Effect of Laser Photocoagulation Before Vitrectomy to Hypoxia Inducible Factor-1alfa and Intracellular Adhesive Molecule-1 in Diabetic Patients: A Randomized Clinical Trial

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ABSTRACT

Purpose: The vitreous concentration of Hypoxia-inducible Factor-1α (HIF-1α) and Intercellular Adhesive Molecule-1 (ICAM-1) were related to the permeability of retinal vessels and the grades of macular edema in proliferative diabetic retinopathy (PDR). Prior studies have showed that pan-retinal photocoagulation (PRP) is beneficial in treating PDR. The aim of this study is to determine how pre-treatment with PRP before vitrectomy affect the vitreous level of HIF–1 and ICAM-1 in patients with PDR.

Materials and Methods: A randomized clinical trial study was conducted to 22 eyes in Cipto Mangunkusumo National General Hospital, Indonesia. At the beginning of PRP, just before vitrectomy, and at 2, 4, and 12 weeks after vitrectomy, central macular thickness (CMT) was measured using optical coherence tomography (OCT). Undiluted vitreous humour was extracted during vitrectomy to obtain HIF-1α and ICAM-1 concentration.

Results: In the control and the photocoagulation group, the average level of HIF-1α (ng/mL) were 0.152±0.015 and 0.164±0.033 respectively. The average level of ICAM-1 (ng/mL) in control group and pre-treated group were 17,840±14,140 and 27,027±10,452 respectively. No statistically significant difference was seen in the level of HIF-1α and ICAM-1 between each group. The correlation between vitreous ICAM-1 and HbA1c was statistically significant (r=0.463, p=0.03). No significant differences for CMT at pre-vitrectomy, or 2 and 4 weeks after vitrectomy. Statistically significant difference was observed at 12 weeks after follow-up (p=0.049). The correlation between vitreous level of HIF-Iα and CMT in the control and laser group are r = 0.447 and r = 0.32, respectively.

Conclusion: Laser photocoagulation 1-2 weeks prior to vitrectomy did not lower vitreous concentration of HIF-1α and ICAM-1.

Keywords: Diabetic retinopathy, Light coagulation, Hypoxia-Inducible Factor 1, Intercellular Adhesion Molecule-1.

INTRODUCTION

Chronic hyperglycaemia causes many complications to the blood vessels. When this condition affects blood vessels in the retina, it will cause diabetic retinopathy (DR).[1] Proliferative diabetic retinopathy (PDR) occurs when neovascularization formed in or around the retina. This condition is the most prevalent cause of vision loss in diabetic patients with diabetic retinopathy.[2,3] PDR accounts for the highest retinal vessel disease found in Cipto Mangunkusumo National General Hospital (RSCM), Indonesia. Between the year 2004 and 2009, out of 3988 DR patients in RSCM, 38.3 % suffered with PDR and in 2010–2012 the percentage of PDR patients increased up to 47.9 %.

The mechanism on how diabetes becomes a risk factor for vascular changes in retinopathy remains inconclusive. Several biochemical mechanisms related to hyperglycaemia have been associated with the pathogenesis

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of DR, including oxidative stress, polyol and hexosamine pathway flux, increased advanced glycation end product (AGE) synthesis, and activation of protein kinase C (PKC) isoforms. However, a general agreement on the relationship between hyperglycaemia and vascular disruptions remains a discussion.[4, 5]

Recently, the focus has been shifted to the effect of hypoxia on the retinal nerve cells with secondary effects on the retinal vasculature. In PDR, the state of ischemia in the retina occurs due to microvascular obstruction and capillary non–perfusion. Increased level of hypoxia has also been linked to increased production of hypoxia–regulated vasoproliferative factors such as vascular endothelial growth factor (VEGF) in DR. Vascular endothelial growth factor (VEGF) is regarded as the main cause of angiogenesis in the retina and it also plays a role in the disruption of the blood retina barrier. It was discovered that VEGF is up-regulated by Hypoxia–Inducible Factor 1 (HIF–1). HIF–1α is an alpha, beta–heterodimeric transcription factor that triggers cellular responses to ischaemic states via the activation of specific genes transcription, such as VEGF.[6,7] Activation of HIF results in a metabolic shift that triggers pathological angiogenesis and degeneration of nerve cells. This activation can be caused by various cellular stresses, such as inflammation, starvation, and hypoxia.[8]

