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RESEARCH ARTICLE

Comparison of catalase, glutathione peroxidase and malondialdehyde levels in tears among diabetic patients with and without diabetic retinopathy

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Abstract

Background Various studies suggest that oxidative stress has a role in the etiology of diabetes mellitus (DM) and its complications. Detection of antioxidant enzymes and malondialdehyde (MDA) level in ocular fluid may provide the possible biomarkers for monitoring the progression of diabetic retinopathy (DR). The aim of this study was to compare catalase, glutathione peroxidase (GPx) and MDA levels in tears among diabetic patients with and without DR.

Methods A cross-sectional study was conducted among type 2 DM patients. The patients were divided into three groups: no DR, non-proliferative DR (NPDR) and proliferative DR (PDR). Tears samples were collected using Schirmer strips for measurement of catalase, GPx and MDA.

Results A total of 171 patients were recruited in this study (no DR, 58 patients; NPDR, 57 patients; PDR, 56 patients). There was significant difference in the mean level of GPx in tears between the three groups (no DR, 658.08 ± 115.70 U/L; NPDR, 653.78 ± 87.90 U/L; PDR, 605.31 ± 107.47 U/L, respectively) before and after adjustment for covariates ($p = 0.013$ and $p = 0.001$, respectively). Bonferroni post-hoc analysis showed PDR group had significantly lower mean GPx level than in no DR (p=0.001) and NPDR (p=0.037) after adjustment for covariates. There was no significant difference of mean catalase and MDA in the tears between the three groups before and after adjustment for covariates.

Conclusion This study demonstrated that diabetic patient with DR is associated with low level of GPx in tears, suggesting that this antioxidant enzyme is a potential biomarker for predicting the presence of DR.

Keywords Diabetic retinopathy · Oxidative stress · Tears · Antioxidant enzymes · Malondialdehyde

Introduction

Diabetic retinopathy (DR) is one of the common causes of blindness among population aged 25–74 years [1]. The prevalence of DR globally was 34.6% and 28% in Asian countries [2, 3]. The prevalence of blindness was 27% in non-proliferative diabetic retinopathy (NPDR) and 6% for proliferative diabetic retinopathy (PDR) among type 2 diabetes mellitus (T2DM) patients [3].

The pathogenesis of the DR is not clearly understood, but the established risk factors include poor blood sugar control, longer duration of diabetes mellitus (DM), increasing age and hypertension can contribute to its development [4]. Studies showed that oxidative stress contributes a significant role in the pathogenesis and its developments of the vascular and the neurological complication [5–8].

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Oxidative stress is an imbalance between the production of free radicals and antioxidant defenses. Free radicals are a group of atoms that have one or more unpaired electrons. Reactive oxygen species (ROS) is one of the types of free radicals. ROS, such as superoxide anion, hydrogen peroxide, and hydroxyl radical, are highly reactive and cause damage to proteins, nucleic acids, and lipids through multiple pathways including protein oxidation, nucleic acid oxidation and lipid peroxidation [9]. Malondialdehyde (MDA) is an end-product of lipid peroxidation. This compound is a reactive aldehyde and is one of the many reactive electrophile species that cause toxic stress in cells. The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism [10].

Antioxidants play significant roles in protecting cells from oxidant stress. There are two types of antioxidant: enzymatic antioxidants and non-enzymatic antioxidants. Superoxide dismutases, catalase and glutathione peroxidase (GPx) are the main enzymatic antioxidants, whereas vitamin E, vitamin C and glutathione are the main non-enzymatic antioxidants. Catalase is found in peroxisomes in eucaryotic cells. It is one of the major antioxidant enzymes with high activity and amounts in the cellular cells. It degrades hydrogen peroxide to water and oxygen, assisting superoxide dismutases in the complete neutralization of ROS [10]. GPx provides a mechanism for detoxification of peroxides in living cells. This reaction plays a crucial role in protecting cells from damage by free radicals, which are formed by peroxide decomposition. GPx reduces peroxides to alcohols, thus preventing formation of free radicals. It also catalyzes the reduction of hydrogen peroxide to stable alcohols and water [10].

