`A COMPARISON STUDY ON AQUEOUS HUMOR TRANSFORMING GROWTH FACTOR-BETA (TGF-β) AND VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) LEVEL IN PRIMARY GLAUCOMA PATIENTS AND CONTROLS

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DISCLAIMER

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

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ABSTRAK

PENGENALAN

Glaukoma boleh dikelaskan kepada dua kumpulan utama mengikut struktur sudut; glaukoma sudut terbuka dan glaukoma sudut tertutup. Seterusnya, glaukoma sudut terbuka dan tertutup ini boleh dispesifikasikan lagi sama ada jenis primer atau sekunder. Glaukoma sudut terbuka primer merupakan jenis glaukoma yang paling lazim. Glaukoma sudut tertutup primer menunjukkan prevalens yang agak tinggi di Asia Tenggara dan boleh menyebabkan kebutaan.

Faktor pertumbuhan transformasi beta (TGF- β) mengawal penghasilan dan pembezaan sel, perkembangan embrio, penyembuhan luka, dan angiogenesis. Faktor pertumbuhan vascular endotelial (VEGF) adalah peptida perangsang yang dihasilkan oleh sel-sel untuk merangsang vaskulogenesis dan angiogenesis. Dalam proses penyembuhan luka, terdapat interaksi kompleks antara mediator-mediator yang terdiri daripada faktor pertumbuhan, sitokin dan kemokin. Pada masa ini, banyak kajian dijalankan untuk mengkaji penglibatan faktor-faktor pertumbuhan TGF- β dan VEGF dalam proses penyembuhan luka selepas pembedahan glaukoma dan juga sebagai faktor dalam patogenesis glaukoma primer.

OBJEKTIF

Kajian ini adalah bagi membandingkan tahap faktor-faktor pertumbuhan TGF-β dan VEGF dalam cecair akueus pesakit glaukoma primer dan kawalan.

KAEDAH KAJIAN

Satu kajian rentas telah dijalankan di antara November 2013 sehingga Februari 2017 di Hospital Universiti Sains Malaysia. Secara keseluruhan, 63 mata daripada 63 pesakit yang menjalani pembedahan katarak atau pembedahan primer trabekulektomi dimasukkan ke dalam kajian ini.

Pengambilan pesakit dibahagikan kepada dua kumpulan iaitu glaukoma primer (POAG dan PACG) dan kawalan. Sampel cecair akueus secara prospektif dikumpulkan pada awal pembedahan daripada 32 mata yang terdiri daripada kumpulan glaukoma primer (16 POAG dan 16 PACG) dan juga dari 31 mata kawalan yang menjalani pembedahan katarak dan trabekulektomi primer. Kepekatan tahap TGF-β dan VEGF diukur dengan menggunakan ujian esei imunoserapan berkaitan enzim (ELISA).

KEPUTUSAN

Kepekatan median TGF-β dalam cecair akueus mata dengan POAG adalah 2432.50 ± 3233.57 pg / ml, 1787.40 ± 1650.37 pg / ml dalam PACG dan 1571.40 ± 409.20 pg/ml dalam kawalan. Tahap median TGF-β dapat diperhatikan jauh lebih tinggi dalam kedua-dua kumpulan glaukoma primer (POAG dan PACG) berbanding dengan kawalan (POAG, $p = 0.004$ dan PACG, $p = 0.015$). Tetapi tidak terdapat perbezaan yang signifikan di antara pesakit POAG dan pesakit PACG ($p = 0.300$).

Tidak terdapat perbezaan yang signifikan dalam tahap median VEGF di antara kedua-dua kumpulan glaukoma primer berbanding dengan kumpulan kawalan ($p = 0.085$), atau pesakit POAG dan pesakit PACG ($p = 0.043$). Kepekatan median VEGF dalam cecair akueus mata POAG adalah 518.58 ± 542.61 pg / ml, 307.69 ± 194.39 pg / ml dalam PACG dan 330.20 ± 202.40 pg / ml dalam kawalan.

KESIMPULAN

Tahap TGF-β dalam cecair akueus adalah jauh lebih tinggi di dalam mata glaukoma primer berbanding dengan kawalan. TGF-β dalam cecair akueus berkemungkinan tinggi memainkan peranan dalam patogenesis POAG dan PACG. Tahap VEGF dalam cecair akueus tidak berbeza dengan ketara antara kedua-dua mata dalam glaukoma primer berbanding dengan kawalan, atau antara mata dengan POAG dan PACG.

ABSTRACT

INTRODUCTION

Glaucoma can be classified into two main groups according to the angle structure; closed angle glaucoma and open angle glaucoma. Open and closed angle glaucoma can further be classified into primary or secondary glaucoma. Primary open angle glaucoma (POAG) is the most common type of glaucoma. Primary angle closure glaucoma (PACG) has been noted to have a relatively high prevalence in Southeast Asia, and to be a leading cause of blindness in this region.

Transforming growth factor (TGF- β) regulates the proliferation and differentiation of cells, embryonic development, wound healing, and angiogenesis. Vascular growth endothelial factor (VEGF) is a signaling peptide produced by cells to stimulate both vasculogenesis and angiogenesis. In the process of wound healing, there is a complex interplay of mediators comprised of growth factors, cytokines and chemokines. Understanding the role of growth factors such as TGF-β and VEGF in the ocular wound healing cascade may increase the success rate of glaucoma filtering surgery and knowledge on pathogenesis of primary glaucoma.

