Evaluation of Vascular Endothelial Growth Factor Level in Tears and Aqueous Among Patients with Non-proliferative Diabetic Retinopathy

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DISSERTATION PROTOCOL

DISCLAIMER

I hereby clarify that the work in this dissertation is of my own except for quotations, some figures, and summaries which have been duly acknowledged. I declare that I have no financial of interest in the instruments and the computer software used in this study.

(Dr Azima Ahmad Shahrudin)

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Abstract

Introduction

Diabetic retinopathy is a reversible cause of blindness with early detection and treatment. Vascular endothelial growth factor (VEGF) plays an essential role in the pathogenesis of blindness-related diseases, such as diabetic retinopathy (DR). VEGF is largely produced intraocularly such as in vitreous and aqueous. As tears sampling is less invasive, evaluating the VEGF level in tears may provide an association of the disease process with ocular surface fluid.

Objectives

This study aims to evaluate the VEGF level in tears and aqueous among diabetes mellitus (DM) patients with non-proliferative diabetic retinopathy (NPDR) and their correlation.

Method

A cross-sectional study was conducted in Hospital Universiti Sains Malaysia and Hospital Raja Perempuan Zainab II from July 2019 until November 2021. Fifty-one diabetic patients with no diabetic retinopathy (DR), 45 patients with NPDR, and 54 non-DM patients were enrolled in this study. Type 2 DM patients and non-DM patients that were planned for cataract surgery and fulfilled the selection criteria were included in the study. Tears were collected using a Schirmer strip before the operation and aqueous were collected via cornea paracentesis during cataract surgery. The concentration of VEGF was determined using an ELISA kit test.

Results

The mean VEGF concentration in tears was 46.9 ± 18.7 pg/ml in DM patients with NPDR, 46.7 ± 23.3 pg/ml in patients with no DR, and 40.1 ± 20.6 pg/ml in non-DM. There was no significant difference in mean VEGF level in tears between the three groups before and after adjusting for covariates ($p = 0.180$ and $p = 0.155$ respectively). VEGF concentration in aqueous was 217.5 ± 89.2 pg/ml in patients with NPDR, 174.3 ± 75.1 pg/ml in DM patients with no DR group, and 140.7 ± 41.9 pg/ml in the non-DM group. There was a significant difference in mean VEGF level in aqueous between the three groups before and after adjusting for age, duration of DM, level of HbA1c, hypertension, dyslipidemia, and status of smoking ($p < 0.001$ and $p = 0.004$, respectively). Post hoc analysis showed VEGF level in NPDR was significantly higher than in no DR ($p = 0.012$) and non-DM ($p = 0.033$). There was a significant weak correlation of VEGF levels between tears and aqueous among diabetic patients ($r = 0.201$, $p = 0.049$).

Conclusions

This study demonstrated that aqueous levels of VEGF were significantly higher in DM patients with NPDR than in no DR and non-DM. The level of tears VEGF has little or no relationship to the level of aqueous VEGF. These findings suggest that aqueous VEGF may reflect the DR status.

Abstrak

Pengenalan

Retinopati diabetis adalah punca kebutaan yang boleh dirawat dengan pengesanan dan rawatan awal. Vascular endothelial growth factor (VEGF) memainkan peranan yang penting dalam mekanisma penyakit kebutaan seperti retinopati diabetis. VEGF banyak terhasil di dalam bola mata, iaitu di dalam vitres dan cecair akues. Memandangkan pengambilan sampel air mata adalah tidak invasif, menilai paras VEGF dalam air mata boleh mengenalpasti hubungkait proses penyakit dengan cecair di permukaan bola mata.

Objektif

Kajian ini bertujuan untuk menilai paras VEGF di dalam air mata dan cecair akues di kalangan pesakit retinopati diabetik non-proliferatif dan hubungan di antaranya.

Kaedah

Sebuah kajian keratan rentas telah dijalankan di dua buah hospital utama di Malaysia; Hospital Universiti Sains Malaysia dan Hospital Raja Perempuan Zainab II bermula Julai 2019 sehingga November 2021. Kajian ini melibatkan 51 pesakit diabetis mellitus (DM) yang tidak mempunyai retinopati diabetis, 45 pesakit DM dengan non-proliferatif retinopati diabetis, dan 54 pesakit yang bukan DM. Pesakit DM jenis dua dan pesakit bukan DM yang akan menjalani pembedahan katarak serta memenuhi kriteria kelayakan dipilih sebagai peserta kajian. Sampel air mata dikumpulkan melalui kertas Schirmer sebelum pembedahan dan cecair akues diambil melalui luka pembedahan ketika pembedahan katarak dilakukan. Nilai VEGF diukur dengan menggunakan ujian kit ELISA.

