ORIGINAL ARTICLE

Antioxidant Enzymes in Tears Among Malay Age-related Macular Degeneration Patients

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ABSTRACT

Introduction: Age-related macular degeneration (ARMD) is an ocular degenerative disorder that associated with impairment of central vision. Oxidative stress plays an important role in the pathogenesis of ARMD. The aim of this study was to determine the level of antioxidant enzymes (catalase and glutathione peroxidase) in tears among Malay ARMD patients. **Methods:** A cross sectional study was conducted between September 2015 and November 2017 among Malay ARMD patients. Schirmer paper was used to collect the tear samples. The level of catalase and glutathione peroxidase level in tears was evaluated using commercially available oxidative stress marker kits. **Results:** A total of 136 Malay ARMD patients were recruited into the study with 68 controls. Mean tear catalase and glutathione peroxidase levels were significantly lower in ARMD patients (1348.97 SD 109.11 μM and 453.87 SD 41.96 U/L respectively) as compared to the control group (1453.38 SD 38.87 μM and 502.28 SD 34.29 U/L respectively) ($P<0.001$ and $P<0.001$ respectively). Late ARMD has lower mean of catalase level in tears as compared to early ARMD ($P=0.044$). Based on subtypes of neovascular late ARMD, neovascular ARMD has lower mean catalase level in tears compared to idiopathic polypoidal choroidal vasculopathy (IPCV) ($P=0.031$). **Conclusion:** This study showed that antioxidant enzyme might play an important factor in the pathogenesis of ARMD.

Keywords: Age-related macular degeneration, Tears, Catalase, Glutathione peroxidase

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INTRODUCTION

Age-related macular degeneration (ARMD) is an ocular degenerative disorder that occur at macular area and associated with impairment of central vision. The risk of ARMD increases with age, especially in women of 75 years and above. With the aging populations, ARMD will become an increasingly prevalent and important condition worldwide. Wisconsin Age-Related Maculopathy Grading Scheme (WARMGS) (1) grading system classified ARMD into early ARMD and late ARMD. Features of early ARMD include drusen and pigmentary changes of the retinal pigment epithelium (RPE). On the other hand, late ARMD consists of either geographic atrophy or neovascular ARMD. Idiopathic polypoidal choroidal vasculopathy (IPCV) is considered a variant of subtypes of neovascular ARMD characterized by polypoidal lesion with or without branching vascular network originating from choroidal vessels (2,3).

The pathogenesis of ARMD is likely to be multifactorial as it involves a complex interaction of genetic, metabolic, hormonal, functional and environmental factors, which remains poorly understood (4-7). The most recent studies suggest that oxidative stress plays an important role in the pathogenesis of ARMD (4,8-10). Oxidative stress oocur when there is an imbalance between free radicals and antioxidants. Free radicals are molecules with unpaired electron such as reactive oxygen species.

Reactive oxygen species degrade polyunsaturated lipids to form malondialdehyde. Level of malondialdehyde is an indicator of oxidative stress. Antioxidants are substances that neutralise free radical. The major antioxidants are superoxide dismutase, catalase and glutathione peroxidase. The increased oxidative stress and impaired antioxidant defenses are some contributory factors to the initiation and progression of ARMD (8).

Previous studies evaluating the antioxidant levels in the serum of ARMD patients have reported conflicting results (10-12). Systemic disease is one of the factor that influence the level of antioxidants in serum (13-16). Therefore, identification of the serum antioxidants is not reflecting the ocular disease. Thus, identification and quantification of serum antioxidants level to evaluate the activity of ocular disease may need highly sensitive assay systems.

Quantification of antioxidants level in the aqueous or vitreous humour, may be a better representative of ARMD. This is because antioxidants levels in vitreous or aqueous humour are more in proximity to the retinal itself and better reflects the pathophysiological process. However, quantification of antioxidant level in aqueous or vitreous is relatively invasive, as it requires intracameral or intravitreal tapping, with all the antecedent risks of surgery complication.