OBJECTIVE

To compare the transforming growth factor- beta (TGF-β) and vascular growth endothelial factor (VEGF) level in aqueous humor of primary glaucoma patients and controls.

METHODOLOGY

This prospective cross-sectional study was conducted between November 2013 and February 2017 at Hospital Universiti Sains Malaysia. In total, 63 eyes of 63 patients undergoing cataract surgery or primary augmented trabeculectomy surgery were included in this study.

Recruited subjects were classified into primary glaucoma (POAG and PACG) group and control group. Aqueous humor samples were prospectively collected at the beginning of surgery from 32 eyes with primary glaucoma (16 POAG and 16 PACG) and from 31 control eyes that underwent cataract operation and primary augmented trabeculectomy. The concentration of TGF-β and VEGF levels was measured by using enzyme-linked immunosorbent assay test (ELISA).

RESULTS

The median concentration (interquertile range (IQR) of TGF-β in the aqueous humor of eyes with POAG was 2432.50 ± 3233.57 pg/ml, 1787.40 ± 1650.37 pg/ml in PACG and 1571.40 ± 409.20 pg/ml in controls. Median levels of TGF-β were observed to be significantly higher in the POAG group and PACG group compared to controls ($p = 0.004$ and $p = 0.015$). However there was no significant difference between POAG and PACG patients ($p = 0.300$).

There was also no significant differences in median levels of VEGF between patients with primary glaucoma compared to controls ($p = 0.085$), or between POAG and PACG patients ($p = 0.043$). The median concentration of VEGF in the aqueous humor of eyes with POAG

was 518.58 ± 542.61 pg/ml, 307.69 ± 194.39 pg/ml in PACG and 330.20 ± 202.40 pg/ml in controls.

CONCLUSION

The aqueous humor TGF-β level was significantly higher in eyes with primary glaucoma compared to control. TGF-β in the aqueous humor may play a role in the pathogenesis of POAG and PACG. The aqueous humor VEGF level did not differ significantly between eyes with primary glaucoma compared to controls, and between eyes with POAG and PACG.

Chapter 1
Introduction

1.1 Primary glaucoma

Glaucoma is an optic neuropathy usually associated with typical visual field defects (Prum *et al.*, 2016). It may be classified into two main groups; closed angle glaucoma and open angle glaucoma. Open and closed angle glaucoma is further classified as primary or secondary glaucoma.

Primary open angle glaucoma (POAG) is defined as characteristic glaucomatous optic nerve damage in an open angle eye with no identifying pathology, with elevated intraocular pressure (Ophthalmology, February 2016). The pathogenesis of POAG includes microcirculatory deficiency at the optic nerve head, or extracellular matrix factors (ECM)(Kountouras *et al.*, 2004; Kwon *et al.*, 2009). These factors may play a combined role (Ray and Mookherjee, 2009).

Primary angle closure glaucoma (PACG) is characterized as chronic, progressive visual field loss and optic nerve cupping, often associated with an elevated IOP due to the presence of iridotrabecular contact (ITC) by gonioscopy, which can either be appositional or synechial, in the absence of underlying secondary ocular disease (European Glaucoma Society, 2014). The rise in IOP is due to poor aqueous outflow through the trabecular meshwork leading to a build-up in aqueous within the eye, hence increased in IOP (Niwas *et al.*, 2016).

1.2 Prevalence of Primary glaucoma

POAG and primary angle closure glaucoma (PACG) are the commonest type of glaucoma. WHO estimated the number of people worldwide affected by glaucoma to be about 20 million in 1993 (Thylefors and Negrel, 1994). Out of these, POAG is responsible for 3.0 million global blindness and PACG is responsible for 2.0 million blindness worldwide.

1.2.1 Prevalence of Primary Open Angle Glaucoma.

After cataracts, glaucoma is the second leading cause of blindness in the world (Kingman, 2004). Open-angle glaucoma is the most common type of glaucoma among populations of European or African descent, whereas angle-closure glaucoma is more common among populations of Asian descent (Kingman, 2004; Tham *et al.*, 2014). Worldwide in 2015, there were an estimated 57.5 million people with open-angle glaucoma, and this number is projected to increase to 65.5 million by 2020 (Kapetanakis *et al.*, 2016). It is estimated that there are 2.8 million people with open-angle glaucoma in the United States (US) (Quigley and Broman, 2006), and that the number will increase to 3.4 million in 2020 (Friedman *et al.*, 2004).

1.2.2 Prevalence of Primary Angle Closure Glaucoma

Angle-closure glaucoma is more prevalent in populations of Asian descent, whereas open-angle glaucoma is more common in populations of European or African descent (Tham *et al.*, 2014). Because PACG appears to be more visually damaging, the risk of blindness associated with PACG is higher than for POAG (Foster and Johnson, 2001). Foster and Johnson have suggested that PACG will probably account for more than 90% of the bilateral glaucoma blindness in China (Foster and Johnson, 2001). There is limited data on the incidence of new cases of glaucoma in Asia. In Singapore, the incidence of acute symptomatic angle closure was highest in Chinese people (12.2/100 000 per year), followed by Malays (6.0/100 000 per year) and Indians (6.3/100 000 per year) (Seah *et al.*, 1997; Wong *et al.*, 2000).