Keputusan

Nilai purata tahap VEGF di dalam air mata adalah 46.9 ± 18.7 pg/ml untuk pesakit DM dengan non-proliferatif retinopati diabetis, 46.7 ± 23.3 pg/ml di kalangan pesakit tanpa retinopati diabetis dan 40.1 ± 20.6 pg/ml untuk pesakit bukan DM. Tiada perbezaan signifikan terhadap nilai purata VEGF di antara ketiga-tiga kumpulan sebelum dan setelah diubahsuai berdasarkan kovariat seperti umur, tempoh penyakit diabetik, paras HbA1c, hipertensi, kolesterol dan status merokok (masing-masing $p = 0.180$ and $p = 0.155$). Nilai purata VEGF di dalam cecair akues ialah 217.5 ± 89.2 pg/ml untuk pesakit non-proliferatif retinopati diabetis, 174.3 ± 75.1 pg/ml untuk pesakit DM tanpa retinopati dan 140.7 ± 41.9 pg/ml untuk pesakit bukan DM. Terdapat perbezaaan signifikan terhadap nilai purata VEGF di dalam cecair akues di antara ketiga-tiga kumpulan sebelum dan setelah diubahsuai berdasarkan kovariat seperti umur, tempoh penyakit diabetik, paras HbA1c, hipertensi, kolesterol dan status merokok (masing-masing $p < 0.001$ and $p = 0.004$). Analisis Post Hoc menunjukkan nilai purata tahap VEGF dalam non-proliferatif retinopati diabetis adalah lebih tinggi signifikan berbanding dengan pesakit DM tanpa retinopati ($p = 0.012$) dan pesakit bukan DM ($p = 0.033$). Tahap VEGF di antara air mata dan cecair akues di kalangan pesakit diabetik mempunyai hubungan signifikan yang lemah ($r = 0.201$, $p = 0.049$).

Kesimpulan

Kajian ini menunjukkan tahap VEGF di dalam cecair akues di kalangan pesakit DM dengan non-proliferatif retinopati diabetis adalah lebih tinggi berbanding pesakit DM tanpa retinopati dan bukan DM. Nilai VEGF di dalam air mata mempunyai hubungan yang lemah dengan nilai VEGF di dalam cecair akues. Kajian ini mencadangkan nilai VEGF yang terhasil di dalam cecair akues menunjukkan aktiviti penyakit retinopati diabetis.

CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

1.1 Study Introduction

Diabetes mellitus (DM) is one of the non-communicable diseases that can cause premature death affecting the working-age population in Malaysia (NHMS, 2015). Diabetes has been developing at an alarming rate in Malaysia, with a relative increase of 15%. It was recorded at 15.2% in 2011 and increased to 17.5% in 2015 (NHMS, 2015). The amount is estimated to be increased to 366 million worldwide in 2030 (Wild S et al., 2004).

Diabetic retinopathy (DR) is a vision-threatening complication of DM. Proliferative diabetic retinopathy (PDR) and diabetic macular oedema is the most common cause of vision loss in the diabetic patient (Fong DS et al., 2004). Blindness leads to emotional distress and poor quality of life.

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that is produced by ocular tissues in response to retinal hypoxia. VEGF is produced by ganglion cells, endothelial cells, Muller cells, astrocytes, and retinal pigment epithelial cells. In DR, hyperglycemia-induced capillaropathy causes ischemia leading to upregulation of VEGF. VEGF causes blood-retinal barrier disruption at the level of vascular endothelium (Antonetti et al., 1999) will eventually lead to macular oedema. VEGF also causes increased proliferation of endothelial cells resulting in neovascularization. Thus, VEGF plays an essential role in the pathogenesis of DR.

Several studies had found increased intraocular VEGF levels in patients with diabetes (Aiello LP et al., 1994; Adamis et al.,1994). Adamis found that VEGF level in aqueous and vitreous in a patient with PDR was higher than a patient with non-proliferative diabetic retinopathy (NPDR), quiescent PDR or non-diabetic. However, the concentration of VEGF in the posterior

segment is greater (60%) than aqueous humour when collected simultaneously (Adamis et al.,1994).

Ang found that the VEGF level in tears was elevated significantly in patients with NPDR and PDR (114.9 ± 8.6 pg/mL and 149.5 ± 10.4 pg/mL, respectively) compared to patients with no DR (41.2 ± 11.3 pg/mL, p < 0.001) (Ang WJ et al., 2019).   However, in his study, the NPDR patients were not staged according to severity. He also found that there was a fair correlation between serum and tears VEGF among diabetic patients (p = 0.015, r = 0.263). (Ang WJ et al., 2019). Therefore, VEGF can be detected in tears in response to DR.

1.2 Study Rationale

This study is designed to evaluate the VEGF level in tears and aqueous among Type 2 diabetes mellitus (T2DM) patients with NPDR and to determine the correlation of VEGF levels between tears and aqueous. If the level of VEGF in tears and aqueous is comparable, VEGF level in tears can be used as a biomarker for the prediction of the severity of DR since VEGF level from an intraocular fluid such as aqueous and vitreous sampling is invasive and carries the risk of infection and other complications. Besides, the VEGF level in tears is useful in predicting the risk of DR in patients with poor fundus view due to media opacity.

