Increased Systemic Oxidative Stress and DNA Damage in Patients with Exudative Age-Related Macular Degeneration

Eksudatif Tip Yaşa Bağlı Makula Dejenerasyonu Tanılı Hastalarda Artmış Sistemik Oksidatif Stres ve DNA Hasarı

Emine Kılıç Toprak[1], İbrahim Toprak[2], Volkan Yaylalı[2], Yasin Özdemir[1], Burak Oymak[1], Melek Bor Küçükatay[1], Vural Küçükatay[1]

[1] Pamukkale University, Faculty of Medicine, Department of Physiology, Denizli, Turkey.
[2] Pamukkale University, Faculty of Medicine, Department of Ophthalmology, Denizli, Turkey.

Abstract

Objective: The purpose of this study was to determine the levels of systemic oxidative stress and genotoxicity (DNA damage) in patients with exudative age-related macular degeneration (AMD) and to investigate the possible role of these factors in development of advanced stage wet AMD.

Material-Method: The study comprised 26 patients with exudative AMD (68.9±1.9 years of age) and 26 age- and sex-matched (64.8±2.2 years of age) healthy controls (p=0.268, p=0.258, respectively). Serum total antioxidant status (TAS) and total oxidant status (TOS) were measured with a commercial kit. Oxidative stress index (OSI) was calculated. DNA damage was evaluated using comet analysis following lymphocyte isolation from venous blood. Parameters including tail length and tail moment were evaluated for quantitative analysis of DNA damage.

Results: The AMD group had lower TAS and higher OSI values than in the control group, while TOS values did not differ between the two groups (p=0.006, p=0.039 and p=0.319, respectively). Furthermore, tail length (p<0.0001) and tail moment (p=0.005) were significantly higher in the AMD group compared to the control group.

Conclusions: Although multiple factors play a role in pathogenesis of AMD, increased systemic oxidative stress and DNA damage might be an important risk factor for development of exudative AMD. In addition, investigating these factors in patients with early AMD might be helpful for developing new preventive therapies.

Keywords: Comet Assay, DNA Damage, Macular Degeneration, Oxidative Stress.

Özet

Amaç: Bu çalışmanın amacı eksudatif tip yaşa bağlı makula dejenerasyonu (YBMD) tanılı hastalarda sistemik oksidatif stres ve genotoksisite (DNA hasarı) düzeylerini belirleyerek bu faktörlerin ileri evre yaş tip YBMD gelişimindeki olası rolünü araştırmaktır.

Materyal-Metot: Çalışmaya eksudatif YBMD tanılı 26 hasta (68,9±1,9 yaş) ve 26 yaş ve cinsiyet uyumlu (64,8±2,2 yaş) sağlıklı kontrol grubu (p=0,268, p=0,258) dahil edildi. Serum total antioksidan durumu (TAS) ve total oksidan durumu (TOS) ticari bir kit kullanılarak ölçüldü. Oksidatif stres indeksi (OSİ) hesaplandı. DNA hasarı, venöz tam kandan lenfosit izolasyonunu takiben Comet (kuyruklu yıldız) analizi kullanılarak değerlendirildi. Kuyruk uzunluğu ve kuyruk momenti dahil olmak üzere parametreler, DNA hasarının kantitatif analizi için değerlendirildi.

Bulgular: YBMD grubunda kontrol grubuna göre daha düşük TAS ve daha yüksek OSİ değerleri mevcut iken, TOS değerleri iki grup arasında farklılık göstermedi (p=0,006, p=0,039, p=0,319, sırasıyla). Ayrıca, YBMD grubunda kuyruk uzunluğu (p<0,0001) ve kuyruk momenti (p=0,005) kontrol grubuna göre anlamlı olarak yüksek bulundu.

Sonuç: YBMD patogenezinde birçok faktör rol oynamasına karşın, artmış sistemik oksidatif stres ve DNA hasarı eksudatif YBMD gelişimi konusunda önemli bir risk faktörü olabilir. Ayrıca, bu faktörlerin erken evre YBMD hastalarında da araştırılması yeni önleme tedavilerinin geliştirilmesi bakımından yol gösterici olabilir.

Anahtar kelimeler: Comet Analizi, DNA Hasarı, Maküler Dejenerasyon, Oksidatif Stres.

