EVALUATION OF SERUM VITAMIN D AND RETINAL NERVE FIBRE LAYER THICKNESS AMONG TYPE 2 DIABETES MELLITUS PATIENT WITH NON PROLIFERATIVE DIABETIC RETINOPATHY

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DISCLAIMER

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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ABSTRAK

Pengenalan:

Neurodegenerasi retina merupakan proses perubahan yang terawal daripada mikroangiopati dalam penyakit Diabetes Mellitus jenis 2. Selain daripada homeostasis kalsium dan tulang metabolisme, peranan vitamin D juga dikenali sebagai agen pelindung saraf dan berfungsi "anti-angiogenesis". Walau bagaimanapun, pemahaman berkenaan hubungan di antara serum vitamin D dan neurodegenerasi retina masih berkurangan. Oleh itu, kajian ini akan mengkaji ketebalan lapisan saraf retina yang diukur dengan teknik yang tidak invasi ini, untuk menilaikan neurodegenerasi retina dan hubungannya dengan serum vitamin D di kalangan pesakit Retinopati Diabetes Tidak Proliferatif (RDTP).

Objektif:

Objektif kami adalah untuk membandingkan purata tahap serum vitamin D dan ketebalan lapisan saraf retina dalam golongan pesakit RDTP dan pesakit yang tiada Retinopati Diabetes (RD). Kajian ini juga menilai hubungan antara tahap serum vitamin D dengan ketebalan lapisan saraf retina di antara pesakit RDTP.

Kaedah:

Kajian keratan rentas telah dijalankan antara March 2017 dan November 2018 di kalangan 78 pesakit DM jenis 2 dari Klinik Oftamologi, Hospital Universiti Sains Malaysia yang memenuhi syarat-syarat penyertaan kajian. Peserta ini dibahagikan kepada dua kumpulan yang terdiri daripada 39 peserta masing-masing, iaitu kumpulan RDTP dan kumpulan tiada RD. Kelas penyakit RD adalah mengikuti "International

Clinical Diabetic Retinopathy Disease Severity Scales". Ketebalan lapisan saraf retina diukur dengan menggunakan mesin "Spectral Domain Optical Coherence Tomography" (SD-OCT). Serum vitamin D diuji dengan memakai "electro-chemiluminescence binding assay kit" (ECLIA). Paras serum vitamin D adalah mengikuti "Endocrine Society Clinical Practice Guideline". Paras serum vitamin D ≤20 ng/ml adalah berkuranngan; 21-29 ng/ml adalah sederhana berkurangan, manakala, paras vitamin D ≥30 ng/ml adalah mencukupi. Analisis statistik dilakukan dengan menggunakan Pakej Statistik untuk Sains Sosial (SPSS Inc Version 25). Kajian ini memperolehi kelulusan dari HREC ("Human Ethics Research Committee" dari Hospital Universiti Sains Malaysia) [USM/JEPeM/ l6100410].

Keputusan:

Dalam kajian ini, umur pesakit terdiri daripada golongan 34 ke 60 tahun. Purata umur pesakit yang terlibat dalam kumpulan RDTP adalah 55.95 tahun, manakala bagi kumpulan tiada RD adalah 52.72 tahun. Kebanyakan pesakit terdiri daripada perempuan (62.8%) dan berkaum Melayu (88.5%). Glikosolasi haemoglobin (HbA1c) adalah 9.13% dan 7.8% dalam kumpulan RDTP dan kumpulan tiada RD masing-masing. Purata serum vitamin D adalah 20.71±10.40 ng/ml dalam RDTP dan 20.93±7.65 ng/ml dalam tiada RD. Purata keseluruhan ketebalan lapisan saraf retina dalam kumpulan RDTP adalah 89.26±9.62 µm dan 91.28±8.00 µm dalam kumpulan tiada RD. Walaupun umur dan HbA1c menunjukkan perbezaan ketara [umur, p=0.015; HbA1c, p=0.004] di antara kumpulan, tetapi tiada perbezaan yang ketara dalam perbandingan serum vitamin D dan kuadran-kuadran ketebalan lapisan saraf retina di antara dua kumpulan, selepas umur dan HbA1c diselaraskan. Ketebalan lapisan saraf retina adalah lebih nipis berbanding dengan kumpulan tiada RD, kecuali kuadran "temporal". Purata ketebalan

lapisan saraf retina dalam kumpulan RDTP dan kumpulan tiada RD adalah dalam berikutan: “global” (RDTP = 89.26[9.62]µm; tiada RD = 91.28[8.00]µm), “superior” (RDTP = 110.49[21.06]µm; tiada RD = 115.15[17.50]µm), “nasal” (RDTP = 65.44[10.31]µm; tiada RD = 68.54[8.25]µm), “inferior” (RDTP = 108.00[23.78]µm; tiada RD = 115.67[14.66]µm), “temporal” (RDTP = 67.80[10.84]µm; tiada RD = 65.03[10.72]µm). Hubungan yang bersongsangan lemah ditunjukkan di antara serum vitamin D dengan ketebalan “inferior” kuadran dalam RDTP *(r*= -0.37, *p*=0.021). Walau bagaimanapun, tiada hubungan berkaitan yang ketara di antara serum vitamin D dengan “global” ketebalan lapisan saraf retina dalam kumpulan RDTP dan kumpulan tiada RD.