A study done by Atalla et al (17) showed that abundance of antioxidant enzymes were found in epithelial structures of the eye, such as cornea epithelium, ciliary epithelium, lens epithelium and RPE. Antioxidant level in tears may be a reflection of the antioxidant level in cornea epithelium because cornea epithelium is directly in contact with tears and is continuously debrided into tears due to aging process. Similarly, antioxidant level in vitreous may be closely similar to antioxidant level in the retina. Increased oxidative stress has been reported in the vitreous fluid in proliferative diabetic retinopathy patients, and oxidative stress has been speculated to be related to retinal cell damage in such patients (18). Thus, reduced antioxidant capacity in tears may reflect a reduced antioxidant capacity in the vitreous as well.

Antioxidant levels in tears have been shown to reflect that of the aqueous fluid. In a study conducted by Horwath-Winter et al (19) it was found that the concentrations of uric acid in tears was almost equal with the concentration of uric acid in aqueous (19). Ang et al demonstrated that there was a fair correlation between serum and tear vascular endothelial growth factor (VEGF) levels among diabetic patients. Evaluation of antioxidants levels in tears may provide a non-invasive method to assess the intraocular oxidative stress level (20).

There is a strong biological correlation between oxidative stress and progression of ARMD. Previous studies have assessed the concentrations of antioxidants such as ascorbic acid, catalase, and superoxide dismutase in tears of normal patients and other diseases, but there have been no previous studies on antioxidant levels in the tears of ARMD patients (21-23). Among ARMD patients, the levels of catalase, glutathione peroxidase and malondialdehyde was measured only in the serum plasma (10-12). Catalase and glutathione peroxidase are common antioxidants in the human body, while malondialdehyde is an end product of lipid peroxidation. However, no study has assessed the levels of these antioxidants in the tears of ARMD patients or on healthy control groups. Thus, our aim of this study was to determine the level of antioxidant enzymes (catalase and glutathione peroxidase) and also malondialdehyde (end product of lipid peroxidation) in the tears of ARMD patients. The outcome of this study may help in developing a predictable antioxidant level for strategising therapeutic modalities based on the underlying pathology.

MATERIALS AND METHODS

A cross-sectional study was conducted in two centres from September 2015 to November 2017. The samples size was calculated using G Power 3.1.9. A total of 136 patients with ARMD were acquired to participate in this study (68 patients with early ARMD and 68 patients with late ARMD) and a control group of 68 patients (24).

In this study, the severity and subtypes of neovascular late ARMD was classified based on WARMGS (1). ARMD was classified into early and late ARMD. Early ARMD characterized by presence of drusen, hyperpigmentation or hypopigmentation of RPE. Whereas, late ARMD consists of either geographic atrophy or neovascular ARMD. There are three subtypes of neovascular ARMD; typical neovascular ARMD, IPCV and retinal angiomatous proliferation.

This study was conducted among Malay population. For this study, Malay patients were identified as those who speak Malay as their first language, who descend from at least two generations, practice Malay customs and who were Muslim (25).

A newly diagnosed Malay ARMD patients aged 45 years old and above with clear media were included. ARMD patients that undergone laser photocoagulation therapy, photodynamic therapy, intravitreal anti-VEGF injection or taking any topical or systemic supplement for ARMD were excluded from the study. Other exclusion criteria included patients with other macular pathology, glaucoma, ocular infection and inflammation, disorder of optic nerve, previous history of intraocular surgery of less than 6 months, severe dry eye and history of taking regular topical eye drops. Patients with systemic vasculitis diseases such as systemic lupus erythematosus, giant cell arteritis, Takayasu's disease or taking antioxidant medication within a 3 month period

were also excluded. The control group was also from the Malay population with no ARMD. The exclusion criteria for the control group were the same as that of the study group.

The demographic data, medical and ocular history were obtained through direct questioning of patients and from patients medical records. The procedure of the study was explained to the selected patients. All the selected patients were required to fill-up the written inform consent.

Fundus examination including optical coherence tomography and fundus fluorescein angiography was performed to confirm the diagnosis of ARMD by independent ophthalmologist. Indocyanine green was done to confirm the diagnosis of IPCV.

