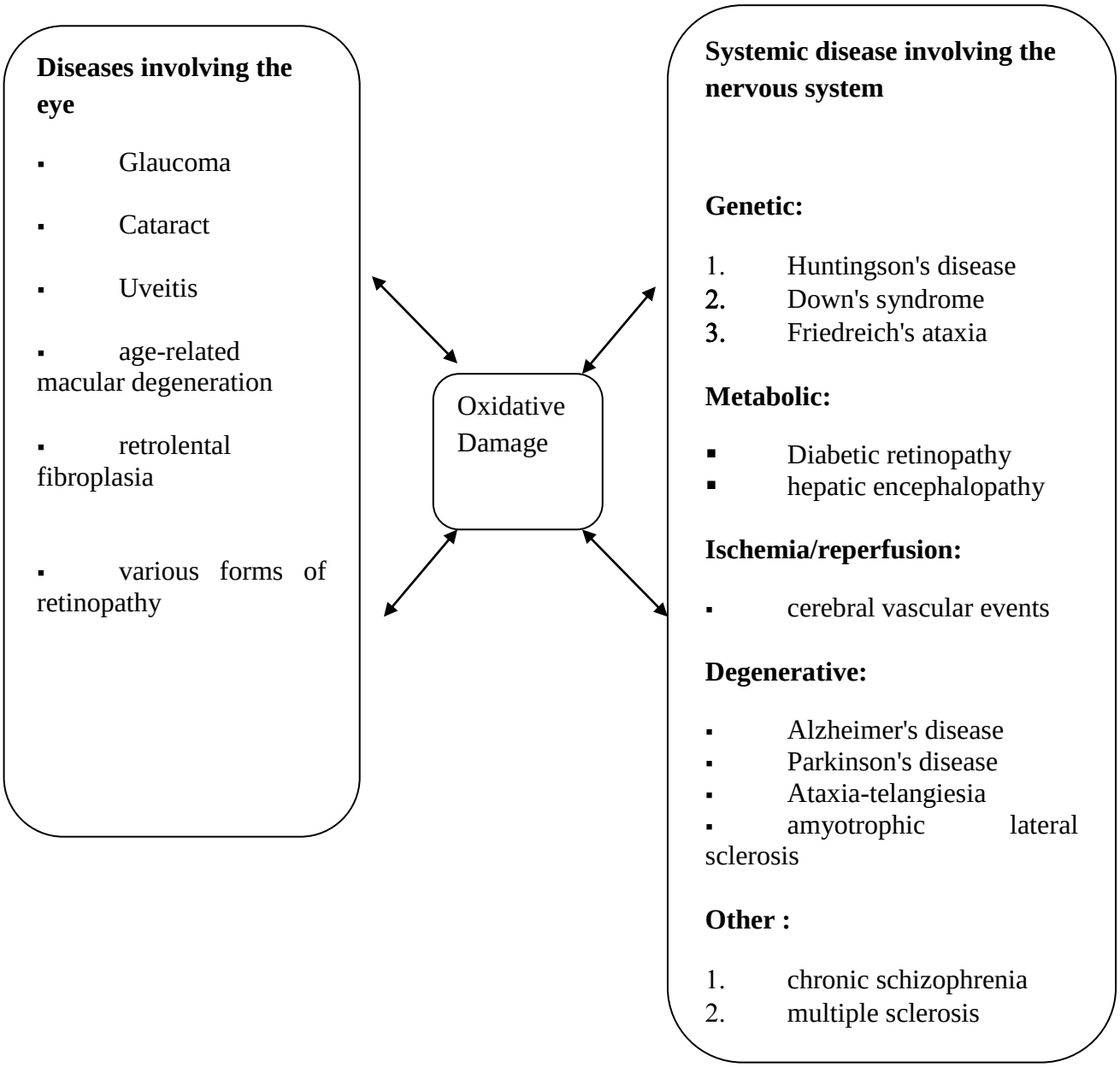

Figure 1.4: Shows that ocular and systemic disease due to oxidative damage. (Adapted from Sacca et al., 2007).