Kesimpulan:

Kajian ini menunjukkan bahawa tiada perbezaan ketara dalam purata serum vitamin D dan ketebalan lapisan saraf retina dalam kumpulan DRTP dan kumpulan tiada RD. Walau bagaimanapun, kajian ini, menunjukkan bahawa serum vitamin D mempunyai hubungan songsang yang lemah dengan ketebalan lapisan saraf retina pada “inferior” kuadran. Tetapi, tiada hubungan yang ketara diperhatikan pada serum vitamin D dengan “global” ketebalan lapisan saraf retina dan yang lain-lain di dalam kedua-dua kumpulan ini. Oleh itu, penyelidikan yang berprospek besar perlu dijalankan untuk menguji ketepatan maklumat tersebut dan peranan serum vitamin D dalam neurodegenerasi serta hubungannya dengan ketebalan lapisan saraf retina di golongan RDTP.

ABSTRACT

Introduction:

Retinal neurodegeneration has been postulated to be an early ocular diabetic change, preceded microangiopathy in patients with type 2 Diabetes Mellitus (DM). Other than calcium homeostasis and bone metabolism, vitamin D is found to serve as neuroprotection and prevention of angiogenesis. There is a lack of study on the relation between vitamin D and retinal neurodegeneration changes. Thus, this study evaluates on Retinal Nerve Fibre Layer (RNFL), a non-invasive technique of assessing retinal neurodegenerative changes in relation to serum vitamin D in Non Proliferative Diabetes Retinopathy (NPDR).

Objective:

Our objectives were to compare the mean level of serum vitamin D and RNFL thickness among type 2 DM patients with non-proliferative diabetic retinopathy (NPDR). We also aim to determine the correlation between serum vitamin D and RNFL thickness among patients with NPDR.

Methods:

A cross-sectional study was conducted from March 2017 to November 2018. We recruited 78 patients with type 2 DM from Ophthalmology Clinic in Hospital Universiti Sains Malaysia who fulfilled the inclusion and exclusion criteria. They were divided

into 2 groups: NPDR group composed of 39 patients with mild or moderate NPDR while no DR group comprised of 39 patients without DR. International Clinical Diabetic Retinopathy Disease Severity Scales was used to grade the DR. Measurements of peripapillary RNFL were performed using Spectral Domain Optical Coherence Tomography (SD-OCT). Serum vitamin D level was analyzed using electro-chemiluminescence binding assay kit (ECLIA) produced by Roche. Definition of vitamin D level is adopted using Endocrine Society Clinical Practice Guideline. Serum vitamin D $\leq$ 20 ng/ml is considered as deficiency; 21-29 ng/ml as insufficiency and $\geq$30 ng/ml as sufficient level. Statistical analysis was done by using Statistical Package for the Social Science (SPSS Inc Version 25). The study protocol followed the tenets of the declaration of Helsinki and was approved by HREC (Human Ethics Research Committee from Hospital USM) [USM/JEPeM/ l6100410].

Results:

The age of the patients ranged from 34-60 years old with the mean age of NPDR group was 55.95 years while the no DR group was 52.72 years. Most of the patients were female (62.8%) and from Malay (88.5%) ethnic. HbA1c levels were 9.13% and 7.8% respectively in NPDR group and no DR group. Both age and HbA1c were statistically significant. The serum vitamin D in both groups were found to have insufficient level with the mean serum vitamin D of 20.71[10.40]ng/ml in NPDR group and 20.93[7.65]ng/ml in no DR group. There was no statistical significance in mean serum vitamin D even after adjusted with age and HbA1c. As for RNFL thickness, the global and quadrantal thickness in NPDR group were general thinner as compares to the no DR group except the temporal quadrant of RNFL. The mean RNFL thickness in NPDR group versus the no DR group were in the following order: global (89.26[9.62]µm vs

91.28[8.00]µm), superior (110.49[21.06]µm vs 115.15[17.50]µm), nasal (65.44[10.31]µm vs 68.54[8.25]µm), inferior (108.00[23.78]µm vs 115.67[14.66]µm), temporal (67.80[10.84]µm vs 65.03[10.72]µm). After adjustment on age and HbA1c, the RNFL thickness between groups were not statistically significant. There were no correlations noted between serum vitamin D and global RNFL thickness in both groups. However, we found a weak inverse correlation between serum vitamin D and inferior quadrant thickness of RNFL in NPDR group (r= -0.37, p=0.021).