Numerous studies in DR patients have described an increase in cellular adhesion molecules, specifically the intercellular adhesion molecule–1 (ICAM–1). This adhesion molecule has an important role in the progression of diabetic retinopathy. A significant rise in ICAM-1 expression was found upon disturbance to the retinal endothelium.[9] The concentration of VEGF and ICAM-1 in vitreous were related to the permeability of the retinal vessel and the grades of diabetic macular edema (DME).[10] The increased activation and expression of ICAM-1 are thought to stimulate leukostasis in diabetic retinopathy.[1]

These novel biomolecular risk factors, HIF–1α and ICAM–1, was not only a potential biomarker, but they also give insights into the advancement of potential target molecules for treating DR that requires vitreous surgery and pan-retinal photocoagulation (PRP). One study showed that pre-treatment with PRP before vitrectomy reduces cytokines activity in DR. However, in several cases, pre-treatment might also results in macular edema.[12] Nevertheless, it will be of great advantage to have a deeper understanding on how these treatment options for PDR, affects those cytokines. The aim of this study is to determine how pre-treatment with laser PRP before vitrectomy affects the level of HIF-1α and ICAM-1 in the vitreous of patients with PDR.

MATERIALS AND METHODS

Study Design

This study is a prospective, post–test only open-labeled randomized clinical trial. This study was conducted from August 2015 to April 2016. The subjects enrolled for this study were diabetic patients with PDR in Cipto Mangunkusumo National General Hospital, Indonesia. Enrolled patients then evaluated for the duration of DM, blood pressure, serum HbA1C level, blood glucose level, and ophthalmic examination which consist of intraocular pressure (IOP), fundus photography, best corrected visual acuity, and central macular thickness (CMT) as measured using optical coherence tomography (OCT), (BCVA) using Snellen Chart (in logMAR).

Inclusion and Exclusion Criteria

DR patients who require pars plana vitrectomy (PPV) and have no history of retinal photocoagulation were included. The criteria included type I/II diabetic patients with age ≥ 18 years old and diagnosed with proliferative diabetic retinopathy that require PPV. The indications of PPV were prolonged vitreous haemorrhage and vitreomacular traction.

Patients with uncontrolled hypertension (blood pressure ≥ 180/110 mmHg), other anterior or posterior segment diseases that could interfere the effect of therapy, any previous surgical/injection/laser treatment to the studied eye, and significant media opacity that could obscure the examination and therapy were excluded.

Patients Enrolments

Consecutive sampling was conducted and subjects were randomized using block randomization into two separate groups: the first group was treated directly with PPV (control) and the second group was pre–treated with PRP. If both eyes were eligible, only one eye with higher degree of DR was chosen to be enrolled. Generation of random allocation, participant enrolment, and assignment of participants into each group were performed by at least two researchers. Thirty-four patients were assessed for eligibility and 12 patients were excluded. Ten out of 12 patients did not meet the inclusion criteria and two patients refused to participate due to house distance and the severity of diabetes.

Institutional ethical clearance was approved by the Ethical Clearance Committee of the Faculty of Medicine, Universitas Indonesia with an ethical approval number of 229/UN2.F1/ETIK/2015. All participants signed an

informed consent form after being explained about the procedure and possible consequences of the experiment.

Subjects who met the inclusion criteria underwent BCVA examination using Snellen chart, fundus photograph with Topcon 3D–OCT 2000, and macula OCT examination using Carl Zeiss Stratus OCT 4. Macula OCT examination was analysed by a certified masked examiner.