Furthermore, the collection of intraocular fluid is ethically possible only if the patient is planned for any surgical intervention. Selected patients with high VEGF levels will be screened further for clinical assessment before treatment commencement. Therefore, early detection of the severity of DR by a non-invasive method will allow early treatment commencement after indicated in clinical assessment to prevent visual loss.

CHAPTER 2

LITERATURE REVIEW

2.0 LITERATURE REVIEW

2.1 Diabetic Retinopathy

DR is the most common microvascular complication of diabetes (Antonetti et al., 2012). DR contributed to 1% of the cause of blindness globally (WHO, 2010). In Malaysia, the prevalence of DR in T2DM was 35% and among the highest after India (42%) in South East Asia (Yang et al., 2019).

2.2 Pathogenesis of Diabetic Retinopathy

The pathogenesis of DR is complex and involved multifactorial pathophysiology. Long-standing hyperglycemia in retinal vasculature may cause inflammation, neuronal dysfunction, oxidative stress, and accumulation of advanced glycation end-products (AGEs). These will result in retinal vascular dysfunction or microangiopathy leading to microvascular leakage and microvascular occlusion.

AGEs caused vascular dysfunction, loss of retinal pericytes and increase vascular permeability in retinal endothelial cells (Shin ES et al., 2016). AGEs caused the breakdown of the blood-retinal barrier by enhancing the translocation of protein kinase C-δ (PKC-δ). AGEs also modify the basement membrane of capillary causing thickening of the basement membrane and leading to microvascular occlusion.

Oxidative stress results in the excess formation of reactive oxygen species (ROS) which is related to vascular dysfunction, including loss of pericytes, formation of acellular capillaries, vascular leakage and thickening of basement membrane (Zheng et al., 2009). ROS is generated by photoreceptors cells in the diabetic retina which was found to be an early source of oxidative stress and local inflammation.

Several pro-inflammatory mediators are known to cause DR such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (cox-2), lipoxygenases (LOs), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and VEGF (Semeraro F et al., 2015).

In DR, microangiopathy and capillary occlusion results in retinal nonperfusion. This hypoxic state will enhance the secretion of VEGF leading to the breakdown of the blood-retinal barrier and the formation of retinal neovascularization (Figure 2.1). VEGF is believed to increase vascular permeability by inducing phosphorylation of tight junction proteins (Zheng et al., 2009). Microvascular leakage is clinically characterized by microaneurysms, retinal haemorrhages, hard exudates, and retinal edema. VEGF is a potent angiogenic factor. The angiogenic role in the retina to which VEGF has been linked is thought to be due to an interaction with angiotensin II (Tarr JM et al., 2013). Microvascular occlusion is clinically characterized by cotton wool spots, arteriovenous shunt and neovascularization.
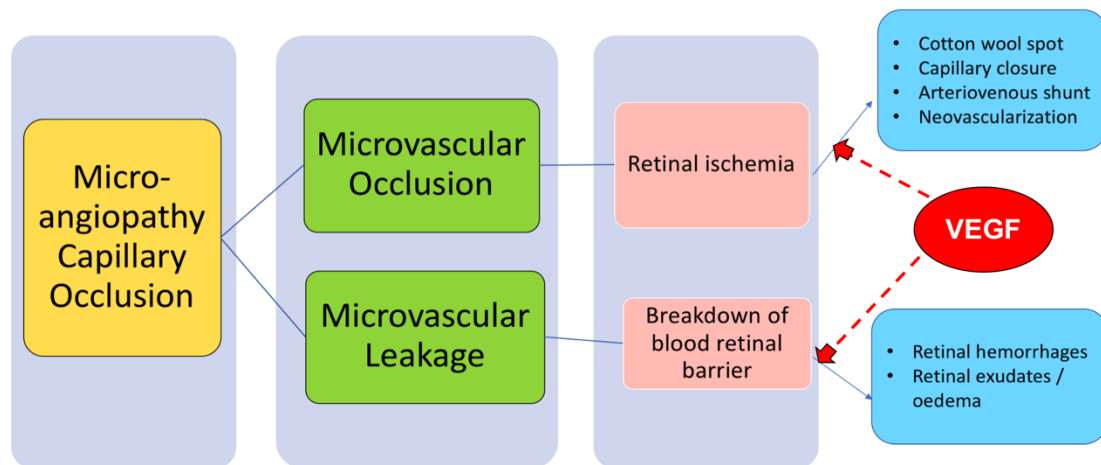
Figure 2.1: Pathogenesis of diabetic retinopathy

2.3 Classification of Diabetic Retinopathy

Based on International Clinical Diabetic Retinopathy Disease Severity Scale (AAO, 2010) DR is classified into no apparent retinopathy, NPDR and PDR (Table 2.1).