Tears was collected using Schirmer strips. Only one eye of the worst severity was selected. For the control group, right eye was selected for standardization. Instillation of topical anesthesia (Alcain, Alcon, Belgium) into the patient's eye was performed prior application of Schirmer strips. Five minutes after instillation of topical anaesthesia, Schirmer strips (Whatman™ Grade 41 Quantitative Filter Paper) was placed at the lateral part of lower eyelid. The Schirmer strips was removed when the amount of wetting was approximately 15 mm. Thereafter, the sample of tear was kept in a plain tube and immediately sent to an immunology laboratory for analysis. The samples can be kept in -80°C freezer for a maximum duration of one year.

The antioxidants selected in this study were catalase and glutathione peroxidase (antioxidant enzyme) and malondialdehyde (end product of lipid peroxidation). All collected samples were soaked with 1000 uL of cold phosphate buffered saline to dissolve the enzyme from the schirmer paper and the samples were refrigerated at 4°C for one day prior to laboratory analysis. The samples were spun vigorously at 1000 microtiter for 30 seconds to mix thoroughly.

Later, the samples were further processed based on different procedures stated on the brochures of EnzyChromTMCatalase Assay Kit (BioAssays, USA), EnzyChromTMGlutathione Peroxidase Assay Kit (BioAssays, USA) and Malondialdehyde Assay Kit (Northwest Life Sciences, USA) respectively. Catalase, glutathione peroxidase and malondialdehyde levels were later confirmed in the computer analysis and recorded.

Ethical approval

Local ethical boards was obtained from Human Research Ethics Committee [Universiti Sains Malaysia (USM)/ Jawatankuasa Etika Penyelidikan Manusia (JEPeM) Registration number: 14110458] and National Medical Research Registry (NMRR) [NMRR-15-1583-27398 Registration of Investigator Initiated Research (IIR)] to conduct this study.

Statistical analysis

Statistical Package for Social Sciences (SPSS) Version 22 was used for statistical analysis. Comparison of sex among study group and control group was done using Chi Square test. Independent T test and ANCOVA (adjusted for age) were used for comparison of mean catalase, malondialdehyde and glutathione peroxidase levels in tears among ARMD patients and control group. P-value < 0.05 was considered as statistically significant.

RESULTS

Demographic data

A total of 204 patients (68 early ARMD, 68 late ARMD, 68 controls) were recruited. Among neovascular late ARMD, there were 37 (54.4%) patients with typical neovascular ARMD and 31 (45.6%) patients were IPCV. There was no geographic atrophy patient and no retinal angiomatous proliferation patient among late ARMD group. ARMD group ranged from 49 to 96 years with mean age of 67.9 SD 8.5 years. Distribution of age and gender among study group, severity of ARMD and subtypes of neovascular late ARMD are shown in Table I.

Table I: Distribution of age and gender among study group, severity of ARMD and subtypes of neovascular late ARMD

Study Group	ARMD n = 136	Control n = 68	P value
Age (years)			
Mean (SD)	67.9 (8.5)	62.3 (10.2)	< 0.001[a]
Range	49 – 96	47 – 80	NA
Sex (n, %)			
Male	68 (50.0)	33 (48.5)	0.843[b]
Female	68 (50.0)	35 (51.5)	
Severity of ARMD	**Early ARMD n = 68**	**Late ARMD n = 68**	**P value**
Age (years)			
Mean (SD)	69.9 (8.5)	65.9 (8.1)	0.006[a]
Range	52 – 96	49 – 85	NA
Sex (n, %)			
Male	31 (45.6)	37 (54.4)	0.303[b]
Female	37 (54.4)	31 (45.6)	
Subtypes Neovascular Late ARMD	**Neovascular ARMD n = 37**	**IPCV n = 31**	**P value**
Age (years)			
Mean (SD)	66.7 (8.4)	64.0 (7.1)	0.164[a]
Range	54 – 85	49 – 75	NA
Sex (n, %)			
Male	21 (56.8)	24 (77.4)	0.073[b]
Female	16 (43.2)	7 (22.6)	