1.3 Pathogenesis of primary glaucoma

The development of glaucomatous optic neuropathy likely results from a variety of factors. Elevated lOP plays a major role in the development of glaucomatous optic neuropathy in most individuals and is considered the most significant risk factor. In general, two hypotheses have emerged to explain the development of glaucomatous optic neuropathy, the mechanical and ischemic theories (Flammer *et al.*, 2002; Flammer and Mozaffarieh, 2007). Both mechanisms might lead to a reduction in axoplasmic flow, interference with the delivery of nutrients or removal of metabolic products, deprivation of neuronal growth factors, oxidative injury and the initiation of immune-mediated damage. Active investigations continue to examine the potential role in glaucomatous optic neuropathy of processes such as excitotoxicity (Casson, 2006), apoptosis, neurotrophin deprivation, ischemia, and autoimmunity.

1.3.1 Mechanical theory

The mechanical theory stresses the importance of direct compression of the axonal fibers and support structures of the anterior optic nerve, with distortion of the lamina cribrosa plates and interruption of axoplasmic flow, resulting in the death of the retinal ganglion cells (Ernest, 1975; Flammer and Mozaffarieh, 2007).

1.3.2 Non mechanical theory

The ischemic theory focuses on the potential development of intraneural ischemia resulting from decreased optic nerve perfusion. This perfusion may result from the stress of lOP on the blood supply to the nerve or from processes intrinsic to the optic nerve. Disturbance of vascular autoregulation may contribute to decreased perfusion and thus to nerve damage(Flammer *et al.,* 2002). The optic nerve vessels normally increase or decrease their tone to maintain a constant blood flow independent of IOP and blood pressure variations (Flammer and Mozaffarieh, 2007). A disturbance in vascular autoregulation may result in decreased optic nerve blood flow from increased lOP. Alternatively, changes in systemic hemodynamics may result in perfusion deficits, even at normal lOP. Such hypothetical derangement could be related to abnormal vessels or to circulating vasoactive substances.

1.4 Treatment modalities of primary glaucoma

1.4.1 Medication

The goal of currently available glaucoma therapy is to preserve visual function by lowering IOP to a level that is likely to prevent further optic nerve damage. The treatment regimen

chosen should achieve this goal with the lowest risk, fewest adverse effects, and the least amount of disruption to the patient's life, taking into account the cost of treatment. Although the goal of treatment is to prevent vision loss, current treatments are aimed at lowering IOP and, in the short term, the efficacy of treatment is gauged according to the IOP level. The concept of target pressure was introduced because some patients require lower IOP levels than do others to stabilize their glaucoma.

In general, the patients who require lower target pressures are those with more advanced optic neuropathy, although patients who develop optic nerve damage in the presence of IOP levels that are never elevated also require low target levels. Despite the significance of the concept of target pressure, it is important not to set a rigid target pressure for the individual patient, as there is no evidence base for doing so. The range should be individualized for the patient, based on the following: IOP level at which damage is thought to have occurred, severity of the damage, life expectancy of the patient, and associated risk factors. The more advanced the disease on initial presentation, the lower the target pressure required for preventing further progression in the average patient. Once the optic nerve is damaged, it can incur additional damage more easily. Once the target pressure range has been determined, the clinician must decide how to achieve this goal, medically or surgically. Regardless of which is chosen, the anticipated benefits of any therapeutic regimen should justify the risks, and regimens associated with substantial adverse effects should be reserved for patients with a high probability of progressive vision loss.

1.4.2 Laser

Laser iridotomy is indicated for primary angle closure (PAC) and primary angle-closure glaucoma (PACG). Laser iridotomy involves the creation of a hole in the peripheral iris by laser (Ritch, 1983; Ritch *et al.*, 1989). The hole provides an alternative pathway for aqueous to flow from the posterior chamber into the anterior chamber, bypassing the pupil. Therefore, iridotomy eliminates pupillary block and prevents forward bowing of the iris as a result of the pressure difference between the two chambers. Iridotomy opens those areas of the angle not involved by PAS and prevents further synechial closure.

Argon laser trabeculoplasty (ALT) has been reported to be a reasonably successful treatment modality (Wishart *et al.*, 1987; Shirakashi *et al.*, 1989). Selective laser trabeculoplasty (SLT) delivers laser energy to pigmented cells in the trabecular meshwork avoiding thermal damage to adjacent cells. In a multicenter prospective study on PACG eyes with high IOP despite iridotomy but with at least 90° of gonioscopically visible pigmented trabecular meshwork, it has shown 20% or more IOP reduction in 54% of eyes at 6 months. The authors suggested selective laser trabeculoplasty to be a safe and effective method of reducing IOP in PACG in which there is a sufficient extent of visible trabecular meshwork (Ho *et al.*, 2009). If the pressure remains uncontrolled and glaucomatous damage develops, filtration surgery is indicated.

1.4.3 Surgery

Surgical treatment for glaucoma is usually undertaken when medical therapy is not appropriate, not tolerated, not effective, or not properly utilized by a particular patient and the glaucoma remains uncontrolled with either documented progressive damage or a very high risk of further damage. Surgery is usually the primary approach for both congenital glaucoma and pupillary block glaucoma. In patients with primary open-angle glaucoma (POAG), surgery has traditionally been considered when medical therapy has failed.

Early studies of trabeculectomy as initial therapy for glaucoma suggested that trabeculectomy might offer better lOP control, reduction in the number of patient visits to the doctor and possibly better visual field preservation. The results of the Collaborative Initial Glaucoma Treatment Study (CIGTS) confirmed that initial surgical therapy achieves better lOP control than does initial medical therapy (Musch *et al.*, 1999). However, this finding did not translate to better visual field stabilization in the average subject because those who received initial surgical treatment had a higher risk of cataract in the longer term. In both groups, there was a low incidence of visual field progression. Based on this study and current practice. most clinicians defer incisional surgery until after an attempt is made to treat with medical therapy (Musch *et al.*, 1999).