2.3.1 No Apparent Retinopathy

No apparent retinopathy is characterized by no abnormalities seen during dilated ophthalmoscopy examination.

2.3.2 Non-Proliferative Diabetic Retinopathy (NPDR)

NPDR occurs as a result of capillaropathy with increased permeability. This is manifested as microaneurysm and dot-hemorrhages and blotchy hemorrhages (Figure 2.2). Furthermore, it also can be presented as hard exudates, venous beading and intraretinal microvascular abnormalities (IRMAs) (Worsley D et al., 2010). NPDR can further be divided into mild, moderate and severe (Table 2.1). IRMA and venous beading are a predictors for PDR. About 50% of severe NPDR patients will develop into PDR within 15 months (ETDRS, 1991).

2.3.3 Proliferative Diabetic Retinopathy (PDR)

PDR is characterized by the formation of new vessels at the disc (NVD) and elsewhere (NVE) (Figure 2.2). New vessels are firstly evident within 45 degrees of the optic disc. There are 15% of patients who developed NVD on the disc or within the one-disc diameter of the optic disc. Another 40% of patients had new vessels appear outside this area (NVE) and 45% had new vessels in both areas (Klein, 1997).

Table 2.1: Classification of DR based on International Clinical Diabetic Retinopathy Disease Severity Scale.

PROPOSED DISEASE SEVERITY LEVEL	FINDINGS
No apparent retinopathy	No abnormalities
Mild NPDR	Microaneurysm only
Moderate NPDR	More than just microaneurysm but less than Severe NPDR
Severe NPDR	Any of the following: 1) >20 intraretinal hemorrhages in each of 4 quadrants 2) Definite venous beading in 2 quadrants 3) Prominent intraretinal microvascular abnormalities (IRMA) in 1 quadrant 4) No signs of proliferative retinopathy
PDR	One or more of the following 1) Neovascularization 2) Vitreous / pre-retinal hemorrhage
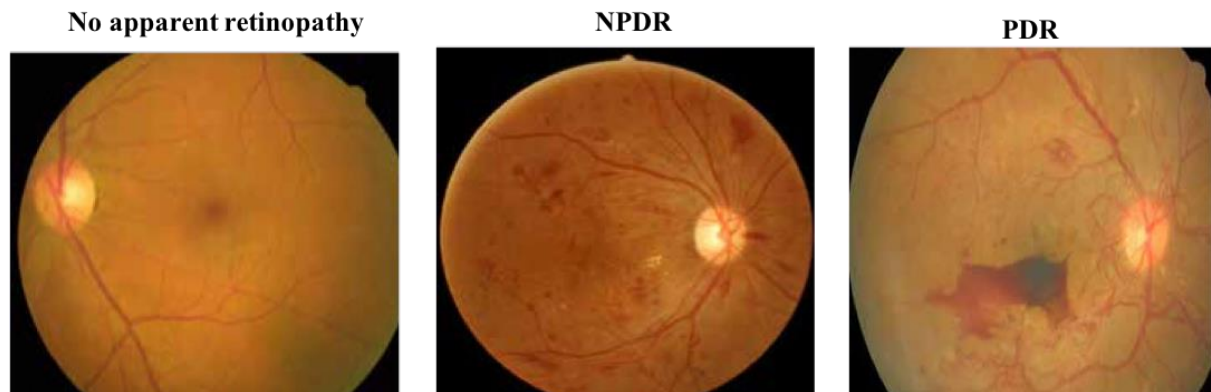
Figure 2.2: Classification of Diabetic retinopathy (Diabetic Retinopathy Screening, 2017).

2.4 Treatment of Diabetic Retinopathy

Management of patients with DR involved determining the stages of DR, prompt ocular treatment, managing the systemic associations and scheduled follow-up examination.

2.4.1 Management of Systemic Associations

The DR progression can be reduced with careful and thorough maintenance of blood glucose in Type 1 DM (T1DM) (DCCT, 1993) and T2DM (UKPDS, 1998). Evidence supported, that tight control of blood pressure among T1DM and T2DM reduces the progression of DR (DCCT 1993; UKPDS, 1998). High levels of total cholesterol and low-density lipoprotein (LDL) are associated with the presence of hard exudates in T2DM in African Americans (Papavasileiou E., 2017). Qader found that there was a significant association between serum cholesterol and LDL with DR. However, there was no association between serum lipid profile with the severity of retinal hard exudates (Qader AMA et al., 2009).

2.4.2 Ocular Treatment

2.4.2.1 Laser Photocoagulation

Laser photocoagulation therapy is directed to treat PDR and severe or clinically significant macular oedema (CSMO). Pan-retinal photocoagulation (PRP) is indicated in PDR where the laser is done at the peripheral retina. The aim is to reduce the amount of angiogenic factors (including VEGF) which is produced by the nonperfused retina. Evidence showed that PRP significantly reduces severe vision loss in eyes with PDR (ETDRS, 1981). Macula laser has shown a greater than 50% reduction in the moderate visual loss in patients with CSMO (ETDRS, 1985).

