COVER LETTER

Date: 11th March 2022

To
The Editor,
Bali Medical Journal
I am enclosing herewith a manuscript entitled:

COMPARISON OF SERUM AND VITREOUS TGF-β1 LEVELS IN PROLIFERATIVE DIABETIC RETINOPATHY WITH AND WITHOUT PANRETINAL PHOTOCOAGULATION LASER THERAPY

Here are attached point by point response to editor and reviewers' suggestion:

1. Ethical clearance number/statement and/or informed consent at the end of the manuscript (Confirmed).
Response:
It was already written in our manuscript.

2. Please state your conflict of interest in the paper. (Confirmed)
Response:
It was already written in our manuscript.

3. Please state the funding (if any) in your paper. (Confirmed)
Response:
It was already written in our manuscript.

4. Please state each author's contribution. (Confirmed)
Response:
It was already written in our manuscript.

5. Based on our proofreading application, we detected 76 critical grammatical errors.
Response: Thank you for your information. Based on your suggestion, we agree to send our manuscript to REVISE for grammar editing. Could you please inform us how to send it to REVISE?

6. Please describe the reason why this article evaluates specifically TGF- β1 instead of another marker.
Response: The selection of the TGF- β1 molecule as a marker in this study was based on the theory that TGF-β1 is a polypeptide member of the transforming growth factor beta family of cytokines. It is a secreted protein that performs many cellular functions, including the control of cell growth, cell proliferation, cell differentiation, and apoptosis (this statement was also added to our revised manuscript line 119-126). As far as the literature search that we did using the pubmed and medline databases, there were only about 247 studies discussing this biomarker where the relationship between TGF- β1 and proliferative diabetic retinopathy was only 77 articles in the last 10 years. The lack of data and the important role of this biomarker are two main reasons for us to select this marker as our research variable.

7. Please describe the selection of 14 patients in this study. Is there any randomization?
Response: In this study, we used a purposive sampling method in determining the sample without randomization. We realize that the limitation of our study is small of samples who met the inclusion criteria. Therefore, we suggested that the further study should recruit more samples and do randomization.

8. The Discussion was too short, please add more discussion regarding this study result.
Response: In this study, we have added some discussion about the overview of the TGF-B molecule (manuscript line 119-130), the relationship between the number of lasers burns and post-laser duration in influencing TGF-B1 levels (manuscript line 131-141) and the relationship between the outcome of PDR patients with serum and vitreous TGF-B1 levels (manuscript line 142-167).

If you have any further information and suggestion, please do not hesitate to inform us. Thank you.

Best regards,

Andi Muhammad Ichsan

ORIGINAL RESEARCH

COMPARISON OF SERUM AND VITREOUS TGF-β1 LEVELS IN PROLIFERATIVE DIABETIC RETINOPATHY WITH AND WITHOUT PANRETINAL PHOTOCOAGULATION LASER THERAPY

ABSTRACT

Background: A long-term diabetic retinopathy will cause an increase of several growth factors expression like Transforming Growth Factor β (TGF-β), a multipotent cytokine that involved in the process of endothelial cell proliferation.

Purpose: This study aims to observe the relationship between TGF-β1 levels in serum and vitreous fluid of Proliferative Diabetic Retinopathy (PDR) patients given Pan Retinal Photocoagulation (PRP) laser therapy.

Method: This was a cross-sectional study involving 14 patients with PDR. TGF-β1 levels of vitreous and peripheral blood were measured using Enzyme linked Immunosorbent Assay (ELISA) method.

Results: Our subjects consisted of 57.1% males with a mean age of 51.8 years, where dyslipidemia was the most common comorbid disease. Mean serum TGF-β1 level was 12,821.43 ± 5,253.16 pg/ml, while the mean value in vitreous was 3,692.86 ± 333.89 pg/ml. Meanwhile, there was no significant difference in serum and vitreous TGF-β1 levels between subjects with and without PRP laser therapy ($p>0.05$).

Conclusion: There were no significant correlation between TGF-β1 levels in proliferative diabetic retinopathy patients with and without pan retinal photocoagulation laser therapy. However, there was a decreasing trend in TGF-β1 levels in the vitreous fluid which indicates that PRP laser therapy has a positive effect on preventing the formation of neovascularization in the eye.