Trabeculectomy is effective for PACG (Sihota *et al.*, 2004; Tham *et al.*, 2006). Trabeculectomy has been shown to have an overall success rate of 68% in controlling IOP. However, compared to primary open-angle glaucoma (POAG), any aqueous-draining procedure in an eye with a shallow anterior chamber and a chronic closed angle poses the risk of further shallowing the anterior chamber or precipitating malignant glaucoma.

Trabeculectomy in PACG is associated with a higher risk of filtration failure, shallow anterior chamber, and malignant glaucoma/aqueous misdirection. As the incidence of PACG increases with age, many patients with PACG have co-existing cataract. Trabeculectomy increases the rate of cataract progression, and a significant proportion of patients will soon need cataract extraction after trabeculectomy.

1.5 Factors affecting the outcome of glaucoma filtering surgery

Successful glaucoma filtering surgery is characterized by the passage of aqueous humour from the anterior chamber to the subconjunctival space, which results in the formation of a filtering bleb. Aqueous in the subconjunctival space may then exit by multiple pathways. Bleb failure most often results from fibroblast proliferation and subconjunctival fibrosis. Factors associated with an increased risk of bleb failure include youth, aphakia, active anterior segment neovascularization, inflammation, previously failed glaucoma filtering surgery, and, possibly, race (Skuta and Parrish, 1987).

1.6 Pathophysiology of wound healing in trabeculectomy

Scarring at the tenon and episcleral level is the main cause of trabeculectomy failure (Skuta, 1987). Glaucoma surgery is different from other types of surgery which complete healing of tissue desirable outcome.

Healing of surgical skin wounds is often considered the prototype repair process that involves both replacement and regeneration (Figure 1.1) (Lama and Fechtner, 2003). During

trabeculectomy, incision is making at conjunctiva and sclera. The initial processes involved in healing are inflammation and coagulation, leading to a cascade of biological events including cellular, hormonal, and growth factor release. These ultimately lead to repair and scar tissue formation. With injury to vascular tissue, leakage of plasma proteins and blood cells occurs. Clotting factor activation leads to conversion of fibrinogen to fibrin. Platelet aggregate on occurs at the site of endothelial cell damage, which exposes subepithelial collagen.

TGF-β release induces mesenchymal cell and fibroblast activation leading them to subsequently reenter the cell cycle, migrate and undergo transformation into myofibroblasts. These transformed cells elaborate a host of mediators which degrade the ECM and components that frequently fail to restore its original organization. ECM remodeling is attributable to excessive accumulation of matrix components consisting of an interlocking meshwork of collagen with other ECM components such as proteoglycans and glycosaminoglycans (GAGs), which are one of its side chain constituents. Characteristic of this remodeling process is tissue granulation accompanied by inflammatory cell influx, neovascularization and altered vascular permeability.

Subsequently, the differentiated myofibroblasts transform the secreted ECM into an actin-based component which creates stronger scar tissue (Desmouliere et al, 1995). Finally, the blood vessels retract over time and fibroblast largely dissapear as the the tissue is remodeled to form a dense collagenous subconjunctival scar.

Desjardins et al described three clinicohistopthologic phases of wound healing. In the first six days after surgery (early healing), fibroblasts had begun to proliferate along the walls of the opening. During the intermediate healing phase (days 7-9), continued proliferation and migration of fibroblasts, which were presumably derived from the adjacent episclera and subconjunctival tissue, were observed. In the late healing phase (days l0-14), were completely closed by granulation tissue.