1.4 Antioxidants

Antioxidant defence mechanisms identified in ocular tissue includes enzymatic antioxidant such as superoxide dismutase, catalase, glutathione peroxidise and non-enzymatic antioxidant such as glutathione, heat shock protein, transferring, ascorbic acid and uric acid (Groussard *et al.*, 2003; Mozaffarieh *et al.*, 2008). It is a molecule stable enough to donate an electron to free radicals and neutralize it (Lobo *et al.*, 2010) Antioxidants inhibit cellular or tissue damage by preventing the formation of radicals, scavenging them. Antioxidants are identified as part of protective mechanism for lens and retina against the damaging effects of ultraviolet radiation, smoking, pollutions or irradiation (Kovacic and Somanathan, 2008).

Superoxide dismutases (SOD) are the enzymes that catalyse the dismutation of superoxide into oxygen (O2) and hydrogen peroxide (H2O) (Fridovich, 1995). There are three major families of superoxide dismutase: Cu/Zn (which binds both copper and zinc), Fe/Mn (which bind either iron or manganese) and Ni type which binds to nickel. There are three forms of superoxide dismutase present in human. SOD1 is located in the cytoplasm, SOD2 is in the mitochondria and SOD3 is found in extracellular (Fukai and Ushio-Fukai, 2011).

Catalase is a ubiquitous antioxidant enzyme found in nearly all living organism (Chelikani *et al.*, 2005). It catalyses the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen.

$$2\ H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2\ H_2O$$

Hydrogen peroxide is harmful to human tissues and cause tissue damage. Therefore, it must be converted quickly to prevent tissues damage. By preventing excessive H_2O_2 build up,

catalase is frequently used by cells. Subsequently, H_2O_2 is decomposed to oxygen and water by catalase to prevent tissue damage.

1.5 Rationale of the study

Oxidative stress has been postulated to play a role in the pathogenesis of glaucoma. Previous studies evaluating the antioxidant levels or oxidative stress in the serum of glaucoma patients have reported conflicting results (Altintas *et al.*, 2005; Chang *et al.*, 2000; Koliakos *et al.*, 2008; Majsterek *et al.*, 2011; Yildirim *et al.*, 2005; Yuki *et al.*, 2010). This may be due to the fact that serum levels of oxidative stress may be affected by the presence of systemic illness (Keaney *et al.*, 2003). As glaucoma is a disease of longevity and systemic diseases are common among the elderly (Ram *et al.*, 1994; Salim and Shields, 2009), the co-existence of glaucoma and systemic diseases is almost inevitable, potentially affect the level of oxidative stress.

Quantification of oxidative stress level in the ocular tissue, i.e. the aqueous, may be more representative of glaucomatous damage. This is because oxidative stress in the aqueous has been postulated to cause damage to the trabecular meshwork, resulting in increased resistance to aqueous outflow and thus increased intraocular pressure. However, measurement of aqueous antioxidant levels is relatively invasive, as it requires paracentesis, with all the antecedent risks of anterior segment surgery. Thus, aqueous collection is only ethically possible if performed during surgical intervention such as cataract and trabeculectomy surgery. Antioxidant levels in tears have been shown to reflect that of the aqueous fluid (Horwath-Winter *et al.*, 2009, Behndiq., 1998). Detection of antioxidant levels in tears may thus be a less invasive, safe and reliable method of assessing the oxidative stress level.

Previous studies have assessed the concentrations of antioxidants such as ascorbic acid, hydrogen peroxidase or ferric reducing ability of plasma (FRAP) in tears of normal patients, but there have been no previous studies on oxidative stress levels in the tears of glaucoma patients. Among glaucoma patients, the levels of SOD, catalase and MDA have been