Keywords: TGF-β1 levels, proliferative diabetic retinopathy, panretinal photocoagulation laser

Fundings: None

Introduction

Diabetes Mellitus (DM) is a metabolic disease characterized by chronic hyperglycemia due to the failure of the pancreas to produce sufficient insulin or the occurrence of cell resistance in peripheral tissues.(1) This condition can cause damage to various organs including the heart, kidneys, and eyes, even become a risk factor for death from complications.(2,3) Epidemiological estimates in several studies predict that patients with DM will reach 380 million in 2025 with 4 million being at risk for visual loss due to Diabetic Retinopathy (DR).(4)

Diabetic retinopathy is classified into an early stage, namely Non-Proliferative Diabetic Retinopathy (NPDR), and an advanced stage, called Proliferative Diabetic Retinopathy (PDR).(5) The case of decrease in visual ability occurs due to two mechanisms, namely increased intraretinal vascular permeability which leads to macular edema and narrowing of the capillary blood vessels' lumen, to cause macular ischemia.(6)

The state of DR in the long term causes an increase in the expression of several growth factors such as Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF), basic Fibroblast Growth Factor (bFGF), and Transforming Growth Factor beta (TGF-β).(7) These biochemical molecules are known to trigger the occurrence of PDR which leads to the threat of permanent blindness in patients.(8) TGF-β is a multipotent cytokine that works through activin receptor-like kinase-1 (ALK1) and 5 (ALK-5).(9) It is involved in the process of endothelial cell proliferation, formation and degradation of extracellular matrix, as well as chemotactic and apoptotic processes that lead to the thickening of the capillary basement membrane and in impaired regulation of systemic blood vessels.(8–10)

The best treatment for RD patients is to control the blood sugar levels,(11) also, the therapeutic outcome is not aimed at curing or restoring visual function but at slowing the progression of vision loss.(12) Panretinal photocoagulation (PRP) laser therapy is reportedly effective for fulfilling these goals.(13) It is

performed when high-risk PDR is found to numb the ischemic area, thereby inhibiting further neovascularization.(14)

This study aims to observe the relationship between TGF-β1 levels in serum and vitreous fluid of PDR patients given PRP laser therapy. The results are expected to form the basis for preventing further complications in DM patients, specifically those already having visual complaints.

Material and methods

Study Design

This was a cross-sectional study involving 14 patients with PDR who had vitrectomy surgery. The history and laboratory tests were investigated to confirm the diabetic status of patients by checking fasting blood sugar and HbA1c levels. Furthermore, routine ophthalmological examinations were carried out including visual acuity test, intraocular pressure (IOP), examination of the anterior segment of the eye with slit-lamp biomicroscopy, as well as the posterior segment using funduscopy. The fundoscopic examination results were stated to be normal for NPDR and PDR, but only patients with PDR were analyzed. Furthermore, an analysis was conducted on the relationship between the duration and number of laser burns given to patients due to changes in TGF-β1 levels examined in the serum and vitreous fluids.

Sample Collection

Vitreous samples were taken using a vitrectomy machine with a volume of 700-1000 µl, while the serum using 3-5 ml blood samples were taken through the median cubital vein. Afterward, they were placed into a vacutainer tube for mobilization and storage.

TGF-β1 Assay

TGF-β1 levels were checked using the human TGF-β1 ELISA reagent kit (Cat. No. MN 55412, R & D Systems, Inc, Minneapolis, USA) where the standard range on the device was 31.2 – 2,000 pg/ml with a detection limit of 4.61 pg/ml.

Processing and Data Analysis

The data were grouped according to the purpose and type of data, then, they were statistically analyzed using SPSS software for Windows ver. 23.0. The normality test showed that the data distribution was abnormal, hence, the Mann-Whitney and the Spearman correlation test were used (Sig. p≤ 0.05).

Results

Observations were made to determine serum and vitreous TGF-β1 levels in patients with PDR with or without laser PRP. It was performed on 14 respondents with PDR and had experienced vitrectomy surgery. The univariate data presented in Table 1 shows that the study subjects consisted of 57.1% males and 42.9% females with a mean age of 51.8 years. The most common comorbid disease was dyslipidemia with a prevalence of 50.0%, the mean serum TGF-β1 level was 12,821.43 ± 5,253.16 pg/ml, while the mean value in vitreous was 3,692.86 ± 333.89 pg/ml.