Pan Retinal Photocoagulation (PRP)

Laser photocoagulation was performed in the dilated pupil of the case group, using Super Quad® 160 lens (Volk Optical Inc, USA). It was performed by one vitreo–retina specialist using laser 532nm (Visulas 532s, Carl Zeiss) with uniform parameters: 200–300µm, exposure time 100ms, 1.5 spot size interval, the number of laser spots were determined by the attending ophthalmologist, total of 500–600 spots on average were usually sufficient to cover the ischaemic area in this study. Power was titrated until it formed a barely visible or light grey laser burn. Additional laser photocoagulation can be performed during or after vitrectomy if necessary. In the control group, no prior laser photocoagulation was carried out.

Vitrectomy and Specimen Collection

Vitrectomy was carried out two weeks after laser photocoagulation of the case group using a standardised three–port pars plana vitrectomy. A 1 mL syringe attached to the vitreous cutter at the beginning of vitrectomy was used to suction 0.5 to 1 mL of undiluted vitreous samples before intra-vitreous infusion of balanced salt solution. Each vitreous sample obtained were divided into two sterile tubes, one for HIF–1α and one for ICAM-1. Those were placed immediately on ice and centrifuged at 13000 G for 5 minutes at 4°C. Supernatants were kept frozen at –80°C until assayed. The same procedures were performed to the control group.

Laboratory Assay

The undiluted vitreous samples were processed using enzyme–linked immunosorbent assay (ELISA) (Elabscience Biotechnology, Guandong, Cina). It was used to measure the concentrations of immunoreactive HIF–1α. This procedure was performed in a two–step sandwich–type immunoassay protocol using microwell plates coated with a HIF–1α antibody and anti–HIF–1α detection antibody labelled with horseradish. The inter–assay and intra-assay coefficient of variation (CV) was 3.5% and 3.3% respectively.

The vitreous concentration of ICAM–1 was measured using ELISA for ICAM–1 (R&D System, Minneapolis, Canada). The assay was run based on the manufacturer's standards. The standard solution was introduced to the wells of a 96–well plate coated with monoclonal antibody. After incubation, the plate was rinsed, and an enzyme labelled antibody was introduced. The incubation process was then continued, the plate was rinsed again, and the sample was added. After the colour changes, the reaction was halted using stop reagent. Absorption spectrophotometer with optical density of 450 to 620 nm was used.

Follow–up Protocol

Follow–up sessions were conducted at 2nd, 4th, and 12th weeks post-surgery. The BCVA and CMT (using OCT examination) were measured for evaluation. Patients were also interviewed for any subjective side effects on each visit.

Statistical Analysis

The outcomes were recorded in mean or median and range. The Statistical Package for Social Sciences Program version 22.0 (SPSS–PC; SPSS Inc, Chicago, Illinois, USA) was used to perform all statistical analyses. The distribution normality was authenticated using of the Shapiro–Wilk normality test. The level of statistical significance for this study is $P<0.05$. The significance of differences between the mean values was tested by the paired t-test. To determine any significant correlations, the Spearman-rank correlation coefficient test was used.

RESULTS

Based on the data collected, female and male ratio was 63.6:36.4. The age of the subjects was between 41- to 66-year-old, mean age at 53.05±5.75 years-old. Baseline clinical characteristics between the two groups showed no statistically significant difference ($p>0.05$), except for the mean HbA1c level ($p=0.007$). HbA1c level in laser photocoagulation group was higher, at 9.64±1.69% (Table 1).

Vitreous Level of HIF–1α and ICAM–1

The concentration of HIF–1α (table 2) in the control group and pre-treated group was 0.152 ± 0.015 ng/mL and 0.164 ± 0.033 ng/mL respectively ($p=0.265$, non-paired t-test). The vitreous concentration of ICAM-1 is 17.840 ± 14.140 ng/mL in control group and 27.027 ± 10.452 ng/mL in pre-treated group ($p=0.099$, non-paired t-test). No significant statistical difference was observed in HIF–1α and ICAM–1 vitreous level between control and pre-treated group (table 2). Clinically, vitreous concentration of ICAM–1 tends to be higher in pre-treated group compared to control group.