Conclusion:

In our study, we demonstrated that both the mean serum vitamin D and RNFL thickness were not statistically significant between the groups before and after adjusted with age and HbA1c. However, there was a weak inverse correlation between serum vitamin D level and RNFL thickness at inferior quadrant in the early stage of NPDR group. Otherwise, there was no relation noted between the serum vitamin D and others quadrantal RNFL thickness in both NPDR and no DR group. RNFL thickness measured by SD-OCT alone may not be suitable to assess retinal neurodegenerative changes in relation to serum vitamin D in NPDR. A large prospective study is needed for further evaluation on the role of vitamin D in retinal neurodegeneration in relation to RNFL thickness in early NPDR.

CHAPTER 1

INTRODUCTION

1.0 Diabetes Mellitus

Diabetes Mellitus (DM) is a non-communicable, chronic metabolic disease characterized by hyperglycemic state with the presence of insulin deficiency or insulin resistance. It has two clinical presentations: Type 1 DM and Type 2 DM. A chronic hyperglycemic state causes cellular dysfunction by increasing accumulation of advanced glycation end products (AGE) and oxidative stress particles. This triggers a local inflammatory cascade leading to increased vascular permeability resulting in macro or micro-vascular complications (Singh *et al*., 2001; Palomer *et al*., 2008).

Prevalence of DM has increased drastically over the years, at an alarming rate. About 366 million people were diagnosed with DM in 2011 and it is estimated that the number of cases will increase to 552 million by 2030 worldwide (Whiting *et al.*, 2011). The data from Ministry of Health, Malaysia has showed a rising trend in prevalence of DM in Malaysia. It was noted there was a shift of the disease trend to the younger age population was noted, while the highest prevalence of DM remained among the elderly group (ages of 60-69 years) (*National Health Morbidity Survey*, 1996; Zanariah *et al.*, 2008). Complications secondary to DM have become the leading cause of morbidity and mortality; causing huge health, financial and social pressures affecting both developed and developing countries.

1.1 Pathogenesis of Diabetes Mellitus

Pathogenesis of Type 1 DM is due to the autoimmune destruction of the insulin producing β islet cell in the pancreas which leads to absolute insulin deficiency in the circulation. These patients require exogenous insulin to facilitate the uptake of glucose molecules into the tissue as an energy source as well as prevent ketosis and decrease

hyperglucagonemia. Hyperglycemia is detectable only when 80-90% of β islet cells are destroyed. Approximately 85% of the patients have circulating islet cell antibodies and anti-insulin antibodies (Khardori, 2018). This condition is associated with other autoimmune conditions like Grave's disease and Hashimoto's thyroiditis (Pilia *et al.*, 2011).

Type 2 DM is a hyperglycemic state resulting from a combination of resistance to insulin action, inadequate insulin secretion and excessive or inappropriate glucagon secretion. Both insulin resistance and inadequate insulin secretion are the basis of type 2 DM. Insulin resistance is defined as a state of reduced responsiveness to normal circulating levels of insulin. Various studies indicate that type 2 DM progresses over a period of worsening insulin action, which begins with peripheral insulin resistance and ends with a loss of insulin secretion (Saltiel, 2000). Other than that, free fatty acids appear to cause insulin resistance by inhibiting the insulin-stimulated tyrosine phosphorylation, preventing the insulin receptor substrate-1 (IRS-1) which is an important mediator to activate insulin dependent glucose transporter (GLUT4). These free fatty acids cause an increase in long chain acyl-Coenzyme A and diacylglycerol, which in turn, triggers serine or threonine kinase cascade and causes reduction in expression of GLUT4 (Dresner *et al.*, 1999).

The hyperglycemic state also accelerates the accumulation of AGE products and oxidative stress species in the cells. Presence of both these particles will trigger the inflammatory cascade locally causing cellular apoptosis and dysfunction (Peppa and Vlassara, 2005; Singh *et al.*, 2001; Stitt, 2001).

1.3 Diabetic Retinopathy

Diabetic retinopathy (DR) is the commonest microvascular complication of DM (Antonetti *et al.*, 2012) and a leading cause of blindness for individuals aged 20 to 64 years (Bunce and Wormald, 2006; Liew *et al.*, 2014; Thylefors *et al.*, 1995). Lee *et al.* (2014) found that DR is associated with overt nephropathy. Thus, DR is used as a predictor to evaluate other diabetic microvascular complications (El-Asrar *et al.*, 2001).