Table 1 Descriptive data of samples

Characteristics	Variable	N	(%)	Description
Gender	Male	8	57.1	
	Female	6	42.9	
Age	<50 years	3	21.4	
	≥50 years	11	78.6	
Comorbidity	Dyslipidemia	7	50.0	
	Hypertension	6	42.9	
	No comorbid	1	7.1	

Serum TGF-β1 levels				
With PRP Laser Therapy	≤12000 pg/ml	3	21.4	Mean of serum TGF-β1 levels of all patients (laser and non-laser): 12,821.43 ± 5,253.16
	>12000 pg/ml	5	35.7	
Without PRP Laser Therapy	≤12000 pg/ml	4	28.6	
	>12000 pg/ml	2	14.3	
Vitreous TGF-β1 levels				
With PRP Laser T**herapy**	≤3,600 pg/ml	6	42,9	Mean TGF-β1 serum of all patients (laser and non-laser): 3,692.86 ± 333.891
	>3,600 pg/ml	2	14,3	
Without PRP Laser T**herapy**	≤3,600 pg/ml	1	7,1	

Table 2 shows the comparison of TGF-β1 levels between patients treated with and without PRP laser therapy. The statistical calculations showed no significant association with $p>0.05$ between serum and vitreous TGF-β1 levels in patients with PDR with or without a history of PRP laser therapy. However, there was a decreasing trend in TGF-β1 levels in the vitreous fluid which indicates that PRP laser therapy has a positive effect on preventing the formation of neovascularization in the eye.

Table 2. Comparative Analysis of Serum and Vitreous TGF-β1 Levels

Variable	Laser History	n	Mean	SD	p*
TGF Serum	Yes	8	14,187.5	5,338.9	0.245
	No	6	11,000.0	4,987.6	
TGF Vitreous	Yes	8	3,587.5	352.3	0.104
	No	6	3,833.3	273.3	

*Mann-Whitney test

Discussion

The number of male patients in this study was more than females, while the mean age was 51.8 years and the most common comorbid disease was dyslipidemia. According to Jeffrey G et al (2011), this is because the development of DR in women can be inhibited by sex hormone receptors. The PDR development can be inhibited by inhibiting hormone receptors, although this mechanism is still unclear.(15) Meanwhile, the age group above 50 years and a history of metabolic syndrome has been reported to be one of the risk factors in the development of DM and PDR.(16)

The mean value of TGF-β1 levels in both groups of patients with or without PRP laser therapy using a vitreous sample was 3,692.86 ± 333.89 pg/ml, while the mean serum level was 12,821.43 ± 5,253.16 pg/ml (Table 1). However, after therapeutic treatment (Table 2), the vitreous TGF-β1 level in the group treated with laser therapy showed lower values compared to patients who did not receive PSP laser therapy. In contrast, the serum samples showed higher values in patients that received PRP laser therapy. Consequently, it was concluded that the administration of laser therapy has a good effect in reducing the level of TGF-β1 locally in the eye but has no significant effect systemically.(17) Administration of PRP laser therapy improves the hypoxic state of the retina and the levels of cytokines in the vitreous fluid, thereby preventing proliferation and further neovascularization.(18) Shimura et al. (2009) stated that PRP laser therapy before vitrectomy surgery can reduce levels of angiogenic factors such as VEGF, IL-6, and TGFβ.(19)

TGF-1 is a polypeptide cytokine of the transforming growth factor beta family. It is a secreted protein that regulates cell growth (Cell cycle regulation and death), cell proliferation, cell differentiation, and tumor suppression. (20–22) TGF-β is released into the extracellular matrix as a latent protein complex integrated to a latency-associated protein and one of four TGF-β binding protein isoforms. TGF-β activation, which is necessary for biological activity, happens via poorly known mechanisms that most likely include proteolytic degradation of the related proteins and release of the TGF-β ligand. TGF-β1 is the most common and widely expressed isoform; most research have explored or used exogenous TGF-β1 as biomarker variable. (23)

It has been proposed that pericyte control of endothelial cell proliferation occurs by activation of pericyte-secreted latent TGF-β, which essentially entails proteolytic release of TGF- β from its binding protein.

Furthermore, among the six known TGF-β subtypes, only TGF-β1 is inhibitory for endothelial cells, and even then only for particular kinds of endothelial cells.(24)

In order to fulfil the tissue's oxygen demands, the retina's oxygenation status controls different growth factors that induce angiogenesis. Sustained hyperglycaemia caused by long-term diabetes causes many metabolic changes that contribute in retinal hypoxia. It would, however, promote some biomarkers such as insulin-like growth factor-I (IGF-I), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), endothelin (ET), pigment epithelium-derived factor (PEDF), and transforming growth factor-β (TGF-β). (25,26)

TGF-β signalling is necessary for retinal pericyte differentiation during retinal vascular development. The absence of TGF-β signalling results in the production of many microaneurysms, leaky capillaries, and retinal haemorrhages. Furthermore, the absence of differentiated pericytes begins a scenario of structural and functional alterations in the retina that are similar to those seen in diabetic retinopathy, indicating a related mechanism.(27)