Introduction

Age-related macular degeneration (AMD) is an important cause of the irreversible visual loss in developed countries. Early AMD is characterized by accumulation of drusen beneath the retinal pigment epithelium (RPE), whereas geographical atrophy (GA) and choroidal neovascularization (CNV) are the hallmarks of advanced AMD (1-3). Treatment of advanced AMD with CNV (wet or exudative type) is mainly based on intra-vitreal injection of anti-vascular endothelial growth factor (VEGF), which plays the key role for CNV development (1-3).

Although inflammation has been blamed as the major factor underlying the pathogenesis of AMD, other contributing factors have not been completely clarified.

Yazışma Adresi / Corresponding: Emine Kılıç Toprak,
Pamukkale University, Faculty of Medicine, Department of Physiology, Kinikli,
20070 Denizli, Turkey.
Tel: +90 258 296 17 02
 Fax: +90 258 296 24 33
E-posta / E-mail: pt_emine@yahoo.com, ektoprak@pau.edu.tr

Based on the current knowledge, excessive amounts of reactive oxygen species (ROS) are produced in RPE due to chronic light exposure. RPE has antioxidant enzymes and molecules (as other cells have in the human body) such as superoxide dismutase (SOD), catalase, glutathione (GSH), carotenoids, vitamin E and C (2-6). Measuring total antioxidant status (TAS) and total oxidant status (TOS) from biological samples are the most practical methods for determining oxidant/ antioxidant balance. These methods reveal cumulative and synergistic action of oxidant and antioxidant compounds, since measuring level of each molecule in oxidative pathway is more expensive and time-consuming (7-8).

It is well known that a decrease in elimination of ROS causes damage in various cellular components such as lipids, proteins and deoxyribonucleic acid (DNA). Genome stability is maintained by DNA repair systems in a healthy organism (9-10). However, with aging, DNA damage accumulates and DNA repair systems slowdown, which lead disorganization of the cell cycle, apoptosis and necrosis (11-12). The amount of DNA damage can be quantified in eukaryotic cells using the comet assay which is based on single cell gel electrophoresis and fluorescent microscopy (13-16).

Molecular pathogenesis of AMD remains unclear and intricate. Although a few numbers of studies investigated components of oxidant/antioxidant pathways and DNA damage/repair systems separately in AMD, there is no comprehensive research study available on this issue to the best of our knowledge (5, 6, 11, 12). Hence, the present study was designed to perform a combined investigation on systemic oxidative stress and genotoxicity in patients with exudative AMD on the basis of serum TOS and TAS measurements as well as comet assay to investigate possible role of these factors in advanced wet AMD.

Material and Methods

The tenets of the declaration of Helsinki were followed and ethics committee approval was obtained before conducting study (Approval number: 2016/06, dated 22 March 2016). The present study included 26 patients with exudative AMD (AMD group) and 26 age and gender matched healthy subjects (control group). The statistical power of the study was 96% at 5% significance level (PASS v11.0.1, NSCC, LLC, Utah, USA).

Written informed consent was obtained from all participants and the study cohort was homogenous regarding race. All cases underwent a complete ophthalmological examination (visual acuity measurement, slit-lamp biomicroscopy, intraocular pressure measurement using Goldmann applanation tonometry, dilated fundoscopic examination with noncontact +90 diopter lens), OCT imaging (Spectralis OCT, Heidelberg Engineering GmbH, Heidelberg, Germany) and fundus fluorescein angiography (FFA). Patients with a diagnosis of exudative AMD in one eye based on dilated fundus examination, OCT imaging (presence of subretinal and/or intraretinal fluid) and FFA (leaking choroidal neovascularization secondary to AMD) were included into the study as the AMD group (over 50 years of age). The control group comprised age- and sex-matched healthy subjects with normal fundus examination and OCT images.

Exclusion criteria were determined as follows, presence of uncontrolled hypertension, renal or hepatic failure, smoking, systemic inflammation, immune suppression, cancer, present antioxidant and/or anti-inflammatory therapies and lymphoproliferative disorders.