ROS generated by high glucose is considered as a causal link between elevated glucose and the other metabolic abnormalities in the development of diabetic complications [11]. Possible sources of oxidative stress in DM include autooxidation of glucose, shifts in redox balances, decreased tissue concentrations of antioxidants such as reduced glutathione and vitamin E, and impaired activities of antioxidant defense enzymes such as superoxide dismutase and catalase [12–14].

Previous studies evaluating the enzymatic antioxidant levels in the serum of DM patients have reported that superoxide dismutase, GPx and catalase were significantly decreased in diabetic patients compared to non-diabetic group [15–17]. Serum superoxide dismutase, GPx and catalase were also significantly decreased in DM patients with DR [18]. In contrary, serum MDA was significantly elevated in DM patients with and without DR compared to non-diabetic group [18].

In the eye, retina is susceptible to oxidative stress in view of high content of polyunsaturated fatty acids and has high oxygen concentration [14]. Thus, lead to lipid peroxidation and play an important role in degenerative ocular diseases such as age-related macular degeneration, cataract, glaucoma, and DR [19]. Therefore, measuring the levels of the oxidative stress biomarkers in ocular fluid such as vitreous or aqueous may directly reflecting the pathophysiological process in the eye with DR. Previous studies reported that vitreous level of MDA was increased in the PDR group compared to NPDR [20] and non-diabetic eye disease [21]. However, there was limited study evaluating MDA or antioxidant activity in aqueous or vitreous due to relatively invasive procedure. Furthermore, aqueous, or vitreous sample collection is only ethically possible if performed during surgical intervention such as cataract and vitreoretinal surgery.

Tears has been shown to have numerous substances which protecting the eye from the pathological process. It also contains numerous enzymatic antioxidants such as catalase and GPx [22], antioxidant including ascorbic acid, glutathione, cysteine, and tyrosine [11], and vascular endothelial growth factor [23, 24]. Antioxidant levels in tears have been shown to reflect that of the aqueous fluid [25]. Detection of antioxidant levels in tears may thus be a less invasive, safe, and reliable method of assessing the oxidative stress level in ocular pathology.

In view of oxidative stress plays a role in pathophysiology of DM and its complications, the aim of this study was to compare catalase, GPx and MDA levels in tears among diabetic patients with and without DR. Tear collection is non-invasive and practically easy. Tear sample may provide as a potential non-invasive technique for monitoring the progression of DR.

Methods

Study design and participants

A cross-sectional study was conducted among T2DM patients from August 2014 to July 2016. The samples size was calculated using G Power 3.1.9. A total of 171 T2DM patients were enrolled in this study. T2DM patients aged between 35–65 years old with both males and females were included.

Those patients with ocular surface disease, glaucoma, retina disease other than DR, previous history of retina laser photocoagulation, ocular trauma or intraocular surgery less than 6 months were excluded from this study. Patients were also excluded if they have uncontrolled hypertension, and systemic conditions that influence the oxidative stress biomarkers such as autoimmune disease, malignant tumors, rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, giant cell arteritis, or taking antioxidant medication within 3 months period from the study.

Those who fulfilled the selection criteria were explained regarding the nature of the study and written consent was obtained. All patients that consented to this study had their

histories taken and underwent systemic examination to assess systemic disorders. The demographic data (age, gender, and ethnicity) and systemic comorbidities (hypertension, hyperlipidemia, ischemic heart disease, chronic kidney disease, and duration of DM) were obtained either from the patients or their medical record.

Then, the patients underwent ocular examination to assess the stages of DR and to rule out other ocular disorders. The classification of DR was based on the International Diabetic Retinopathy Severity scales [26], in which the DR stages was graded as no DR, NPDR, and PDR.

After eye examination, the patients were subjected for tears collection and blood taking for HbA1c measurement. About 2.5 ml venous blood was taken, collected into EDTA tube, and sent to laboratory for HbA1c analysis.

Tear collection and analysis

Tears sample was collected by using Schirmer strips as described by Ang et al. [24]. Right eye was selected for standardization in no DR group. For those with DR, the eye with the worst severity of DR was selected. Then, the Schirmer strips that wetted with tears was placed in a plain tube and kept in -80°C freezer.