As the prevalence of DM is estimated to increase drastically likewise prevalence of complications will be increasing as well. Yau *et al*. (2012) performed a systemic review over 35 studies (1980-2008) providing data from 22,896 individuals with DM and found that the overall prevalence for any form of DR was 34.6%, while proliferative diabetic retinopathy (PDR) was 7.0%. Epidemiology statistics for Asian region was scanty and the overall prevalence of any form of DR was 19.92% and vision threatening DR was 5.25% from the review (Yau *et al.*, 2012).

1.4 Classification of Diabetic Retinopathy

The full disease classification of DR has developed from the original Airlie House Classification. It was later modified for the Early Treatment Diabetic Retinopathy Study (ETDRS) aiming to grade DR in the context of overall severity. However, a simplified version was later developed based on ETDRS. For clinical practice purposes, International Clinical Diabetic Retinopathy and Diabetic Macular Edema Disease Severity Scales is widely used as a reference (Wilkinson *et al.*, 2003) and it is based on the findings of the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) and the ETDRS.

In this study, International Clinical Diabetic Retinopathy and Diabetic Macular Edema Disease Severity Scales was used. It classifies the disease into 3 main groups: non proliferative diabetic retinopathy (NPDR), PDR and advanced diabetic eye disease. For diabetic maculopathy, the disease is grouped into absent or present of the disease. (*Diabetic Retinopathy and diabetic maculopathy classification based on International Clinical Diabetic Retinopathy (American Academy of Ophthalmology. 2002).* 2016).

1.4.1 Non Proliferative Diabetic Retinopathy

Features of NPDR on dilated Ophthalmoscopy:

- Mild NPDR - Microaneurysms only
- Moderate NPDR - More than just microaneurysms but less than severe NPDR
- Severe NPDR - Any of the following:
  - More than 20 intraretinal haemorrhages in each of four quadrants
  - Definite venous beading in two or more quadrants
  - Prominent intraretinal microvascular abnormalities (IRMA) in 1 or more quadrants

1.4.2 Proliferative Diabetic Retinopathy

Features of PDR:

One or both of the features:

- Neovascularization
- Vitreous or preretinal haemorrhage

1.4.3 Advanced Diabetic Eye Disease

Features of advanced diabetic eye disease:

One of the following:

- Formation of fibrovascular tissue proliferation
- Traction retinal detachment due to formation of posterior vitreous detachment
- Dragging of retinal or distortion.
- Rhegmatogenous retinal detachment

1.4.4 Diabetic Maculopathy

Features of diabetic maculopathy:

- Apparently absent : No retinal thickening or hard exudates in posterior pole
- Apparently present : Some apparent retinal thickening or hard exudates in posterior pole
- Present of macular oedema
    - Mild - some retinal thickening or hard exudates in posterior pole but distant from the macula
    - Moderate - retinal thickening or hard exudate approaching the centre of the macula but not involving the centre
    - Severe - retinal thickening or hard exudates involving the centre of the macula

1.5 Pathogenesis of Diabetic Retinopathy

Microangiopathy is the pathophysiology hallmark of DR. Chronic exposure to hyperglycemia and other risk factors (obesity, hypertension and dyslipidemia) are

believed to trigger cascades of biochemical activation and hematological changes that lead to microangiopathy which subsequently leads to thickening of the basement membrane, loss of pericytes, breakdown of blood-retinal barrier and increased vascular permeability (Cheung *et al.*, 2010). This causes abnormal outpouchings of capillaries leading to microaneurysms, retinal haemorrhages, macular edema and hard exudate formation.

Hyperglycemic state also cause abnormal haematological changes with both erythrocytes and leukocytes (Chibber *et al.*, 2007) as they become abnormal and undergo rouleaux deformation, leading to leukostasis causing vessel occlusion and stasis of axonal debris; whilst platelets also become sticky leading to increase in plasma viscosity. This will cause disruption in blood flow to the retinal capillary resulting in retinal hypoxia and eventually lead to over expression of vascular endothelial growth factor (VEGF), causing an imbalance between VEGF and anti-VEGF, resulting in neovascularization to the hypoxic retina. Retinal neovascularization is the hallmark of PDR. It induces the formation of abnormal blood vessels which can bleed easily into the vitreous causing vitreous haemorrhage which later progresses to tractional retinal detachment.

In addition to the hyperglycemic state, there are several biochemical mechanisms proposed to modulate the pathogenesis of DR through effects on cellular metabolism, signaling, and growth factors (Cheung *et al.*, 2010; Goh and Cooper, 2008). These pathways include the accumulation of sorbitol, AGE products, oxidative stress particles, protein kinase C activation, inflammatory cytokines [Eg: Interleukin (IL) -2, IL-6, IL-8, IL-12, Tumour Necrosis Factor (TNF)], and upregulation of both renin-angiotensin

system and VEGF. All these changes will ultimately lead to microvascular damage in response to abnormal leucocyte-endothelial interactions (Goh and Cooper, 2008; Cheung *et al*., 2010; Tang and Kern, 2011).