[a]Independent t-test, [b]Pearson Chi-Square test, p < 0.05 significant
Abbreviation: ARMD: Age-related macular degeneration, IPCV: idiopathic polypoidal choroidal vasculopathy, NA: not applicable

ARMD group has older age mean compared to the control group (67.9 SD 8.5 vs 62.3 SD 10.2 years, P < 0.001). There were equal percentage of men (50%) and women (50%) among ARMD group. Early ARMD group has older mean of age compared to late ARMD group (69.9 SD 8.5 vs 65.9 SD 8.1 years, P=0.006). Patients with IPCV were younger than neovascular late ARMD patients (mean age, 64.0 SD 7.1 vs 66.7 SD 8.4 years). However, there was no statistically significant difference in mean age between these two groups. There was higher percentage of men developed IPCV (77.4%) compared to neovascular ARMD group (56.8%) (Table I).

Antioxidants level in tears

ARMD group has significant lower mean catalase (1348.97 SD 109.11 μM vs 1453.38 SD 38.87 μM, P<0.001) and glutathione peroxidase (453.87 SD 41.96 U/L vs 502.28 SD 34.29 U/L, P<0.001) level in tears compared to the control group (Table II). There was significant mean difference of tear catalase (P=0.001) and tear glutathione peroxidase (P<0.001) between ARMD and control group after age adjustment (Table III). Mean tear malondialdehyde showed no difference between ARMD and controls.

Among ARMD group, late ARMD has significant lower mean of catalase level in tears compared to early ARMD (1309.29 SD 112.47 μM vs 1388.06 SD 100.31 μM, P=0.044) (Table II). There was significant mean difference of catalase level in tears between early ARMD and late ARMD after age adjustment (P=0.029) (Table III). There was no significant difference of glutathione peroxidase and malondialdehyde level in tears between the severity of ARMD.

Table II: Comparison of antioxidant enzymes and malondialdehyde in tears between ARMD and control, severity of ARMD and subtypes of neovascular late ARMD

Tear Oxidative Stress Markers	Catalase (μM)	Glutathione Peroxidase (U/L)	Malondialdehyde (μM)
	Mean (SD)	Mean (SD)	Mean (SD)
Study Group			
ARMD	1348.97 (109.11)	453.87 (41.96)	68.02 (20.97)
Control	1453.38 (38.87)	502.28 (34.29)	54.39 (31.21)
P value	< 0.001	< 0.001	0.148
Severity of ARMD			
Early ARMD	1388.06 (100.31)	460.43 (36.56)	58.79 (39.21)
Late ARMD	1309.29 (112.47)	447.31 (46.09)	77.25 (21.67)
P value	0.044	0.066	0.249
Subtype of neovascular late ARMD			
Neovascular ARMD	1267.27 (128.21)	440.76 (49.22)	80.93 (9.28)
IPCV	1393.24 (53.12)	459.42 (37.22)	70.07 (11.09)
P value	0.031	0.116	0.585

Independent t-test, p < 0.05 significant
Abbreviation: ARMD: Age-related macular degeneration, IPCV: idiopathic polypoidal choroidal vasculopathy

Table III: Comparison of antioxidant enzymes and malondialdehyde level in tears between ARMD and control, severity of ARMD and subtypes of neovascular late ARMD after adjusted for age

Tear Oxidative Stress Markers	Catalase (μM)	Glutathione Peroxidase (U/L)	Malondialdehyde (μM)
	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)
Study Group			
ARMD	1348.51 (1312.91, 1383.53)	453.87 (448.83, 458.91)	68.25 (53.08, 81.52)
Control	1455.33 (1403.90, 1504.79)	504.30 (458.91, 514.38)	54.98 (36.02, 75.83)
F-stat (df)	11.04 (1,203)	65.30 (1,203)	1.02 (1,203)
P value	0.001	< 0.001	0.315
Severity of ARMD			
Early ARMD	1392.03 (1336.65, 1445.44)	458.91 (448.82, 469.00)	58.77 (36.02, 79.62)
Late ARMD	1306.97 (1251.59, 1360.38)	448.83 (438.74, 458.91)	77.73 (54.98, 100.47)
F-stat (df)	4.89 (1,135)	3.08 (1,135)	1.49 (1,135)
P value	0.029	0.082	0.225
Subtype of neovascular late ARMD			
Neovascular ARMD	1263.45 (1196.20, 1860.26)	438.74 (428.67, 453.87)	79.62 (58.77, 100.48)
IPCV	1399.95 (1305.00, 1494.90)	458.91 (438.74, 479.09)	72.04 (41.71, 102.37)
F-stat (df)	5.12 (1,67)	2.64 (1,67)	0.24 (1,67)
P value	0.027	0.109	0.624