Measurement of catalase, GPx and MDA was conducted in Central Research Laboratory. The collected Schirmer strips were soaked with 1000 uL of cold phosphate buffered saline to dissolve the enzyme from the Schirmer strips. Then, the samples were spun vigorously at 1000 microtiter for 30 s to mix thoroughly. Following that, the samples were further processed based on different procedure stated on the brochures individually for each kit. Catalase was analysed using EnzyChrom™ Catalase Assay kit (BioAssays, USA), GPx using EnzyChrom™ Glutathione Peroxidase Assay kit (BioAssays, USA) and MDA using MDA Assay kit (North West Life Sciences, USA).

Statistical analysis

The statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) Version 22. All values were tested for normal distribution and equal variances. Chi Square test and Fisher Exact test were used for comparison of categorical data. Whereas one-way analysis of variance (ANOVA) was used for comparison of numerical data between the different stages of DR groups. Analysis of covariance (ANCOVA) test was used to compare catalase, GPx and MDA levels with covariates between groups. Bonferroni post-hoc comparison was used to compare significant ANCOVA results. It was adjusted for age, duration of DM, HbA1c level, smoking, hypertension, hyperlipidemia, chronic kidney disease and ischemic heart disease. Significance of difference in values was determined by the $p < 0.05$.

Ethics statement

The study was approved by the Institutional Review Board (IRB) of Universiti Sains Malaysia (USM) [ref: USM/Jawatankuasa Etika Penyelidikan Manusia (JEPeM)/276.2.(4)] and followed the tenets of the declaration of Helsinki. Written and informed consent of participants was obtained for each patient prior to the study.

Results

Demographic data and clinical profiles

A total of 171 patients (no DR, 58 patients; NPDR, 57 patients; and PDR, 56 patients) were recruited in this study. Overall, there were 120 (70.2%) males and 51 (29.8%) females with no significant difference of gender distribution between no DR, NPDR, and PDR groups. Most of the patients were Malay (60.2%) and followed by Chinese (24.0%) with no significant difference of ethnicity distribution between the three groups. The mean age was 54.4 ± 13.2 years in the no DR, 58.4 ± 8.6 years in the NPDR, and 52.1 ± 11.6 years in the PDR groups. There was significant difference in mean age between the three groups ($p = 0.013$), in which the NPDR group had the oldest mean age among the three groups. Duration of DM was 9.1 ± 6.0 years in the no DR, 14.9 ± 7.4 years in the NPDR, 14.1 ± 8.8 years in the PDR groups. There was significant difference of duration of DM between the three groups ($p < 0.001$) in which duration of DM were longer in DR group (NPDR and PDR) than in no DR group. There was significant difference in mean HbA1c level among the three groups ($p < 0.001$) where HbA1c was higher in NPDR ($9.3 \pm 2.3\%$) and PDR ($9.2 \pm 2.4\%$) than in no DR ($7.7 \pm 1.8\%$). There was no significant difference of smoking status and comorbidities (hypertension, hyperlipidemia, ischemic heart disease, and chronic kidney disease) between the three groups. The demographic data, clinical profiles, and comorbidities between the three groups among DM patients is shown in Table 1.

Catalase, GPX and MDA level in tears

The mean catalase in the tears was 1596.42 ± 789.24 U/L in the no DR, 1408.21 ± 796.32 U/L in the NPDR, and 1863.67 ± 3172.84 U/L in the PDR group. There was no significant difference of mean catalase in tears between the three groups before and after adjustment for age, duration of DM, HbA1c, smoking, hypertension, hyperlipidemia, chronic kidney disease, and ischemic heart

Table 1 Demographic data, clinical profiles, and comorbidities between the three groups