2.4.2.2 Vitreoretinal Surgery

Surgery remains the cornerstone in managing complicated PDR such as vitreous haemorrhage, traction retinal detachment, combined tractional and rhegmatogenous retinal detachment (CRD), pre-macular haemorrhage and diabetic macular oedema (DMO) (Gupta V et al., 2013). The Diabetic Retinopathy Vitrectomy Study (DRVS) demonstrated that there is an improvement of visual acuity of 20/40 in 25% of patients undergoing early vitrectomy compared to 15% who underwent conventional treatment (DRVS, 1985). Seventy percent of patients in a study showed improvement in visual acuity following vitrectomy for CRD (Yang CM et al., 2008). Pars plana vitrectomy is only indicated for DMO which is caused by vitreomacular traction and taut posterior hyaloid membrane (Lewis et al., 1992)

2.4.2.3 Antiangiogenesis

Anti-VEGF has become the first-line therapy for centrally involved DMO. Multiple trials such as VISTA, VIVID and RESTORE proved that intravitreal Ranibizumab and Intravitreal Aflibercept gain better visual outcomes compared to laser therapy in CSMO (VIVID, VISTA 2015; RESTORE study group, 2011).

2.5 Vascular Endothelial Growth Factor (VEGF)

VEGF is a potent angiogenic factor necessary for vascular endothelial cells. It is a dimeric glycoprotein of about 40 kDa and the activity requires the activation of VEGF receptors. There are 7 types of VEGF which consist of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and PlGF (placental growth factor) (David et al., 2005).

The VEGF gene expression is regulated by several different mechanisms during reduced oxygen tension. Retinal vascular changes such as leukostasis, aggregation of platelets, altered

blood flow, degeneration of pericytes and thickening of basement membranes lead to occlusion of the retinal capillaries. The level of VEGF will be upregulated following retinal hypoperfusion. In addition, hyperglycemia, AGE and proinflammatory cytokine contribute to VEGF secretion (Figure 2.3). Several hypotheses have been suggested for VEGF-A causing dysfunction of the blood-retinal barrier. First, VEGF-A act as a potent mediator for several pro-inflammatory mediators in DR. Second, it has direct phosphorylation of tight junction proteins. Eventually, induction of pericytes degeneration and depletion (Zheng et al., 2009).
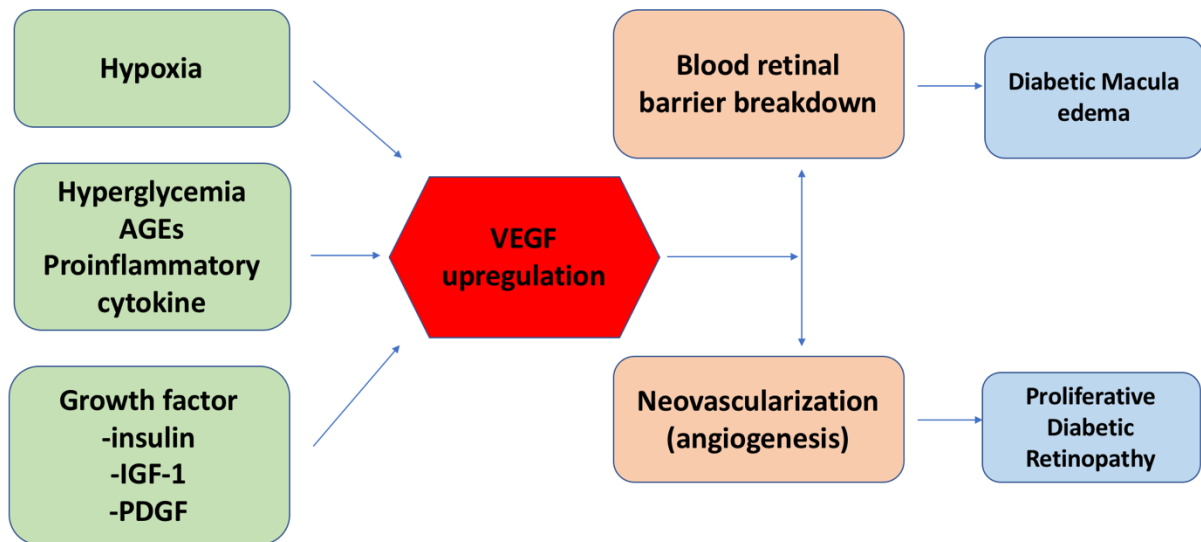
Figure 2.3: Role of VEGF in diabetic retinopathy

Intraocular VEGF level was found to be increased in patients with diabetes (Aiello LP et al., 1994; Adamis et al.,1994). Adamis found that the level of VEGF in an aqueous and vitreous patient with active PDR was higher compared to a patient with NPDR, quiescent PDR or non-diabetic.