Univariate ANCOVA, p < 0.05 significant
Abbreviation: ARMD: Age-related macular degeneration, IPCV: idiopathic polypoidal choroidal vasculopathy

Based on subtypes of neovascular late ARMD, neovascular ARMD group has significant lower mean of catalase level in tears compared to IPCV group (1267.27 SD 128.21 μM vs 1393.24 SD 53.12 μM, P=0.031) (Table II). There was significant mean difference of catalase level in tears between neovascular ARMD and IPCV after age adjustment (P=0.027) (Table III). However, no significant difference of glutathione peroxidase and malondialdehyde level in tears between subtypes of neovascular late ARMD.

DISCUSSION

Oxidative stress is a condition in which there is the state of production of free radicals exceeds the neutralizing capacity by antioxidant defense mechanisms under normal physiological conditions. It is believed that oxidative stress plays an important role in age related degenerative eye disease such as ARMD and cataract.

In ARMD, oxidative stress causes damage to the RPE and Bruch's membrane which are the important structures for the pathogenesis of ARMD (26,27). Retina has high oxygen and polyunsaturated fatty acids concentration that provide high risk to oxidative stress with formation of lipid peroxidation (28). Formation of lipid peroxidation also increases with aging (29). The antioxidants level analysed in this study are catalase, glutathione peroxidase (antioxidant enzyme) and malondialdehyde

(end product of lipid peroxidation).

Study conducted by Klein et al (30) showed that prevalence of ARMD was significantly different between ethnic groups. Prevalence of ARMD was 2.4% in black people, 5.4% among the white population and 4.6% among the Chinese. This study suggested that ethnicity is a significant confounding factor in the development of ARMD. Thus we focused only on Malay subjects for both study group and control group.

Current studies have shown that ARMD group has lower mean for catalase (P=0.001) and glutathione peroxidase level in tears (P<0.001) compared to control group when adjusted for age. Reduced catalase and glutathione peroxidase in tears can possibly be a reflection of low antioxidant level in retina, which may be related to the development and progression of ARMD. This finding could be compared with the animal study done by Robison et al (31). ARMD occurs when there is imbalance between antioxidant system and oxidative damage (32).

In our study, we found out that the mean malondialdehyde level in tears showed no significant difference between ARMD and control group. However, study done by Ateset et al, showed that serum levels of malondialdehyde were significantly higher in the patients with exudative-type ARMD than those in the control group (33). This is probably because malondialdehyde level in tears does not reflect the lipid peroxidation activities in RPE.

Previous studies showed that increasing age, smoking, cardiovascular disease, and prior cataract surgery have significant role in predicting ARMD (34,35). Thus, we have excluded patients with a history of intraocular surgery from this study, in order to minimize the confounding effect on the study outcome.

Oxidative stress is caused by age (36). A strong correlation has been observed between increasing age and oxidative damage to cellular macromolecules (37). Serum malondialdehyde level was strongly correlated with age in men between 0 – 77 years old (38). Moreover, there were significant age-related difference in erythrocyte copper/zinc (cu/zn) superoxide dismutase activity between middle age (55-77 years old) and late middle age groups (70-85 years old) (39). Therefore, for the purpose of this study, the impact of age was controlled for the analysis of oxidative stress between ARMD patients and control group. We have included age as the confounding factor in univariate ANCOVA analysis.