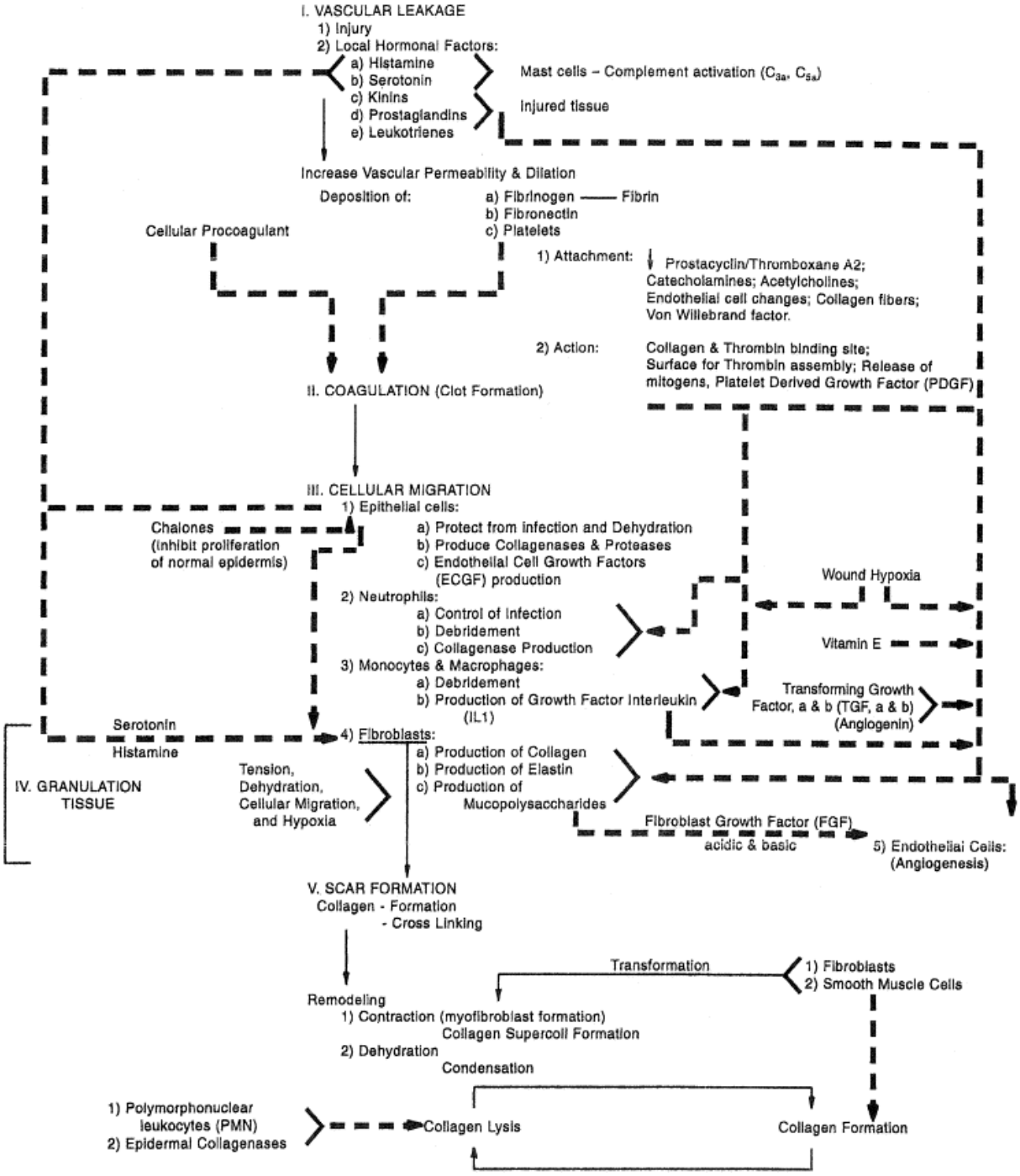

Figure 1.1: Flow chart of the wound healing pathway

1.7 Human aqueous humour

Human aqueous humour is a complex mixture of electrolytes, organic solutes, growth factors, cytokines, and additional proteins that provide the metabolic requirements to the avascular tissues of the anterior segment (Freddo *et al.*, 1990; Barsotti *et al.*, 1992; Freddo, 1993; To *et al.*, 2002). It fills both the anterior and the posterior chambers of the eye, which is located in the space between the lens and the retina, also known as the posterior cavity or vitreous chamber (Figure 1.2). It is produced from the nonpigmented ciliary body epithelium through active transport of ions and solutes and secreted into the posterior chamber (Murray and Bartels, 1993; To *et al.*, 2002; Fitt and Gonzalez, 2006). From the posterior chamber, aqueous flows between the lens and iris into the anterior chamber.

Human aqueous humour exits the anterior chamber via the trabecular meshwork or Schlemm's canal (conventional outflow pathway) and through the ciliary muscle bundles into the supraciliary and suprachoroidal spaces (uveoscleral pathway) (Figure 1.3). A balance between the production and the drainage of human aqueous humour is important for maintaining the normal physiological intraocular pressure that is essential to maintaining the optical and refractive properties of the eye (Mark, 2010).

The protein component of human aqueous humour is minimal, containing between 120 and 500 ng/μL of protein (Tripathi *et al.*, 1989). The proteins in human aqueous humour are thought to arise from plasma as the result of filtration through fenestrated capillaries of the ciliary body stroma via the iris root (Freddo, 1993). However, human aqueous humour is not a simple diffusate of plasma, since it has both qualitative and quantitative differences in protein and ion content in comparison with plasma (Kinsey, 1951; Tripathi *et al.*, 1989). Furthermore,

proteins in human aqueous humour that are secreted from the anterior segment tissues may have a significant role in the pathogenesisof various eye diseases (Klenkler and Sheardown, 2004).

A number of growth factors and their associated receptors, including epidermal growth factor, transforming growth factor-β, keratinocyte growth factor, hepatocyte growth factor, fibroblast growth factor and platelet-derived growth factor have been detected in the human aqueous humour (Klenkler and Sheardown, 2004). On binding to cellular receptors, these factors activate signalling cascades, which regulate functions including mitosis, differentiation, motility and apoptosis. Production of growth factors by corneal cells and their presence in the aqueous humour is essential for maintenance and renewal of normal tissue in the anterior eye and the prevention of undesirable immune or angiogenic reactions (Klenkler and Sheardown, 2004).

Growth factors also play a vital role in corneal wound healing, mediating the proliferation of epithelial and stromal tissue and affecting the remodelling of the extracellular matrix (ECM) (Klenkler and Sheardown, 2004). These functions depend on a complex interplay between growth factors of different types, the ECM, and regulatory mechanisms of the affected cells. Imbalances may lead to deficient wound healing and various ocular pathologies, including edema, neovascularization and glaucoma.