TOS and TAS measurements

The venous blood samples (10 ml) were obtained from all cases after overnight fasting in the morning and collected into plain red top blood collection tubes. Samples were centrifuged at 5018 G-force for 6 minutes and serum was separated and stored at -80°C until laboratory analysis for TOS and TAS measurements. An automated measurement method developed by Erel was used for the TOS and TAS measurements (Rel Assay Diagnostics, Gaziantep, Turkey) (7).

TOS Measurement

The ferrous ion-o-dianisidine sophisticated is oxidized to ferric-ion by oxidants, present in the specimen. Glycerol molecules enhance oxidative reaction. The ferric ion transforms to a colored complex in acidic-solution. The density of color is measured with a spectrophotometer. In the sample, it is accepted to be comparative to the total amount of oxidant molecules. Hydrogen peroxide is used for assay calibration. Results are expressed as micromolar hydrogen peroxide equivalents per liter ($\mu mol\ H_2O_2$ equivalent/l).

TAS Measurement

Fenton reaction produces hydroxyl radical (most potent radical), which reacts with the colorless substrate O-dianisidine. This reaction is resulted in formation of bright yellowish-brown colored the dianisyl radical. Oxidative reactions are initiated following addition of serum sample by the hydroxyl radicals in the reaction mixture are also suppressed by antioxidant components of the sample. It prevents color change, this mechanism is the main idea for measuring TAS of the serum. The results were explicated as mmol Trolox equivalent/l.

Oxidative stress index (OSI)

OSI was calculated following formula (7, 8).

OSI (arbitrary unit, A.U)=TOS ($\mu mol\ H_2O_2$ equivalent/l)/ TAS (μmol Trolox equivalent/l)×100

Comet Assay

Collection of Whole Blood And Isolation of Lymphocytes

Peripheral venous blood was obtained from all subjects into a 10 ml vacutainer tube containing K3EDTA and lymphocytes were separated using Histopaque-1077. Blood was diluted 1:1 with phosphate buffered saline (PBS) and poured directly into the Leucosep® tube. Then it was centrifuged at 800xg and room temperature for 15 minutes. Following removal of buffy coats, it is washed two times with PBS.

Table 1. Comparison of total oxidant status (TOS), total antioxidant status (TAS) and oxidative stress index (OSI) values between the control and AMD groups

	Groups		
Oxidative stress measurements *(mean±standard error)*	**Control Group (n=26)**	**AMD Group (n=26)**	**p value**
TOS *(μmol H_2O_2 equivalent/l)*	4.739±0.391	4.566±0.364	0.319
TAS *(mmol Trolox equivalent/l)*	**1.512±0.064**	**1.132±0.097**	**0.006***
OSI *(arbitrary unit, A.U)*	**0.326±0.036**	**0.786±0.164**	**0.039***

* indicates statistically significant difference (p<0.05) between the two groups (Mann Whitney U test).

Cryopreservation of Cells Prior to Comet Assay

As described by Visvardis et al, after centrifuge process (at 200xg for 5 min) of the cell suspension, cell pellet is suspended at 3x105 cell/ml in ice-cold medium (10% DMSO, 40% RPMI and 50% fetal calf serum) (17). Then cell suspension is transferred to vials (in aliquots of 2x106 cells). After the vials are placed in a Cryo 1°C freezing container, directly transferred into a -80°C freezer to achieve -1°C/min cooling rate. Then, they are stored in -80°C.

Comet Assay

Assay was performed as described by Nandhakumar et al (18). A 37°C water bath was applied to the vials until all ice was melted. Thawed cells were promptly transferred to conic shaped centrifuge tubes, which contain 40% RPMI, 10% dextrose and 15 ml of pre-chilled thawing medium (containing of 50% fetal calf serum). At 200xg and 4°C, cells were centrifuged for 10 min. Cell pellet was suspended in cold PBS with a pH of 7.3 for the comet assay process.