Variables	No DR ($n=58$) n (%)	NPDR ($n=57$) n (%)	PDR ($n=56$) n (%)	p value
Age (year)*	54.4 (13.2)	58.4 (8.6)	52.1 (11.6)	0.013[a]
Gender				
Male	37 (63.8)	45 (79.0)	38 (67.9)	0.186[b]
Female	21 (36.2)	12 (21.0)	18 (32.1)	
Race				
Malay	30 (51.7)	31 (54.4)	42 (75.0)	0.055[c]
Chinese	14 (24.1)	16 (28.1)	11 (19.6)	
Indian	13 (22.5)	10 (17.5)	2 (3.6)	
Others	1 (1.7)	0 (0.0)	1 (1.8)	
Duration of DM (years)*	9.1 (6.0)	14.9 7.4)	14.1 (8.8)	<0.001[a]
HbA1c (%)*	7.7 (1.8)	9.3 (2.3)	9.2 (2.4)	<0.001[a]
Smoking				
No smoking	40 (69.0)	36 (63.2)	34 (60.7)	0.555[c]
Smoking	6 (10.3)	10 (17.5)	6 (10.7)	
Ex-smoking	12 (20.7)	11 (19.3)	16 (28.6)	
Hypertension				
No	14 (24.1)	8 (14.0)	11 (19.6)	0.389[c]
Yes	44 (75.9)	49 (86.0)	45 (80.4)	
Hyperlipidemia				
No	20 (34.5)	15 (26.3)	17 (30.4)	0.636[c]
Yes	38 (65.5)	42 (73.7)	39 (69.6)	
Chronic Kidney Disease				
No	53 (91.4)	51 (89.5)	45 (80.4)	0.173[c]
Yes	5 (8.6)	6 (10.5)	11 (19.6)	
Ischemic Heart Disease				
No	48 (82.8)	52 (91.2)	44 (78.6)	0.170[c]
Yes	10 (17.2)	5 (8.8)	12 (21.4)	

*Mean (Standard deviation)

p values were calculated using [a]One-way analysis of variance (ANOVA), [b]Pearson Chi Square test, and [c]Fisher Exact Test

DM = diabetes mellitus, *DR* = diabetic retinopathy, *NPDR* = non-proliferative diabetic retinopathy, *PDR* = proliferative diabetic retinopathy

disease ($p=0.454$ and $p=0.600$, respectively) (Table 2 and Table 3).

The mean GPx in the tears was 658.08 ± 115.70 U/L in the no DR, 653.78 ± 87.90 U/L in the NPDR, and 605.31 ± 107.47 U/L in the PDR group. There was significant difference of mean GPx in tears between the three groups before and after adjustment for age, duration of DM, HbA1c, smoking, hypertension, hyperlipidemia, chronic kidney disease, and ischemic heart disease ($p=0.013$ and $p=0.001$, respectively) (Table 2 and Table 3). Bonferroni post-hoc analysis showed the PDR group had significantly lower mean GPx level than in the no DR ($p=0.001$) and NPDR ($p=0.037$) after adjustment for age, duration of DM, HbA1c, smoking, hypertension, hyperlipidemia, chronic kidney disease, and ischemic heart disease (Table 4). However, there was no significant difference of mean GPx in tears between no DR and NPDR ($p=0.618$) (Table 4).

The mean MDA in the tears was 26.83 ± 19.15 µM in the no DR, 31.08 ± 23.15 µM in the NPDR, and 26.33 ± 24.30 µM in the PDR group. There was no significant difference of mean MDA in tears between the three groups before and after adjustment for age, duration of DM, HbA1c, smoking, hypertension, hyperlipidemia, chronic kidney disease, and ischemic heart disease ($p=0.459$ and $p=0.720$, respectively) (Table 2 and Table 3).

Discussion

Oxidative stress is a condition when there is an imbalance between the production of free radical and antioxidant defense mechanism. It plays a significant role in certain metabolic diseases such as diabetic eye disease and its complications. Diabetic patient with high level of oxidative stress status contribute to the risk of neuropathy [6], nephropathy [7] and retinopathy [8]. Previous studies had been conducted to look for the correlation of oxidative stress level and antioxidant in serum, vitreous and aqueous among DR patients [21, 27, 28]. This current study is to evaluate the oxidative stress level in tears among diabetic patients.