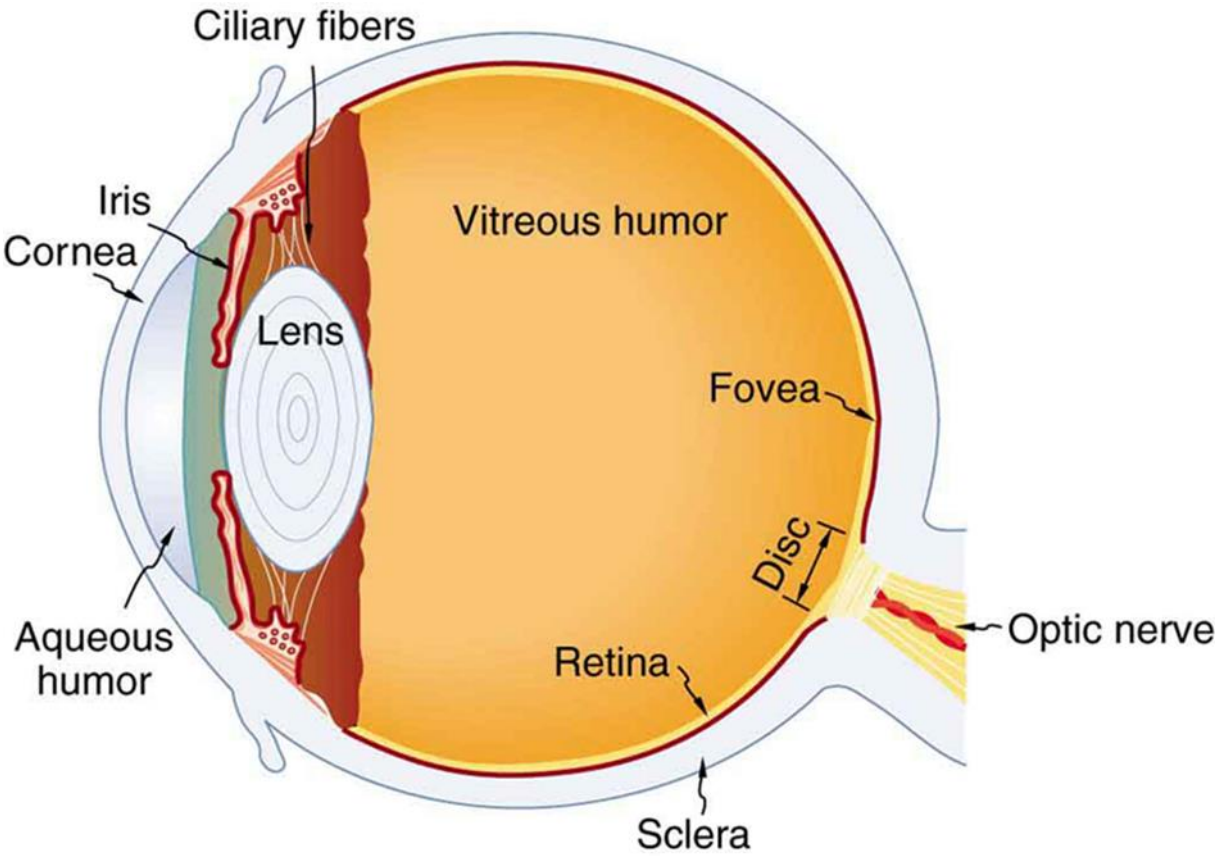

Figure 1.2 : Position of aqueous humour in eyeball

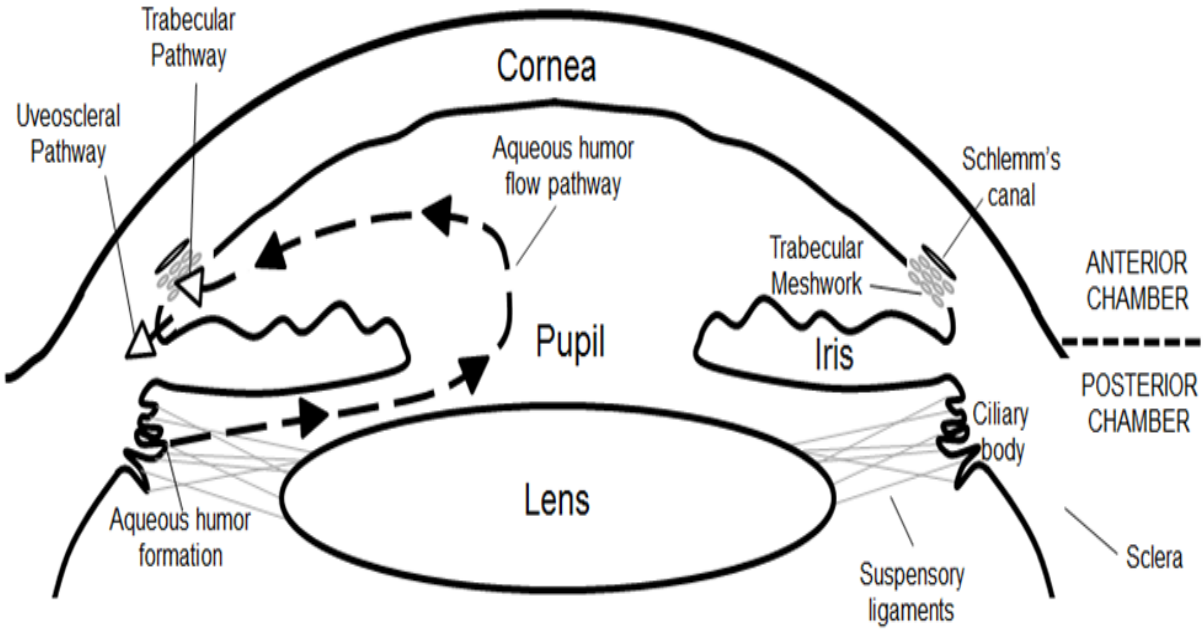

Figure 1.3 : Pathway of aqueous humour (please refer to the arrow)

1.8 Transforming growth factor-beta (TGF-β)

1.8.1 TGF-beta in human aqueous humour

Transforming growth factor-beta (TGF-β) is a member of a family of dimeric polypeptide growth factors. TGF- β regulates the proliferation and differentiation of cells, embryonic development, wound healing, and angiogenesis (Blobe *et al.*, 2000). There are three isoforms of TGF- β: TGF-β1, TGF- β2, and TGF- β3 that have been found in humans (Roberts and Sporn, 1988).