As described by Singh et al, comet assay was performed using an adaptation method under alkaline conditions (19). The 1% low melting point agarose in PBS (pH of 7.4, at 37°C) was used for cell suspension. Then, 100 μl were pipetted on to a precoated-glass (film layer of 1% normal melting-point agarose) microscope slide. The agarose was set on ice (for 10 minutes) and slide was exposed to lysis solution at 4°C for one hour to remove cellular proteins. The lysis solution contains 100 mM Na_2EDTA,10 mM Tris, 2.5 M NaCl, NaOH to pH 10.0, and 1% Triton X-100. Then, processed slides stayed in the alkaline buffer (0.3 M NaOH and 1 mM Na EDTA) for half an hour for alkali unwinding of DNA (to expose alkali labile sites) prior to electrophoresis in the electrophoresis tank. After 30 minutes, electrophoresis was carried out at 25 V, 300 mA for 30 minutes at the same temperature. The slides were gently lifted from the electrophoresis buffer and placed on a staining tray. The slides were washed for three times 5 minutes each with neutralization buffer (0.4 M Tris-HCl, pH 7.5). Subsequently the slides were visualized by fluorescent staining method. Briefly, fifty μL of ethidium bromide stain was dropped onto each slide and covered with a clean cover slip. Before viewing the slides, excess stain from the back and edges of the slides were blotted away. A fluorescent microscope with an excitation filter (515-560 nm with barrier filter of 590 nm) and 20X magnification was used to visualize ethidium bromide-stained slides.

All steps beginning with the isolation of lymphocytes were conducted under yellow light to minimize the possibility of cellular DNA damage. Slides were analyzed microscopically by using Comet IV Computer Software (Perceptive Instruments, UK).

Statistical Analysis

Statistical analysis was performed with the SPSS version 18.0 (Statistical package for social sciences, SPSS Inc., Chicago, IL, USA). Qualitative data were compared between the two groups using the chi square test. Quantitative values were expressed as the mean ± standard error (SE). Comparison of the quantitative data between the two groups was performed using the Mann Whitney U test. Spearman correlation coefficients were used to assess relations among laboratory measurements. A p value <0.05 was accepted as statistically significant at 95% confidence interval.

Results

The AMD group consisted of 26 patients (13 females and 13 males) with a mean age of 68.9±1.9 years and all cases had active CNV based on FFA and OCT images. Twenty-six (18 females and 8 males) age- and sex- matched healthy subjects with a mean age of 64.8±2.2 years comprised the control group. There was no statistically significant difference between the groups in terms of, age and gender (p=0.268, p=0.258, respectively).

In patients with AMD, TAS was significantly lower (p=0.006) and OSI was significantly higher (p=0.039) compared to the healthy subjects, whereas TOS showed similar values (p=0.319) between the AMD and control groups. Table 1 represents comparison of oxidative stress parameters between the two groups.

DNA damage was quantified using alkali comet assay and 50 cells/slide per sample were scored. Tail length (22.17±1.39 vs. 16.33±0.54 μm, p<0.0001) and tail moment (1.32±0.20 vs. 0.96±0.32 μm, p=0.005) were significantly higher in the AMD group than in the control group. Figure 1 and Figure 2 show graphical comparison of the comet analysis results between the control and AMD groups.

Correlation analysis did not demonstrate significant relations (p>0.05) among age, oxidative stress and DNA damage parameters.
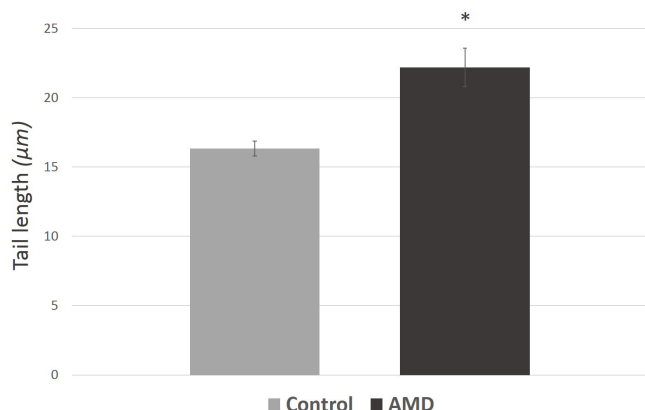
Figure 1. Comparison of tail length values (mean±standard error) between the control (n=26) and exudative AMD (n=26) groups. * indicates statistically significant difference (p<0.0001) between the two groups (Mann Whitney U test).
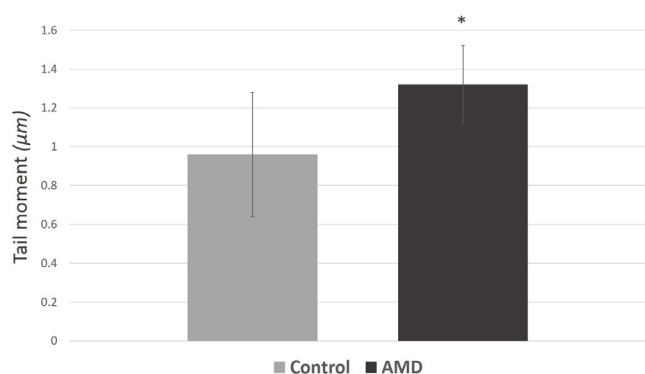
Figure 2. Increased tail moment (mean±standard error) value in patients with exudative AMD (n=26) compared to the healthy controls (n=26). * indicates statistically significant difference (p=0.005) between the two groups (Mann Whitney U test).