Table 2 Comparison of catalase, glutathione peroxidase and malondialdehyde in tears between the three groups

Variables	No DR Mean (SD)	NPDR Mean (SD)	PDR Mean (SD)	p value
Catalase (U/L)	1596.42 (789.24)	1408.21 (796.32)	1863.67 (3172.84)	0.454
Glutathione peroxidase (U/L)	658.08 (115.70)	653.78 (87.90)	605.31 (107.47)	0.013
Malondialdehyde (µM)	26.83 (19.15)	31.08 (23.15)	26.33 (24.30)	0.459

One-way analysis of variance (ANOVA) test, $p<0.05$, significant

Abbreviation:

DR: diabetic retinopathy, *NPDR*: non-proliferative diabetic retinopathy, *PDR*: proliferative diabetic retinopathy

Table 3 Comparison of catalase, glutathione peroxidase and malondialdehyde in tears between the three groups after adjusted for covariates

Variables	No DR Mean (95% CI)	NPDR Mean (95% CI)	PDR Mean (95% CI)	*p* value
Catalase (U/L)	1446.09 (646.38, 2245.80)	1346.87 (510.19, 2183.55)	1731.71 (985.24, 2478.19)	0.600
Glutathione peroxidase (U/L)	703.45 (662.64, 744.26)	676.72 (634.03, 719.42)	625.62 (587.53, 663.71)	0.001
Malondialdehyde (μM)	23.65 (14.49, 32.80)	27.48 (17.90, 37.06)	25.54 (17.00, 34.09)	0.720

Analysis of covariance (ANCOVA) test adjusted for age, duration of DM, HbA1c, smoking, hypertension, hyperlipidemia, chronic kidney disease, and ischemic heart disease, $p < 0.05$, significant

Abbreviation:

DR: diabetic retinopathy, *NPDR*: non-proliferative diabetic retinopathy, *PDR*: proliferative diabetic retinopathy

Table 4 Bonferroni post-hoc comparison of mean glutathione peroxidase in tears after adjusted for covariates

Variable	Group	Mean difference (95%, CI)	*p* value
Glutathione peroxidase (U/L)	No DR – NPDR	26.73 (-24.18,77.63)	0.618
	No DR – PDR	77.83 (27.96, 127.70)	0.001
	NPDR – PDR	51.11 (2.232, 99.98)	0.037

Bonferroni post-hoc comparison test adjusted for age, duration of DM, HbA1c, smoking, hypertension, hyperlipidemia, chronic kidney disease, and ischemic heart disease, $p < 0.05$, significant

Abbreviation:

DR: diabetic retinopathy, *NPDR*: non-proliferative diabetic retinopathy, *PDR*: proliferative diabetic retinopathy

Oxidative stress is increased in diabetic eye disease. Superoxide dismutase, GPx, glutathione reductase and catalase are the enzymatic antioxidants that responsible for the removal of free radicals. In this study, we evaluated GPx and catalase level in tears. We found that there was significant lower level of GPx in tears in the PDR than in the no DR group. Among DR patients, the GPx level in tears was significantly lower in the PDR than in the NPDR. There have been no previous studies that compared the level of GPx in tears between different stages of DR. However, there has been a study comparing the level of GPx in the tears in other retinal disorder [22]. Koh et al. [22] evaluated GPx level in tears among age-related macular degeneration (ARMD) patients and found that the level of GPx in the tears was significantly lower in patients with ARMD than those in control group. Study done by Said et al. [29] evaluated GPx in serum found that serum GPx was decreased in diabetic patients with retinopathy than those without retinopathy.

Serum GPx has shown to have significant correlation with the severity of DR [30, 31]. The chronic glycation state of the diabetic patients causes more oxidative stress and reduced amount of antioxidant to reduce its detrimental effect. However, the actual mechanism the cause of reduce level of GPx in diabetic patients is unknown. Darmaun et al. [32] postulated that the depletion of glutathione arises from increased glutathione utilization but not due to decrease in the rate of synthesis. Beside DM itself, there are multiple factors that can influence the level of GPx in body fluid such as systemic comorbidities, glycemic control, duration of DM, and smoking status [33, 34].