1.8.2 Factor affecting the TGF-beta in human aqueous humour

Several studies have demonstrated an elevation of TGF-β level in human aqueous humour (Jampel *et al.*, 1990; Tovar *et al.*, 2014). Common causes of elevated TGF- β level in human aqueous humour include primary glaucoma (Inatani *et al.*, 2001; Ochiai and Ochiai, 2002), diabetic eyes (Ochiai and Ochiai, 2002; Min *et al.*, 2006), secondary open angle glaucoma complicated with uveitis (Min *et al.*, 2006), high myopia (Zhu *et al.*, 2016) and pseudoexfoliation syndrome (Kara *et al.*, 2014).

1.8.3 TGF-beta level and primary glaucoma

TGF-β has been implicated in the pathogenesis of primary glaucoma (Tamm and Fuchshofer, 2007; Fuchshofer and Tamm, 2012). The role of TGF-β in the pathogenesis of POAG has been explained by its induction of extracellular matrix (ECM) expression in trabecular meshwork (TM) cells and optic nerve head astrocytes. Increased ECM in the TM causes increased resistance to aqueous humour outflow, causing higher intraocular pressure (IOP).

Concurrently, mechanical deformation of optic nerve head axons due to the increased ECM may impair axonal transport, which is worsened by the increased IOP. This raised IOP itself has been hypothesized to induce further expression of activated TGF-β in trabecular meshwork cells and optic nerve head astrocytes, thus propagating a vicious cycle (Tamm and Fuchshofer, 2007; Fuchshofer and Tamm, 2012).

1.9 Vascular endothelial growth factor (VEGF)

Growth factors are a broad category of endogenous molecules that promote cell proliferation or differentiation. These factors include vascular endothelial growth factor (VEGF), fibroblast growth factor and transcription factors such as hypoxia inducible factor (Unger *et al.*, 1994; Vincent *et al.*, 2000; Samani *et al.*, 2007). VEGF is a signaling peptide produced by cells to stimulate both vasculogenesis and angiogenesis.

1.9.1 VEGF in human aqueous humour

VEGF, which stimulates the growth of vascular endothelial cells and increases vascular permeability, is significantly increased in human eyes with glaucoma (Hu *et al.*, 2002). Increased levels of VEGF mRNA and protein are stimulated by hypoxia and ischemia (Aiello *et al.*, 1995; Kuroki *et al.*, 1996). The expression of VEGF is potentiated in response to hypoxia, by activated oncogenes, and by a variety of cytokines. VEGF induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis (Neufeld *et al.*, 1999).

1.9.2 Factor affecting the VEGF in human aqueous humour

Ocular VEGF levels are elevated in diseases such as rubeotic glaucoma (Sone *et al.*, 1996), uveitic eyes, acute primary angle-closure eyes (Huang *et al.*, 2016), type 2 diabetes, proliferative diabetic retinopathy , diabetic macular edema (Costagliola *et al.*, 2013) and choroidal neovascularisation (Tong *et al.*, 2006). Van Bergen et al showed VEGF can cause the scarring and failure of filtering surgery (Van Bergen *et al.*, 2011). Another study by Li et al showed that antiVEGF used in filtering surgery can reduce scarring (Li *et al.*, 2009) .

1.9.3 VEGF level and primary glaucoma

Growth factors identified in aqueous humour of eyes with primary glaucoma include VEGF (Hu *et al.*, 2002), transforming growth factor-beta (Inatani *et al.*, 2001), fibronectin (Babizhayev and Brodskaya, 1989), transferrin (Tripathi *et al.*, 1992) and CD44s (Knepper *et al.*, 2002).

1.10 Enzyme-linked immunosorbent assays (ELISAs)

1.10.1 Principle of ELISA

ELISA combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme. ELISA can provide a useful measurement of antigen or antibody concentration. The ELISA can be used to detect the presence of antigens that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen.

A general principle of ELISA is a five-step procedure (Figure 1.4 & Figure 1.5). An anti-human TGF-β or VEGF coating antibody is adsorbed onto microwells (coat the microtiter plate wells with antigen). A biotin-conjugated anti-human TGF-β or VEGF antibody is added and binds to human TGF-β or VEGF captured by the first antibody (block all unbound sites to prevent false positive results). Following incubation unbound biotin conjugated anti-human TGF-β or VEGF antibody is removed during a wash step (secondary antibody conjugated to an enzyme). Streptavidin- HRP is added and binds to the biotin conjugated anti-human TGF-β or VEGF antibody. Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells. A coloured product is formed in proportion to the amount of human TGF-β or VEGF present in the sample or standard (positive reaction). The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human TGF-β or VEGF standard dilutions and human TGF-β or VEGF sample concentration determined.

Compared to other immunoassay methods, there are many advantages of ELISA. ELISA tests are more accurate. They are considered highly sensitive, specific and compare favorably with other methods used to detect substances in the body, such as radioimmune assay tests (Mossmann *et al.*,1989). ELISA possesses the added advantages of not needing radioisotopes (radioactive substances) or a costly radiation counter (a radiation-counting apparatus).