Based on the scatter plot diagrams (in figure 1), the

Table 1: *Baseline Characteristics.*

Characteristics	Groups		p
	Control (n=11)	Pre-treated (n=11)	
Age (years) ±SD	53.36±7.58*	52.73±3.43*	0.802[a]
Gender Male Female	 6 (54.5%) 5 (45.5%)	 2 (18.2%) 9 (81.8%)	0.201[b]
DM duration (years) ±SD	12.36±7.86*	12 (5;15)**	0.974[c]
Total Cholesterol (mg/dL) ±SD	214.82±48.41*	247.55±72.07*	0.226[a]
Sistolic (mmHg) ±SD	155.45±15.72*	155.91±22.45*	0.957[a]
Diastolic (mmHg) ±SD	82.73±11.90*	83.64±8.09*	0.836[a]
HbA1c(%)±SD	7.90±0.89*	9.64±1.69*	0.007[a]
CMT baseline (µm) ±SD	939.18±408.02*	834.73±319.39*	0.511[a]
CMT pre–vitrectomy (µm)±SD	905.82±391.24*	939.64±360.30*	0.835[a]
BCVA baseline (logMAR)±SD	1.92±0.10*	1.87±0.10*	0.219[a]
BCVA pre–vitrectomy(logMAR) ±SD	1.92±0.10*	1.87±0.10*	0.219[a]

[a]Non–paired t–test, [b]Chi Square, [c]Mann Whitney, *mean±SD, **Median (min;max), LogMAR: *Logarithm of the Minimum Angle of Resolution*

Table 2: *Vitreous level of HIF-1 alpha and ICAM-1.*

Variables	Group		
	Control	Pre-treated	p
Vitreous level of HIF-1 alpha (ng/mL)	0,152±0,015	0,164±0,033	0,265[a]
Vitrous level of ICAM-1 (ng/mL)	17,840±14,140	27,027±10,452	0,099[a]

[a]Unpaired t-test

concentration of vitreous HIF-1alpha increased as the level of HbA1c increases. In a similar fashion, the concentration of ICAM-1 tends to increase as the level of HbA1c increases. The HbA1c level has a positive correlation with vitreous ICAM–1 level, r = 0.463, p = 0.03 (see Fig.1). Even though, this correlation is statistically not significant, the same conclusion can be drawn between HIF-1 alpha and HbA1c (r=0.014, p=0.612).

Central Macular Thickness (CMT)

Statistical analysis was performed in 19 patients with CMT measurement from OCT examination on the 2nd, 4th, and 12th week follow up. OCT examination could not be performed in three patients due to vitreous opacity, cataract, and loss to follow-up. In the pre-treated group, CMT before vitrectomy was at 973 µm. At two, four, and twelve weeks after vitrectomy, CMT slowly decreases to 376, 251, and 232 µm, respectively. Meanwhile, in the control group, the baseline CMT was slightly lower at 934 µm. At two, four, and twelve weeks after vitrectomy, the CMT were 405, 270, and 286 µm, respectively. The CMT in the control group reduced progressively after vitrectomy however at the 12th week it was slightly higher than the pre-treated group. There was no statistically significant difference for CMT at baseline, pre–vitrectomy, 2 and 4 weeks after vitrectomy. However, statistically significance difference between the CMT of the two groups were achieved at 12 weeks of follow-up after vitrectomy (p=0.049). A linear graph of CMT changes at each follow up showed that pre-treated group had lower CMT than control group (figure 2).

Intragroup analysis showed that in pre–treated group, CMT was increased from 858.80±325.98 µm to 965.90±368.52 µm (p=0.077) after 1–2 weeks. In contrast, CMT in the control group showed only slight decrease from 958.78±419.48 µm to 934.44±410.42 µm during the 1–2 weeks prior to vitrectomy.