This study also showed that, early ARMD group has statistically significant older mean of age compared to late ARMD group (P=0.006). This is probably due to earlier presentation of late ARMD patients to hospital because of poor vision, as a result of haemorrhage, exudation and macular scarring from late ARMD. In contrast, early ARMD patients may have minimal or no eye symptom. Early ARMD is frequently detected as an incidental finding during eye assessment, especially when patients developed cataract as they grow older. You et al (40) also found that the incidence of early ARMD is associated with older people. On the other hand, Yasuda et al (41) reported that old age is associated with the incidence of late ARMD.

Apart from age, oxidative stress have also been noted to vary according to gender (42). Our current study found no significant difference in gender distribution between ARMD and control group. The difference in gender distribution between early ARMD and late ARMD were not statistically significant. In women, the anti-oxidant properties in estrogen help to protect the retina. On the other hand, there is low level of estrogen in post-menopausal women that lead to formation of ARMD due to reduction of anti-oxidant. However, meta-analysis by Rudnicka et al (35) suggested that prevalence of neovascular ARMD is higher in women than men.

The Age-Related Eye Disease Study (AREDS) (43) and study done by Thornton et al (44) revealed that smoking is one of the risk factors for development of ARMD. However, the data collected is based on subject-reported smoking experience and the report might provide overestimation or underestimation of data pertaining to smoking. Besides, passive smokers may also carry the similar risk as active smokers in the development and progression of ARMD. However, it is technically difficult to quantify the amount of exposure to smoking. Thus, it could be difficult to assess the confounding effect of smoking and passive smoking on the tear antioxidants level in our study subjects.

IPCV is a subtype of neovascular ARMD and it is more common among the Asian people (45). Both have features of choroidal neovascularization but with different underlying mechanism. The study shows that, 45 years old was our lowest age variable point in view of patients affected by IPCV and they were generally younger than those with choroidal neovascularization secondary to ARMD (46). Our study found that neovascular ARMD group has significant lower mean of catalase compared to IPCV group after age adjustment. However, there were no significant differences between malondialdehyde and glutathione peroxidase levels in tears between subtypes of neovascular and late ARMD. The association between oxidative stress and IPCV requires further clarification, although this could be technically difficult.

Antioxidants level in tears have been shown to reflect that of the aqueous fluid (21). Detection of antioxidants level in tears may thus be a less invasive, safe and reliable method of assessing the oxidative stress. To the best of our knowledge, there is no study that evaluates

the antioxidants level in the tear film in ARMD patients. Evaluating the antioxidants level in tears is non-invasive and easy. Perhaps, this technique may be a potential tear antioxidants biomarker for the detection of development and progression of ARMD. Although, we are aware that antioxidants level in tears may be affected by many other factors such as ultra-radiation, pollutions and smoking.

This study only evaluated the antioxidants level in the tear film. In future, evaluating antioxidants level in the aqueous or vitreous and comparing antioxidants level to tear film and serum levels may give a more comprehensive picture of the oxidative stress activity in the eye. Vitreous sample probably can be collected during intravitreal procedure of anti-VEGF injection. However, this can only be done to patients with late ARMD that indicated for intravitreal anti-VEGF injection.

The correlation between systemic illness and ARMD was not included in our study. This is mainly because most of the patients belong to the elderly group who are most commonly associated with certain systemic illness. Thus, it is not possible to exclude all patients with systemic illness as our study subjects. However, systemic co-morbidities such as diabetes mellitus, hypertension, renal failure, stroke and cardiovascular disease are postulated to have some association with ARMD (47,48). Thus, the evaluation of correlation between ARMD and certain systemic illnesses such as diabetes mellitus, stroke and cardiovascular disease are ideal in future studies. Besides, old age is also one of the factors that can influence the oxidative stress level.

Tear film measurement of antioxidants level revealed the fluid content of the substances which probably do not reflect the actual activities of the oxidative stress inside the eyes. A more precise cellular measurement can perhaps be applied to measure antioxidants level as most of the oxidative activities occur inside the cells.

Detailed history on lifestyle, pollution, irradiation and medication intake should be obtained and taken into account during analysis as all these factors can alter the outcome of oxidative stress status in the body.

CONCLUSION

This study showed that antioxidant enzyme might play an important factor in the pathogenesis of ARMD.

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