The high sensitivity of ELISA, comes from the enzyme as a reporting group. As is known to all, the enzyme is an organic catalyst, a small amount of which could induce a large span of catalytic reactions to produce observable chromogenic reaction phenomenon. Therefore, this system is often taken as the amplification system of enzyme. By ELISA, a tracer of the

antigen or antibody is achieved in the cell or subcellular level, also, antigen or antibody quantification can be done in the microgram or even nanogram levels.

Specificity of ELISA is because of the selectivity of the antibody or antigen. Actually, the binding of antigen or antibody only occurs in the epitope of an antigen or antigen-binding site of an antibody. Since, there is a complementary relationship between epitope and antigen-binding site both in chemical structure and spatial configuration, the reaction between antigen and antibody shows a strong specifity. These advantages of ELISA make it an useful biotechnical tool with many appllicaitons, either in scientific research or clinical diagnosis of diseases or conditions.

A major limitation of testing aqueous humor is that only small sample volumes (typically 50 to 150 μL of fluid) can be obtained from human eyes; these amounts are barely sufficient to test a few cytokines using ELISA techniques. Multiplex bead immunoassays is another test which allow for simultaneous detection of multiple cytokines in small volume clinical samples (Takai *et al.*, 2012).

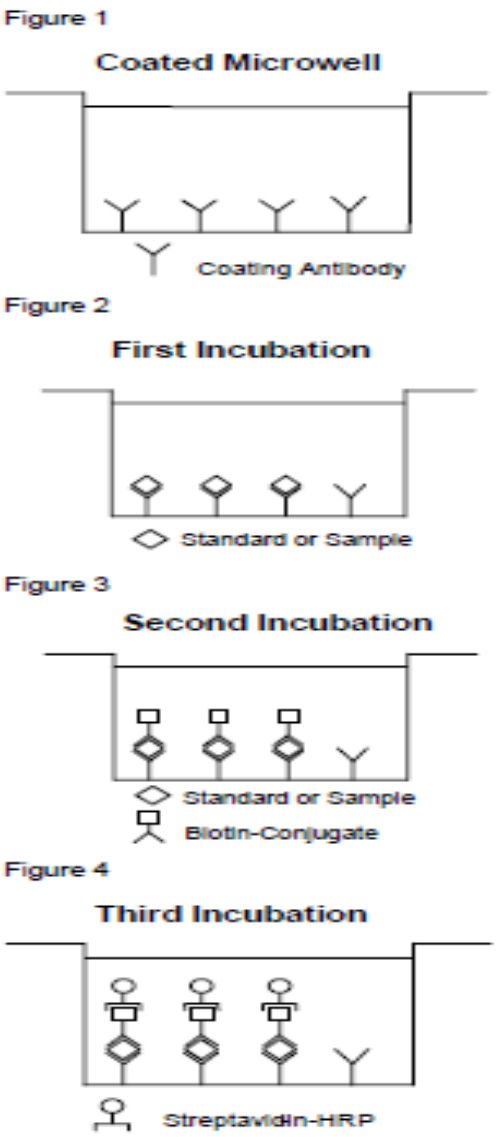

Figure 1.4 Principle of ELISA test for VEGF and TGF

Figure 5

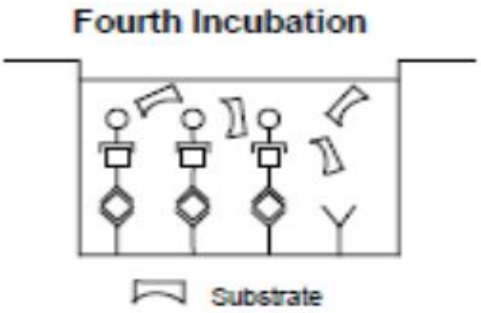

Figure 6

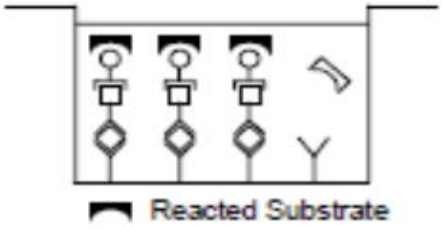

Figure 1.5 Principle of ELISA test for VEGF and TGF

1.11 Rationale of the study

TGF-β and VEGF are among the markers that being investigated as susceptibility markers and markers to determine the success of glaucoma surgery (Reichel *et al*., 1996, Cordeiro *et al.,* 2000, Park *et al*., 2013). In the process of wound healing, there is a complex interplay of mediators comprising of growth factors, cytokines and chemokines which also include TGF-β and VEGF. Based on the conjunctival impression cytology technique, TGF-β has been found to be significant higher in both POAG and PACG patients compared to controls in Asian patients (Ng *et al.,* 2015). Several studies have shown that the inhibition of VEGF results in reduction of scar formation at the trabeculectomy bleb and improves the success of glaucoma surgery (Li *et al*., 2009, Van *et al.,* 2011). TGF-β and VEGF is found in aqueous humour and may also contribute to both the pathogenesis of glaucoma and the outcome of glaucoma surgery particularly in our population. There were very limited published data on the level of TGF- β and VEGF in aqueous humour of glaucoma patients in Asian region. This study may improve our understanding of the pathogenesis of primary glaucoma and also factors contribute to the outcome of glaucoma filtering surgery in this region.

1.11 References

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