2.6 Ocular Tears

2.6.1 Tears Production and Drainage

The tear film is a complex layer secreted by various glands and ocular tissue. It is produced mainly by the lacrimal gland, Meibomian gland and conjunctival goblets cells. Previously it was known to have 3 layers; lipid the outermost, aqueous the middle layer and mucin the innermost. Evidence showed a 2-phase model of the tear film which comprised of a lipid layer that overlies the mucoaqueous gel, as shown in Figure 2.4, (Wilcox MDP et al., 2017).

There are 2 types of tears secretion; basal and reflex. Both are secreted in response to autonomic input which includes sympathetic and parasympathetic stimulation and also hormonal control. Reflex secretion is induced by physical irritation, psychogenic factor and bright light. Meanwhile, evidence showed temperature-sensitive corneal sensory nerves that regulate basal lacrimation secretion (Parra A et al., 2010).

Figure 2.4. Two-phase model of the tear film (Wilcox MDP et al., 2017)

Blinking plays a key role in tear film renewal, distribution and drainage. Tears is distributed in the tear meniscus, preocular film and conjunctival sac. Tears are pushed from lateral to t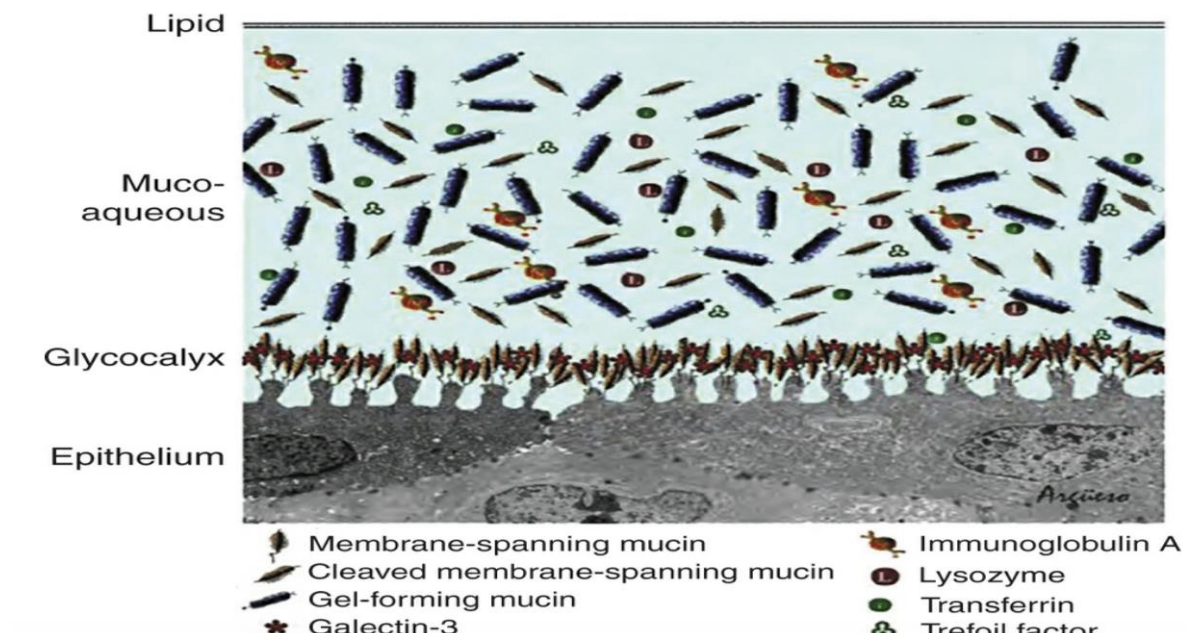he nasal side of the lid margin during blinking. Tears are sucked into the canalicular system upon the opening of the eyelids due to the elastic expansion of the lacrimal papillae and expulsed along the nasolacrimal duct upon the closure of the eyelids due to sac compression (Rosengren-Döane, 1981).

2.6.2 Composition of Tears and Its Function

Tears are essential in providing ocular surface comfort and maintaining ocular surface health. As the cornea is avascular, the epithelium depends on tears to supply the glucose, electrolytes and growth factors (Foster JB et al., 2013). The lipid layer contains a mixture of polar and neutral lipids which are responsible to reduce the surface tension of tear film (Miler D, 1969), maintaining a hydrophobic barrier that prevents tears overflow (Forrester AD et al., 2015) and preventing evaporation.

On the other hand, the mucoaqueous layer has several functions such as providing antibacterial and antiviral defense, interacting with the tear lipid to reduce surface tension and lubricating the eye (Vikram SB et al., 2019). The mucin components as well as protein, electrolytes, water and carbohydrate coat the superficial cornea in a polar glycocalyx. Several proteins were identified in mucoaqueous components that possess anti-bacterial activity such as lactoferrin, lipocalins and lysozyme. Lysozyme and lipocalins (Dartt DA, 2011) are the most abundant protein found in tears. There are also several proteins that serve as tears biomarkers such as VEGF.