TGF-β is a neuroprotective protein that contributes in the recovery from diverse neural damage. The activation of stress response proteins and the metabolic activity, such as aldehyde dehydrogenase 3A1 (ALDH3A1), hemeoxygenase-1 (HO-1), nuclear factor erythroid 2-related factor (Nrf2), and hypoxia-inducible factor (HIF), was linked to TGF-β1 mediated antioxidant signalling. It was also shown that TGF-β protects retinal ganglion cells (RGCs) from hyperglycaemia-induced damage by activating the antioxidant system, implying a possible anti-diabetic therapy for the treatment of diabetic retinopathy.(28)

According to Zorena et al. (2013), the threshold serum TGF-β1 values that showed a discriminative potential to predict the existence of DR were 443 pg/ml. It was determined by analyzing the receiver operating characteristic (ROC) curves. The computed sensitivity and specificity were 72% and 88%, respectively. TGF-β1 serum concentrations may be an additional criterion in predicting the incidence of DR, according to these findings.(29)

Serum TGF-β1 levels can be influenced by various other factors such as hypertension and dyslipidemia. (30,31) In conditions of hyperglycemia, TGF-β1 levels also increase, this is in line with several other studies which stated that elevated levels of TGF-β1 are also found in other systemic diseases such as diabetic nephropathy, lung and autoimmune diseases, cancer, cardiovascular disease, hyperglycemia, and hypercholesterolemia.(20,32–34)

Moreover, there are also a correlation between laser burning and duration on TGF-β1 levels. Measurement of laser timing in study by Xu et al. (2018) showed that after PRP laser therapy, TGF-β1 levels in the vitreous returns to normal in 3 weeks.(35) Previous study also showed that laser burn ranging from 1,200-1,500 and a spot size of 500 µm reduces oxygen demand outside the retina up to 20% as well as the levels of growth factors in the vitreous.(36) The number of burns in this range can be beneficial as it balance oxygen demand and availability to reduce hypoxic areas. Consequently, this lowers the levels of angiogenic factors including TGF-β1.(37,38) Meanwhile, the number of burn calculation is expected to provide information about the effect of PRP laser therapy which is believed to prevent severe visual loss for up to 2 years after therapy.(39,40)

Conclusion

Based on the results, there was no significant difference in serum and vitreous TGF-β1 levels between subjects with and without PRP laser therapy. However, the trend of TGF-β1 levels in subjects with PRP laser therapy was lower than those without panretinal photocoagulation laser therapy.

Acknowledgments

The author would like to thank and appreciate the team of nurses and staff of Hasanuddin University Hospital and JEC Orbita who were involved in the preparation, treatment and follow up of this patient.

Disclosure

Statement of Ethics

This study protocol was reviewed and approved by The Ethics Committee of Medical Research, Faculty of Medicine, Hasanuddin University with approval number: 515/UN.4.6.4.5.31/PP36/2021.

Conflict of Interest Statement

The authors state there is no conflict of interest in writing this article.

Funding Sources

This manuscript did not receive any funding.

Data availability statement

Not applicable

Author Contributions

HSM, RZA, AMI: conception or design of the work, performing the medical examination, analysis and interpretation of the data, laboratory examination and drafting the work. BD, JS: Supervision and quality check of the medical examination, caring for patients, performing follow-up after surgery. AS, ICI: project administration, data statistic evaluation, drafting the work and final check for publication.

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RESPONSE TO EDITOR AND REVIEWERS

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Here are attached point by point response regarding our manuscript submission:

1. Ethical clearance number/statement and/or informed consent at the end of the manuscript (Confirmed).

2. Please state your conflict of interest in the paper. (Confirmed)

3. Please state the funding (if any) in your paper. (Confirmed)

4. Please state each author's contribution. (Confirmed)

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Response: The selection of the TGF- β1 molecule as a marker in this study was based on the theory that TGF-β1 is a polypeptide member of the transforming growth factor beta family of cytokines. It is a secreted protein that performs many cellular functions, including the control of cell growth, cell proliferation, cell differentiation, and apoptosis (this statement was also added to our revised manuscript line 119-126). As far as the literature search that we did using the pubmed and medline databases, there were only about 247 studies discussing this biomarker where the relationship between TGF- β1 and proliferative diabetic retinopathy was only 77 articles in the last 10 years. The lack of data and the important role of this biomarker are two main reasons for us to select this marker as our research variable.

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Thank you.

Best regards,

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