Catalase is a common antioxidant enzyme which also responsible for the removal of free radicals. We found that there was no significant difference of catalase level in tears between DR and no DR groups before and after adjustment for covariates. There have been no previous studies that compared the level of catalase in tears among DR. Study done by Koh et al. [22] evaluated catalase level in tears among ARMD patients found that there was significantly lower catalase level in tears in ARMD patients than those in control group. In contrast, Pavlov et al. [35] demonstrated that a significant elevation of catalase level in tears among hypertensive retinopathy. Few studies reported that diabetic patient was associated with decrease level of serum catalase [36, 37]. However, other studies found that there was increased level of catalase in serum [38] and saliva [39] among diabetic patients. We postulated that inconsistent finding of catalase level in the body fluid could be related to the severity of concomitant local disorders or systemic comorbidities.

Retina has high oxygen and polyunsaturated fatty acids concentration that provide high risk to oxidative stress with formation of lipid peroxidation [22]. MDA is the end products of lipid peroxidation. In our study, there was no significant difference of MDA level in tears between no DR, NPDR and PDR. Koh et al. [22] also found no significant difference of MDA level in tears between ARMD patient and control groups. In other ocular fluid, Mancino et al. [20] demonstrated that the level of vitreous MDA in the PDR group was significantly

higher compared to controls and to NPDR patients. Increased MDA levels in serum have been reported in DM [40] and diabetic patients with neuropathy [41], retinopathy [42], nephropathy [41, 43] and coronary artery disease [41]. Diabetic patient is associated with high level of MDA due to an increased oxidative stress through lipid peroxidation activity [44].

There are few limitations in our study. The antioxidant system is comprised of a wide variety of components. In current study, due to limited fund, only catalase, GPx, and MDA were evaluated. In future studies, we recommend including other oxidative stress biomarkers such manganese-superoxide dismutase, and glutathione reductase. Furthermore, we evaluated the oxidative stress level in tears only. Comparing oxidative stress level between tears and serum or other ocular fluid (aqueous or vitreous) was not investigated. Thus, a thorough comparative analysis with a larger group of patients should be performed to demonstrate the correlation between oxidative stress biomarkers in tears and ocular pathologies. Finally, detailed history on lifestyle, pollution, irradiation, medications intake should be obtained and considered during analysis as all these factors can altered the level of oxidative stress level in the body. Beside DM, other ocular and systemic comorbidities influence the production of oxidative stress biomarkers. Levels of oxidative stress biomarkers in tears may not be only attributed to DM, but also arise owing to other systemic comorbidities, since we also included patients with hypertension, hyperlipidemia, ischemic heart disease, and chronic kidney disease. These findings could compromise the clinical significance. In future studies, we recommend excluding DM patients with concomitant other comorbidities.

Conclusion

Our finding demonstrated that diabetic patient with DR is associated with low level of GPx in tears, suggesting that this antioxidant enzyme is a potential biomarker for predicting the presence of DR. Diabetic patients with concomitant systemic comorbidities, could compromise the clinical significance. Therefore, systemic conditions that influence the oxidative stress biomarkers level should be considered in the real-world clinical settings when evaluating these biomarkers.

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Authors' contributions **KKH:** Conceptualization, data curation, formal analysis, methodology, writing—original draft. **EZ:** Conceptualization, data curation, formal analysis, funding acquisition, methodology, writing—review & editing, supervision, project administration. **HH:** Conceptualization, formal analysis, methodology, supervision. **AACB:** Conceptualization, data curation, formal analysis, methodology, supervision. **CHCM:** Conceptualization, data curation, formal analysis, methodology, supervision.

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Data availability All the data and materials are contained within the manuscript.

Code availability Not applicable.

Declarations

Ethics approval The study was approved by the Institutional Review Board (IRB) of Universiti Sains Malaysia (USM) [ref: USM/ Jawatankuasa Etika Penyelidikan Manusia (JEPeM)/276.2. (4)] and followed the tenets of the declaration of Helsinki. Written and informed consent of participants was obtained for each patient prior to the study.

Consent to participate Not applicable.

Consent for publication All authors approved the manuscript for publication.

Competing interests The authors declare that they have no competing interests.

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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