2.6.3 Ocular Tear Film and Biomarkers

Local biomarkers are more reliable in indicating retinal pathology. However, the method to collect intraocular fluid was invasive. A few systemic and ocular biomarkers of DR were recognized such as C-reactive protein (CRP), TNF-α, IL-1β, IL-6, IL-8, IL-12 and VEGF (Kastelan S et al., 2020). Tears are the most readily available ocular specimens providing information regarding anterior segment pathology.

Method of collecting the tears such as Schirmer strip, mini sponges, fire-polished microcapillary tube and storage method could influence the results. Rentka used a capillary micropipette to collect tears from the inferior temporal meniscus, then PBS-T buffer was used to transfer to the Eppendorf tube and stored at -80^0C (Rentka A et al., 2015). Sheikhrezaee used a Schimer strip to collect the unstimulated tears and then soaked them with PBS to free their protein and stored them at -40^oC (Sheikhrezaee et al., 2019). Furthermore, elution with tri-distilled water followed by centrifugation can also extract protein from Schirmer strips (Farias E, 2013). VEGF detection can be unsuccessful if any steps from sample processing go wrong. There are various methods to determine the VEGF level that including ELISA (enzyme-linked immunosorbent assays) and biosensors.

Several studies showed tears have become DR biomarkers (Park et al., 2008; Costagliola et al., 2013; Ang et al, 2019). The levels of nerve growth factor (NGF) in tears and serum were significantly higher in a patient with DR (Park et al., 2008). Furthermore, Costagliola found that level of cytokine TNF-α was also higher in diabetic patients with DR. The VEGF levels in tears were significantly elevated in patients with retinal vein occlusions (Kasza et al., 2105). Tears VEGF levels were also significantly elevated in a patient with DR (Ang WJ et al., 2019) However, a reduced level of VEGF in tears was found in patients with systemic sclerosis

(Rentka et al., 2015). Some procedures like photorefractive keratectomy (PRK) may increase the VEGF level due to inflammation(Vesaluoma et al, 1997). Refractive surgery decreases the tear film stability due to damage to corneal afferent nerves that disrupt the ocular surface lacrimal gland feedback system. Konomi found that patients returned to preoperative values about 3-9 months after surgery (Konomi K et al., 2008).

CHAPTER 3

OBJECTIVES

3.0 OBJECTIVE

3.1 Research Questions

    3.1.1   Is there any significant difference of the mean VEGF levels in tears between DM with NPDR, DM with no DR and non-DM patients?

    3.1.2   Is there any significant difference of the mean VEGF levels in aqueous between DM with NPDR, DM with no DR and non-DM patients?

    3.1.3   Is there any correlation between mean VEGF levels in tears and aqueous among DM patients?

3.2 Research Hypothesis

    3.2.1   There is a significant difference in the mean VEGF levels in tears between DM with NPDR, DM with no DR and non-DM patients.

    3.2.2   There is a significant difference in the mean VEGF levels in aqueous between DM with NPDR, DM with no DR and non-DM patients.

    3.2.3   There is a correlation between VEGF levels in tears and aqueous among DM patients.

3.3 Objectives

3.3.1 General Objective

    To evaluate the VEGF levels in tears and aqueous among diabetic patients with NPDR.

3.3.2 Specific Objectives

i.   To compare the mean VEGF levels in tears between DM with NPDR, DM with no DR and non-DM patients.

ii.   To compare the mean VEGF levels in aqueous between DM with NPDR, DM with no DR and non-DM patients.

iii.   To determine the correlation between VEGF levels in tears and aqueous among DM patients.

CHAPTER 4

RESEARCH METHODOLOGY

4.0 METHODOLOGY

4.1 Study Design

A prospective cross-sectional study was conducted in Ophthalmology Clinic, Hospital Universiti Sains Malaysia (USM) and Hospital Raja Perempuan Zainab II (HRPZ II) from July 2019 to November 2021. The study population involved all T2DM patients with NPDR and no DR who were going for cataract surgery during the study duration and fulfilled the selection criteria. A control group of non-DM patients who attended Ophthalmology Clinic, Hospital USM and HRPZ II and were going for cataract operation was recruited for the study for comparison.

4.2 Ethical Approval

This study was conducted after approval by the local Human Research Ethics Committee (Universiti Sains Malaysia [USM]/ Jawatankuasa Etika Penyelidikan Manusia [JEPEM]; Registration Number: 20010061 (Appendix A) and Medical Research Ethics Committee (NMRR-20-1496-55228-IIR) (Appendix B), with the highest respect for subjects according to the study protocol, the ethical principles that have their origin in the Declaration of Helsinki, and the International Conference on Harmonisation (ICH) – Harmonised Tripartite Guideline for Good Clinical Practice (GCP).