DR has been primarily observed as a retinal microangiopathy disorder. However, retinal neurodegeneration has recently emerged as a potential cause of diabetic ocular complications which precedes DR (Sohn *et al.*, 2016). A significant decreased in the nerve fibre layers, ganglion cell layers, and inner plexiform layers were demonstrated over time in patients without DR and minimal DR (Sohn *et al.*, 2016). Other studies conducted by Kern and Barber (2008), Antonetti et al. (2012) and Bogdanov et al. (2014) also demonstrated both neural apoptosis and glial cell reactivity are associated in the pathogenesis of retinal neurodegeneration and appears to be independent of vascular changes in the retina (Fletcher *et al.*, 2008; S Stem and W Gardner, 2013).

1.6 Treatment of Diabetic Retinopathy

Timely panretinal laser photocoagulation remains the mainstay treatment for sight threatening PDR. Although laser therapy is destructive in nature, it aims to promote regression by reducing the retinal hypoxic drive and expression of VEGF in order to halt the progression of retinal neovascularization. Optimising blood glycemia and blood pressure play major roles in preventing disease progression into complications (Stratton *et al.*, 2001; Porta and Bandello, 2002; Vinik and Vinik, 2003). However, systemic control of the disease is challenging and often complex when patient's compliance is poor.

Intravitreal anti-VEGF and grid laser therapy are management options for diabetic maculopathy. Intravitreal anti-VEGF has been approved to treat diabetic macular oedema and has showed visual improvement and reduction in central macular thickness (Nguyen *et al.*, 2012). However, the long term safety in patient with diabetes has not yet been established (Wirostko *et al.*, 2008) and the effect of the medication is not sustainable and patients may need several injections per year to maintain their vision. Adverse events and complications of intravitreal anti-VEGF injection include infection, iatrogenic traumatic cataract, vitreous haemorrhage, retinal detachment and arterial thromboembolic events.

In advanced diabetic eye disease with extensive tractional retinal detachment or unresolved vitreous haemorrhage, vitreoretinal referral is warranted for pars plana vitrectomy, endolaser and retinal detachment surgery if indicated.

1.7 Serum Vitamin D

Vitamin D is a lipid soluble steroid hormone. It has two forms: Ergocalciferol, or vitamin D_2 which is present in dietary form from plants, egg yolk and deep-sea fish and Cholecalciferol, or vitamin D_3 which is synthesized in the skin by sunlight with the presence of ultraviolet B rays (UVB). About 80-90% of vitamin D is acquired via synthesis from the skin after sun exposure whilst only 10-20% of the required vitamin D is obtained from diet (Holick MF, 2007).

After exposure to sunlight, the cholesterol precursor-7 dehydrocholesterol which is available in the skin will be converted to vitamin D_3 in the presence of UVB. Vitamin D_3 is an inactive form, which binds with the vitamin D binding protein to be transported

to the liver to undergo hydroxylation to 25-hydroxy-vitamin D_3 (25(OH)D). This 25(OH)D is an inactive form of vitamin D circulating in the body. This is later converted to an active compound 1,25 dihydroxy vitamin D ($1,25(OH)_2D$) or calcitriol in the kidney. The process of hydroxylation is catalysed by 1α-hydroxylase. Calcitriol can both inhibit renal 1-αhydroxylase and stimulate the 24-hydroxylase enzymes, to maintain the circulating levels within limited boundaries and preventing excessive vitamin D activity or signaling (Aranow, 2011).

Since the synthesis of vitamin D requires UVB light, vitamin D production is influenced by altitude, climate and the use of sunscreen. UVB light can be blocked by clouds and application of sunscreen. Darker skin with heavy pigmentation can result in reduced vitamin D production as melanin absorbs UVB radiation, thus shielding the UV rays from penetrating the epidermal layer to photolyze provitamin D_3, thereby inhibiting the production of vitamin D (Tsiaras and Weinstock, 2011).

1.7.1 Sampling Serum Vitamin D

Vitamin D status is established by measuring the levels of 25(OH)D in the blood. This 25(OH)D serves as a suitable biomarker, representing the total circulating reservoir of vitamin D in the body as compare to $1,25(OH)_2D$, due to its innate form in the circulation and having a longer half-life in plasma up to 2-3 weeks (Holick MF, 2007). There are various vitamin D assay kits currently available, with different approach methods to separate the vitamin D binding protein from 25(OH)D and thus sensitivity level of each test differs. The Roche Modular Analytics E-170 Total Vitamin D assay is an automated direct competitive electro-chemiluminescence immunoassay that detects 25(OH) vitamin D_2 and 25(OH) vitamin D_3 in serum with the assay range

between 7.5 nmol/l to 175 nmol/l. It met the vitamin D External Quality Assessment Scheme (DEQAS) accuracy criteria, given a bias of less than 20% in 80% of tested samples (Chen *et al.,* 2012).