To investigate the relationship between CMT at the time of pre vitrectomy, HIF–Iα, and ICAM–1, correlation coefficient was calculated (Figure 3). There was a positive correlation between the level of HIF–Iα in the vitreous and

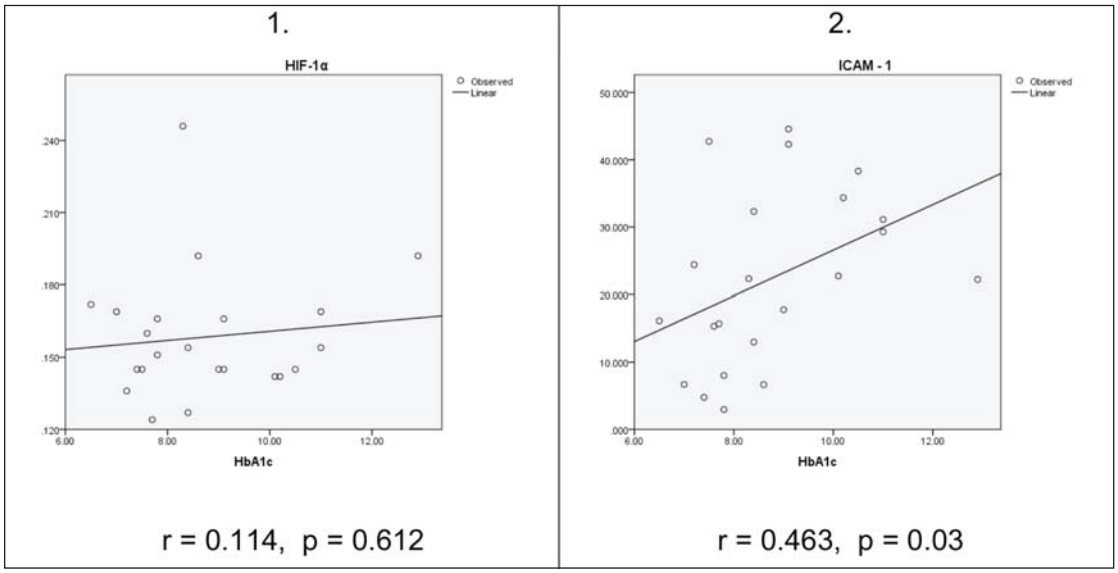

Figure 1: *Graph of (1) HIF-1 aplha and (2) ICAM–1 in the vitreous concentration sample of PDR patients against the concentration of HbA1c (r = 0.114, p = 0.612 and r = 0.463, p = 0.03).*

CMT in both groups (r = 0.447, p=0.168 and r=0.32, p= 0.543, rescpectively). Even though, it was not statistically significant.

COMPLICATIONS

There were seven cases of complication in this study. One patient had vitreous haemorrhage, one patient had hyphema, one patient had retinal detachment, two patients developed cataract, and two patients had glaucoma. Table 3 shows the number of intra-operative and post-operative complications for each group.

DISCUSSION

The mean concentration of HIF–1α (ng/mL) in the vitreous were 0.152±0.015 and 0.164±0.033, in the control and pre-treated group, respectively. The difference was not statistically significant (p=0.265, p>0.05). There was no previous study that compared these two groups. Numerous reports have described an increase in intravitreal HIF–1α concentration in patients with diabetic retinopathy. Loukovaraa et al[7] reported that the mean HIF-1 α were higher in PDR compared to controls with 0.53 ± 0.34 and 0.13 ± 0.04 ng/mL, respectively (p = 0.009). Wang et al[19] showed intravitreal HIF–1 α median level was greater in diabetic patients with PDR, which was 0.466 (0.346 to 1.449). These findings were similar to our baseline characteristics of HIF-1 α concentration in PDR patients which were 0.152 ± 0.015 ng/mL (control group) and 0.164 ± 0.033 ng/mL (laser PRP group).