Discussion

RPE is exposed to excessive amounts of ROS due to chronic light exposure and high oxygen consumption. However, various antioxidant enzymes and defense mechanisms protect the RPE against oxidative damage (9, 20, 21). On the other hand, AMD has been suggested to be related with oxidative stress, as in many ocular diseases, whereas it is also well-known that AMD is a multifactorial disease and underlying mechanisms are still unclear (1-5).

In the present study, patients with AMD had lower TAS and higher OSI values than in the healthy subjects, whereas these patients had similar TOS values with the controls. OSI is a commonly used formula that provides more reliable data regarding the balance between oxidative stress and antioxidant defense (7, 8). In our study, higher OSI value in the AMD group might be important for demonstrating imbalance between systemic oxidant and antioxidant systems supporting decreased antioxidant defense even these patients had similar TOS values with the healthy controls. On the other hand, a lower TAS value in the AMD group might be

due to consumption of antioxidant molecules and enzymes in order to maintain oxidant status in a physiological level. The current literature represents conflicting results on TOS and TAS values in AMD. A study by Elbay et al. showed that patients with exudative AMD had higher serum TOS and lower TAS levels compared to those of the healthy controls and the researchers suggested that increased oxidative stress and decreased antioxidant defense might be involved in AMD progression (5). Ugurlu et al. reported similar results with Elbay et al. suggesting increased TOS level in advanced AMD (5, 6). They also demonstrated reduced paraoxonase 1 (PON1) activity and total thiol status (TTS), which are important antioxidants, in an exudative AMD group (with CNV or disciform scar), whereas TAS value was reported to be similar with the healthy subjects.

Genomic instability is generally compensated by DNA repair systems in a healthy individual. However, it has been reported that diminished antioxidant defense and DNA repair systems, which leads damaged genome and subsequent cell loss, contribute pathogenesis of many diseases (9, 20, 21). In the current study, alkali comet assay was used, which has been shown to be reliable, sensitive and cost effective method for detecting DNA strand breaks, alkali labile sites in DNA and incomplete excision repair sites at the level of a single cell. This assay is based on measuring the extent of electrophoretic migration of negatively charged DNA fragments, which is directly proportional to DNA damage. Under fluorescent microscopy, damaged DNA fragments move to the anode freely and resemble the tail of a comet (22-24). In our study, patients with AMD had significantly higher tail length and tail moment values than in the control group. These results might be suggestive for increased DNA damage in patients with exudative AMD compared to those of the healthy subjects. Higher tail length value is known to be the sign of increased DNA breaks. However, tail moment, which is derived from the tail length and %DNA in the tail, shows more stable predicting performance for DNA damage according to most researchers (25, 26).

In the literature, there are a few numbers of studies investigating DNA damage in patients with AMD using comet assay. A study by Szaflik et al. showed higher percentage of tail DNA in an AMD group (included dry and wet AMD) and they suggested that AMD patients had increased DNA damage (11). However, they reported no significant difference regarding DNA damage between patients with dry and wet AMD. Similarly, Wozniak et al. demonstrated higher endogenous oxidative DNA damage (higher %tail DNA) and lower efficacy of DNA repair in patients with wet AMD compared to those of the healthy controls (12).

The results of our study are in agreement with the above mentioned studies regarding increased cumulative DNA damage in AMD based on the comet analysis. However, our study differs from previous reports by also investigating systemic oxidant/antioxidant status in exudative AMD.