Serum 25(OH)D concentration of ≥30 ng/ml is considered as sufficient; 21-29 ng/ml as insufficient and ≤20 ng/ml is considered as deficient based on guidelines implemented by American Endocrine Society Clinician Vitamin D guideline (Holick *et al.,* 2011).

1.7.2 Vitamin D and Diabetes Mellitus

Unlike other vitamins which act only as cofactor enzymes, vitamin D acts as a ligand and exerts its effect on tissues by binding to the vitamin D receptors (VDR). Currently, VDR has been identified not only in pancreatic β islet cell, it is also has been identified in other cells in the body, involving 38 sites (Haussler *et al.,* 2008). Interaction of vitamin D and VDR in pancreatic β islet cell can reduce insulin resistance (Pittas *et al.,* 2007) and improve insulin secretion (Gedik and Akahn, 1986). Thus, it was postulated that vitamin D can modulate the pathogenesis of DM, by exerting a protective role in glycemic control in subjects with and without DM as shown by Hutchinson et al. (2011). When vitamin D and VDR interaction take place at the pancreatic β islet cell, there is a surge of calcium intracellularly. This intracellular calcium is important to facilitate the secretion of insulin from pancreatic β islet cell via non-selective voltage-dependent calcium channel and act on the β-cell calcium dependent endopeptidase, to stimulate the conversion of proinsulin into insulin (Sergeev and Rhoten, 1995; Chua *et al.,* 2004). A double-blinded placebo trial by Mitri et al. (2011), shown 13% reduction in risk of type 2 DM with a short-term supplementation with vitamin D >500 IU/day for 14 weeks.

More recent study also concluded that supplementation vitamin D with 4000 IU/day had significantly reduced the HbA1c level among type 2 DM patient (Mirhosseini *et al.*, 2017).

Apart from this, type 2 DM associated with obesity proposed to be related with low degree of chronic inflammatory process involving several biochemical mechanisms which will cause upregulating of cytokines and interleukins (Cheung *et al.,* 2010; Goh and Cooper, 2008). TNF and IL-6 were found elevated in obese patient. It is known that TNF and IL-6 are regulated by Nuclear Factor kappa Beta (NF-kB). NF-kB is the transcription factor of proinflammatory mediator, and it is present inside the macrophages and monocytes. In euglycemic condition, insulin binds to its receptor and triggers tyrosine phosphorylation of IRS which induces insulin action on glucose intake and inhibits gluconeogenesis in liver (Zeyda *et al.,* 2009). But in hyperglycemic state, upregulation of protein kinase C will cause IRS under seline phosphorylation and result in failing insulin receptor signal and cause insulin resistance. On the other hand, there are high expression of TNF and IL-1β into the circulation by high NF-kB following hyperglycemic state. These circulating TNF and IL-1β will bind to cell membrane and cause insulin resistance (Arkan *et al.,* 2005; Regor and Hotamisligil, 2011). As for IL-6, it will lead to IRS degradation and raise insulin resistance (Lebun and Obberghen, 2008). Since VDR is found in macrophage, interaction between vitamin D and VDR will reduce the NF-kB release and subsequent reduce the productions of IL-1β, IL-6 and TNF which can lower the insulin resistance (Cohen-Lahav *et al.,* 2006).

1.7.3 Vitamin D and Diabetic Retinopathy

Seven studies and one meta-analysis study comparing 11 cross sectional studies established that there is an inverse or negative correlation between serum vitamin D levels with the severity of the DR (Alcubierre *et al.*, 2015; Bener *et al.*, 2018; He *et al.*, 2014; Herrmann *et al.*, 2015; Jee *et al.*, 2014; Reheem and Fattah, 2013; Shimo *et al.*, 2014; Zhang *et al.*, 2017). Low vitamin D level might be a risk factor for development and progression of DR (Reheem and Fattah, 2013). However, the observations were not consistent to demonstrate vitamin D deficiency could be related to DR. Other studies performed by Alam et al. (2016) in the UK, Bonakdaranet et al. (2015) in India and Patrick et al. (2012) in USA found that there were no correlations between serum vitamin D levels and the severity of DR.

Likewise, in DR, hyperglycemic state will induce several pathway alterations involving: first, the raised of polyol pathway; second, accumulation of AGE production; third, upregulating protein kinase C and lastly, increased activation of hexosamine pathway. These will subsequently draw more oxidative stress like reactive oxygen species (ROS) to the site and result in increased vascular permeability causing endothelial pericytes loss, vascular occlusion, and ischemia. Subsequently, led to upregulation of proangiogenic and inflammatory factors such as vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), stromal derived factor-1 (SDF-1), TNF, IL-1β and IL-6, which further escalate neovascularization in the retinas (Kaul *et al.*, 2010; Rangasamy *et al.*, 2012).