Table 3: *Complications.*

Complications	Groups ∑(%)		Total
	Control	Pre-treated	
Vitreous Haemorrhage	–	1	1(4.5%)
Hyphema	1	–	1(4.5%)
Redetachment	1	–	1(4.5%)
Cataract	1	1	2(9.1%)
Glaucoma	1	1	2(9.1%)

There were several factors that may contribute to the insignificance difference of intravitreal concentration of HIF–1α between groups. First, this study has a relatively small sample size. The outcome of this study might not be the same with a larger sample size. Second, the laser photocoagulation group has worse glycaemic control compared to control group. This condition might interfere the level of HIF–1α vitreous before treatment. Another factor that might contribute to the difference is the laser parameter that was used in this study. The total laser spots were 500–600 which was lower than the recommended amount by ETDRS protocol[14] which was (1200–1600 spots). This amount, even though lower, was deemed sufficient to treat PDR according to the discussion held by the attending ophthalmologist. The presence of fibrovascular membrane and vitreous haemorrhage might also disturb the laser visualization. Hence, this condition can affect the expected retinal oxygen tension after laser photocoagulation

The physiological mechanism of photocoagulation is known to affect oxygen tension. Melanin in the retinal pigment epithelium absorbs physical light energy. The

neighbouring photoreceptors are terminated, and glial scar will form. Hence, the oxygen expenditure in the outer retina will be lowered. Oxygen that was usually transferred from the choriocapillary into the retina can now diffused through the laser photocoagulation-induced scars in the photoreceptor layer without being absorbed by the photoreceptors' mitochondria. This oxygen influx in the inner retina releases it from the state of ischaemic, increasing the oxygen tension.[20-22]

The mean intravitreal concentration of ICAM–1 (ng/mL) were 17.840±14.140 and 27.027±10.452 in the control and photocoagulation group, respectively. However, this difference was not statistically significant. The observed trend showed that the level of ICAM–1 in the vitreous was greater in the laser photocoagulation group than in the control group. Chidlow et al proved that laser photocoagulation induced proinflammatory cytokines that cause leukocyte adhesion.[23] Our study includes PDR patient with fibrovascular membrane that cause vitreomacular traction. Pathogenesis of vitreomacular traction remains unclear. One study proved Advanced Glycation End–products (AGEs), which are found on the posterior vitreous cortex and internal limiting membrane (ILM), might be responsible for the structural changes found in the posterior hyaloid that enhances the vitreomacular adherence between ILM and posterior hyaloid.[24,25]

In this study, both groups experienced reduction in CMT (figure 3). It was also shown that CMT was positively correlated with the level of HIF–1α but not with the level of vitreous ICAM–1. There were no other previous reports that proved the effect of these two–biomolecular agents to the CMT. An increase of CMT in laser photocoagulation group after treatment compared to baseline is showed in figure 2. Study by McDonald et al stated that 75 of 175 eyes (45%) had macular edema after panretinal laser photocoagulation.[26] Macular edema occurs immediately