4.3 Selection Criteria

The study group included all T2DM patients that were planned for cataract operation. Any patients with the ocular surface disease (eg Sjogren's syndrome), severe dry eye and postocular surface procedure (eg: excimer laser photorefractive keratectomy) were excluded from the study. Other ocular exclusion criteria were the eyes on topical drugs (steroid, antiglaucoma and nevanac), intravitreal therapy and laser photocoagulation therapy within 3 months before

recruitment, and eyes with a history of trauma or intraocular surgery (post-cataract surgery, pars plana vitrectomy and a scleral buckle), patient with histories of any ocular inflammatory disease like uveitis and patient with systemic conditions that upregulate VEGF such as bronchial asthma, hematological disorder and malignancies.

Non-diabetic patients that were planned for cataract operation were selected for the control group. The exclusion criteria for the control group were the same as the study group.

4.4 Sample Size Estimation

Objective 1: To compare the mean VEGF level in tears between DM with NPDR, DM with no DR and non-DM patients.

Objective 2: To compare the mean VEGF level in aqueous between DM with NPDR, DM with no DR and non-DM patients.

Sample size estimation for objectives 1 and 2 was calculated based on G*power software version 3.1.9.4.

Statistical Test: one-way ANOVA

Input parameters:

α error probability   : 0.05

Effect size f   : 0.3

Power (1-β err prob)   : 0.8

Number of groups   : 3

Total of sample size   : 111

Output parameters:

Noncentrality parameter λ   : 9.99

Critical F   : 3.0803

Numerator df   : 2

Denominator df   : 108

Actual power   : 0.8034

Sample size for each group (n): 111 ÷ 3 + (10% drop out) = 41

DM with NPDR = 41, DM with no DR = 41, Non-DM = 41

Objective 3: To determine the correlation between mean VEGF level in tears and aqueous among DM patients.

Sample size estimation was calculated based on G*power software version 3.1.9.4.

for Pearson correlation.

Test Ho : Rho alt = Rho null, usually null Rho is 0

Input parameters:

Tails   : two

 effect size (ρ) : 0.3

α   : 0.05 (two sided)

Power   : 0.8

Output parameters:

Noncentrality parameters δ   : 2.8477

Critical t   : 1.990

Df   : 80

Total sample size   : 82

Actual power   : 0.8

Estimated sample size calculated for diabetic groups (no DR and NPDR) = 82

Sample size for each diabetic group (n) = 82 ÷ 2 + (10% drop out) = 45

The estimated required sample size for each diabetic group, n = 45

Therefore the sample size for this study is = 123

(DM with NPDR: 41, DM with no DR: 41, and Non-DM: 41)

4.5 Sampling Method

A nonprobability sampling method was applied to the participant who attended the Ophthalmology Clinic Hospital USM who are fulfil the criteria throughout the duration of the study.

4.6 Definition of Term

4.6.1 Type 2 Diabetes Mellitus

In this study, the T2DM patient was defined as a participant who had a fasting venous plasma glucose (FPG) of 7.1 mmol/L or more, or random venous plasma glucose (RPG) of more than 11.1 mmol/L (Clinical Practice Guideline, 2011). The participants who had FPG less than 7.1mmol/L and RPG less than 11.1mmol/L will be selected as a control group.

4.6.2 Diabetic Retinopathy

The International Clinical Diabetic Retinopathy Disease Severity Scale (AAO, 2010) will be used to classify the patient into no DR and NPDR.

    i.   No diabetic retinopathy

- diabetic patient who does not have any fundus abnormality.

ii.   NPDR

- A diabetic patient who is presented with intraretinal haemorrhages, hard exudates, microvascular abnormalities (including microaneurysms and venous beading) and absence of neovascularization.

4.6.3 Vascular Endothelial Growth Factor (VEGF)

VEGF is a dimeric glycoprotein, with a molecular weight of 45 kDa that plays a key role in vascular endothelial cell growth and angiogenesis (Ferrara N, 1989). It can be present in the tears, aqueous and vitreous. Oxygen tension has a significant impact on VEGF mRNA expression, with VEGF gene expression being boosted in hypoxic environments (Schweiki D et al., 1992). The retinal pigment epithelium, endothelial cells, pericytes, glial cells, and ganglion cells are all capable of producing VEGF. Because of anterior hyaloid is in contact with the aqueous, VEGF and other cytokines can be detected in the aqueous. According to the Michaelson theory, VEGF is a soluble factor that is freely diffusible, produced in the hypoxic retina, and encourages neovascularization both locally and at a distance (Michaelson IC., 1948). The level of VEGF-A will be evaluated in this study.

4.7 Research Tool

4.7.1   Instruments

Snellen chart was used for visual acuity. Slit-lamp biomicroscope was used to examine the anterior segment. Binocular indirect Opthtalmoscope (BIO) (Keelers's ophthalmic instruments, USA) was used to examine the fundus. Slit-lamp biomicroscope with Volk superfield NC (non-contact)/ 78 Diopter/ 90 Diopter condensing lens was used to examine the fundus. Fundus camera (Kowa Technology, USA), was used for documentation of fundus finding**.**