It was in the recent decades that researchers found there were alterations in the retinal neural function in early DM. Muller cell is crucial in maintaining and supporting the

retinal neuron function. It's also plays a major role in regulating retinal blood flow and fluid balance in the neuron (Bringmann and Wiederman, 2012). So, when the Muller cell function is compromised due to DM, the conversion of glutamate to glutamine will be reduced (Mizutani *et al.,* 1998; Lieth *et al.,* 2000). This led to excessive glutamate causing uncontrolled influx of intracellular calcium ions which was neurotoxin to the neuron and leading to neuron apoptosis (Orrenius and Nicotera, 1994). Other than this, Muller cell also underwent reactive gliosis result in upregulation of aberrant glial fibrillary acidic protein (GFAP) which cause increased in VEGF and other inflammatory mediators which further compromised the vascular permeability. Moreover, oxidative stress and AGE products accumulations will also cause oxidative damage to the retinal neuron (Shamsul and Alhomida, 2014). Retinal neural such as ganglion cells, Muller cells and the inner retinal fibre layers consequently underwent apoptosis before the evidence of microangiopathy and these neurons loss will be reflected by thinning of the retinal nerve fibre layer (RNFL) and ganglion cell layer (Lieth *et al.,* 2000; Ng *et al.,* 2016; Chihara *et al.,* 1993; Vujosevic and Midena, 2013).

Following the discovery of VDR in the photoreceptors, horizontal cells, bipolar cells, ganglion cells and inner and outer plexiform layers in human retinas and animal models (Craig *et al.,* 2008), the anti-inflammatory role of vitamin D has been evaluated in DR. It has been found that vitamin D reduced the production of proinflammatory mediators such as IL-2, IL-6, IL-8, IL-12 and TNF in the eye (Lefebvre d'Hellencourt *et al.,* 2003; Mitri *et al.,* 2011; Takahashi *et al.,* 2002; D'ambrosio *et al.,* 1998 ; Palomer *et al.,* 2008). These proinflammatory cytokines like IL-1β, IL-6 and TNF played critical role in the progression of DR (Vincent and Mohr, 2007; Behl *et al.,* 2008; Kocak *et al.,* 2010). Animal interventional studies performed on rats with DM by Albert et al. (2007) and

Ren et al. (2012) showed that vitamin D inhibited the expression of VEGF in retinal tissues and was DR protective, respectively. Likewise, in the mouse model of ischemic retinopathy, vitamin D was found to inhibit neovascularisation in the retina (Albert *et al.,* 2007). Recent study by Lu et al. 2018 demonstrated that vitamin D protected the retina by inhibiting the activation of some intracellular inflammatory mediators or pathways such as reactive oxygen species (ROS) and TRX-interacting protein (TXNIP)/NOD-like receptor family, pyrin domain-containing 3 (NLRP3) pathway in diabetic rats.

1.8 Retinal Nerve Fibre Layer

The RNFL is a layer of non-myelinated axonal ganglion cells which converge at the optic nerve head and passes through the lamina cribrosa forming myelinated axons and continues as the optic nerve (Atlas, 2007). It is the innermost retinal neuron layer, lying below the internal limiting membrane. It is commonly measured over the optic nerve head, where the superior and inferior halves of the retina do not cross the midline; while the macular fibres are distributed horizontally by forming papillomacular bundle. Distribution of the retinal nerve fibre is unequal around the optic nerve head. The neuroretinal rim thickness follows the ISNT rule in decremental order, whereby the inferior rim is thicker than superior rim, and the superior rim is thicker than the nasal rim and followed by temporal rim (Harizman *et al.,* 2006).

Quigley et al. (1982) found that 40% of the nerve are irreversibly damaged before peripheral vision loss can be clinically demonstrated. The advent of optical coherence tomography (OCT) offers a non-invasive, reliable tool for the early detection of ganglion cell damage by measuring the RNFL thickness.

1.8.1 Vitamin D and Retinal Nerve Fibre Layer

As recent studies have elucidated, retinal neurodegeneration precedes DR in diabetics (Vujosevic and Midena, 2013). Evidences found by researchers showed that retinal neuron undergone apoptosis secondary to accumulation of the AGE products, and upregulations of VEGF in the glial cells of retina as mentioned earlier (Amin *et al.*, 1997). Vitamin D deficiency was found to have related with high mortality secondary to macrovascular complication. It was demonstrated that vitamin D had inhibitory role by exerting its action binding on the cyclic AMP response element binding protein in the renin gene promotor to halt the renin gene transcription (Li *et al.*, 2011). Upregulation of the renin-angiotensin system will also upregulate the inflammatory cells within the vessel wall. This will affect the endothelial cell dysfunction and increase in vascular permeability and disrupt the microcirculation of the optic nerve head (Gungor *et al.*, 2015). In their animal model study, Li et al. (2004) found that vitamin D regulates the renin-angiotensin system for the optimization of blood pressure.