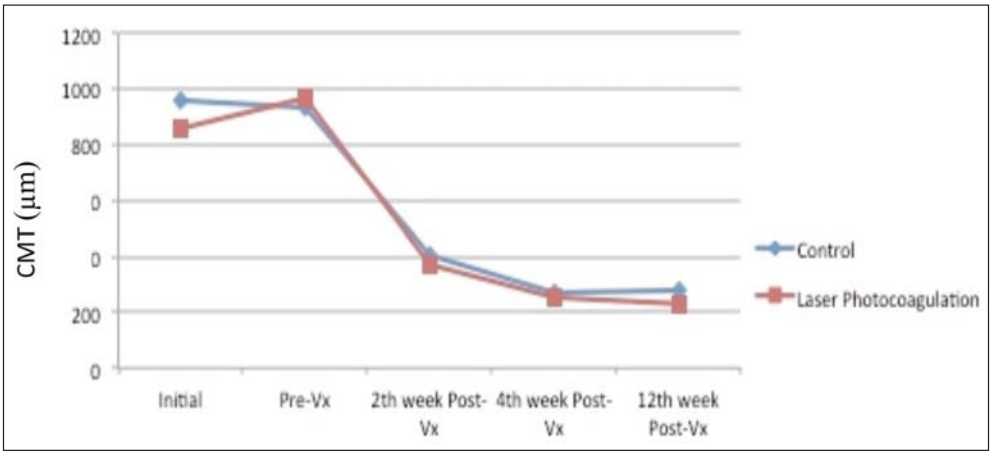

Figure 2. *Changes of CMT between the two groups during baseline, pre–vitrectomy, 2nd, 4th, and 12th week of follow-up. (*Vx = vitrectomy).*

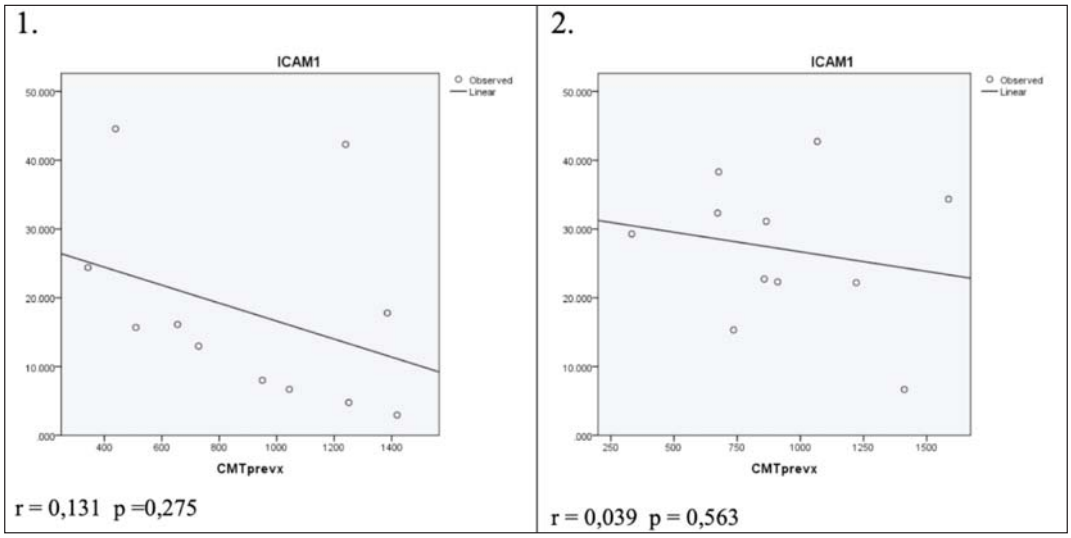

Figure 3: *Graph of CMT pre-vitrectomy against ICAM-1 concentration in vitreous in a) control group and b) laser photocoagulation group.*

after laser treatment, but it is temporary. Likewise, a study by Shimura et al showed that laser photocoagulation group before vitrectomy experienced a significant increase in macular thickness compared to the group that did not receive the laser pre-vitrectomy. The pathogenesis of post-laser photocoagulation macular edema is associated with inflammatory reactions in the retina.[27]{Heng, 2013 #76}

This study has several limitations. First, the possibility for recall error from the patients' data on DM durations as they were self-reported by the patients. Second, the baseline of HbA1c showed significant difference between the two groups (p=0.007). These could lead to undetected false baseline characteristics that could affect the concentration of HIF–1α and ICAM–1. Third, the small sample size could also explain the insignificant difference found in several findings. Further studies with a bigger sample size should be carried out to examine the effects of laser photocoagulation and vitrectomy with adjustable parameter. A more accurate measurement of vitreous HIF–1α and ICAM–1 concentration might be obtained from a longer duration of follow up.

CONCLUSION

Laser photocoagulation 1-2 weeks prior to vitrectomy did not lower vitreous concentration of HIF-1α and ICAM-1. The level of HIF–1α in the vitreous was positively correlated with CMT, whereas no such relationship was observed between the concentration of ICAM–1 and CMT.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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