In the animal study by Riaz et al. (1999), vitamin D was found to be involved in regulating neurotrophin and neuronal calcium homeostasis, thus offering neuroprotection to the retinal neuron. The neuroprotection role of vitamin D was later elaborated by Kalluef et al. (2004), and noted vitamin D reduced the reactive oxygen species, nitric oxide and hydrogen peroxide that could possible cause neuronal cells and endothelial cells damage which triggered VEGF and other growth factors over expressed. VEGF is known for its action as angiogenesis when responded to cellular hypoxic injury. In addition to that, VEGF is expressed in normal condition in a balance level to maintain the neuron function by improving the survival of ganglion neuron and

to stimulate axonal growth (Imai *et al.,* 2009). The overexpression of VEGF is the pathognomonic disease progression of DR.

1.8.2 Diabetic Retinopathy and Retinal Nerve Fibre Layer

Pathogenesis of DR has evolved from microangiopathy disease to microvascular neurodegeneration disease characterised with impairment of neurovascular coupling at the retina in type 2 DM. Evidence of neurodegeneration was first detected by multifocal electroretinogram (mfERG) with abnormal amplitude and delay latency (Antonetti *et al.,* 2012; S Stem and W Gardner, 2013). It was also stated that neurodegeneration in DR can occur even before the clinical evidence of microaneurysm (Harrison *et al.,* 2011). Thus, a reproducible and quantitative method on assessing the retinal thickness may be pivotal to aid in managing DR.

However, there are many factors that may contribute to a less accurate retinal thickness. Conditions like underlying optic neuropathy, high myope with peripapillary atrophy and glaucoma neuropathy will contribute to thinner RNFL. Most of the studies show significant thinning of the RNFL in DM patient with preclinical DR or NPDR, however, there were confounding factors which limiting the strength of meta-analysis studies (Chen *et al.,* 2015). Whether the RNFL in type 2 DM patient without DR is much thinner than the normal population is still unclear and the usefulness as screening tool for early neurodegeneration in DR patient is still debatable (Skarf, 2002). Albeit, neurodegeneration in retinal cause retinal neuron undergo apoptosis and eventually atrophy, the early phase of the disease as in NPDR may have subclinical microvascular damage which compromised vascular permeability that occur over the peripapillary region may have account for the RNFL thickness as well.

Rationale of the study

DR has lately been recognised as a neurovascular disorder whereby neurodegeneration may precede microangiopathy (Lynch and Abramoff, 2017; Sohn *et al.,* 2016). Various molecular studies have been done to investigate and truly understand the effects of metabolic disturbance induced by hyperglycemia in type 2 DM. Muller cells which serve as the main supportive cell to both the retinal neuron and retinal vessels undergo gliosis in hyperglycemic conditions. It sustains damage by the accumulation of AGE products, ROS and hydrogen peroxide particles. There is an imbalance between neurotrophin and neurotoxin in the retina and with high expression of VEGF following tissue injury and Muller cell gliosis, leading to the accumulation of glutamate which is excitotoxic to the retina. This will cause more damage to the neurons and subsequently the neurons undergo apoptosis.

The role of vitamin D is not only confined to bone metabolism and calcium homeostasis. Presence of VDR in the retinal neuronal layer is postulated to be related to the role of vitamin D in DR (Bouillon *et al.*, 2008; Johnson *et al.,* 1996; Verstappen *et al.,* 1986). Vitamin D has been postulated to be neuroprotective and prevent angiogenesis in the retinal cells by immunomodulating and down regulating the expression of proinflammatory cytokines, NF-kB. It also acts as an antioxidant towards ROS and hydrogen peroxide particles to reduce local tissue damage and to preserve the neurovascular coupling in the retina.

Thus far, there are no clinical signs to suggest the presence of retinal neurodegeneration. It was initially detected by electroretinogram in DM patients (Antonetti *et al.,* 2012; S Stem and W Gardner, 2013). Currently, SD-OCT is commonly used to quantify

neuronal loss and monitor progression in cases of optic neuropathy. Measurement of RNFL thickness is a simple, non-invasive test which is readily available in ophthalmology clinics. It provides an objective, quantifiable result, is easy to operate and it is non-operator dependent.

SD-OCT can be used as a screening tool to demonstrate reduced RNFL thickness in diabetic patients with NPDR and concurrent vitamin D deficiency. Reduced RNFL thickness in such patients could be an indicator of early retinal neurodegeneration and offer prognostic value for visual potential. Vitamin D supplementation may be a potential treatment strategy to ameliorate diabetic neurodegeneration in high risk patients.

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