Conclusion

In conclusion, patients with exudative AMD seem to have

increased systemic oxidative stress and DNA damage, which might contribute to development of advanced wet AMD. However, further studies are necessary to investigate these factors in earlier stages of AMD, which might be valuable for developing preventive strategies in AMD.

References

1. Seddon JM. Macular Degeneration Epidemiology: Nature-Nurture, Lifestyle Factors, Genetic Risk, and Gene-Environment Interactions-The Weisenfeld Award Lecture. Invest Ophthalmol Vis Sci 2017; 58(14): 6513-28.

2. Gorusupudi A, Nelson K, Bernstein PS. The Age-Related Eye Disease 2 Study: Micronutrients in the Treatment of Macular Degeneration. Adv Nutr 2017; 8(1): 40-53.

3. Datta S, Cano M, Ebrahimi K, Wang L, Handa JT. The impact of oxidative stress and inflammation on RPE degeneration in non-neovascular AMD. Prog Retin Eye Res 2017; 60: 201-18.

4. Du Z, Wu X, Song M, Li P, Wang L. Oxidative damage induces MCP-1 secretion and macrophage aggregation in age-related macular degeneration (AMD). Graefes Arch Clin Exp Ophthalmol 2016; 254(12): 2469-76.

5. Elbay A, Ozer OF, Akkan JC, Celik U, Kutlutürk I, Koytak A, et al. Comparison of serum thiol-disulphide homeostasis and total antioxidant-oxidant levels between exudative age-related macular degeneration patients and healthy subjects. Int Ophthalmol 2017; 37(5): 1095-101.

6. Uğurlu N, Aşık MD, Yülek F, Cagil N. Oxidative stress and anti-oxidative defence in patients with age-related macular degeneration. Curr Eye Res 2013; 38: 497-502.

7. Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem 2005; 38: 1103-11.

8. Horoz M, Bolukbas C, Bolukbas FF, Aslan M, Koylu AO, Selek S, et al. Oxidative stress in hepatitis C infected end-stage renal disease subjects. BMC Infect Dis 2006; 6: 114.

9. Regulski MJ. Cellular Senescence: What, Why, and How. Wounds 2017; 29(6): 168-74.

10. Menck CF, Munford V. DNA repair diseases: What do they tell us about cancer and aging? Genet Mol Biol 2014; 37(1 Suppl): 220-33.

11. Szaflik JP, Janik-Papis K, Synowiec E, Ksiazek D, Zaras M, Wozniak K, et al. DNA damage and repair in age-related macular degeneration. Mutat Res 2009; 669: 169-76.

12. Wozniak K, Szaflik JP, Zaras M, Sklodowska A, Janik-Papis K, Poplawski TR, et al. DNA damage/repair and polymorphism of the hOGG1 gene in lymphocytes of AMD patients. J Biomed Biotechnol 2009; 2009: 827562.

13. Kumaravel TS, Vilhar B, Faux SP, Jha AN. Comet Assay measurements: a perspective. Cell Biol Toxicol 2009; 25: 53-64.

14. Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations Mol Biotechnol 2004; 26: 249-61.

15. Xu B, Kang L, Zhang G, Wu J, Zhu R, Yang M, et al. The changes of 8-OHdG, hOGG1, APE1 and Pol β in lenses of patients with age-related cataract. Curr Eye Res 2015; 40(4): 378-85.

16. Valavanidis A, Vlachogianni T, Fiotakis C. 8-hydroxy-2'-deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 2009; 27: 120-39.

17. Visvardis EE, Tassiou AM, Piperakis SM Study of DNA damage induction and repair capacity of fresh and cryopreserved lymphocytes exposed to H_2O_2 and gamma-irradiation with the alkaline comet assay Mutat Res 1997; 383: 71-80.

18. Nandhakumar S, Parasuraman S, Shanmugam MM, Rao KR, Chand P, Bhat BV. Evaluation of DNA damage using single-cell gel electrophoresis (Comet Assay). J Pharmacol Pharmacother 2011; 2: 107-11.

19. Singh NP, Stephens RE, Schneider EL. Modifications of alkaline microgel electrophoresis for sensitive detection of DNA damage. Int J Radiat Biol 1994; 66: 23-8.

20. Sachdeva MM, Cano M, Handa JT. Nrf2 signaling is impaired in the aging RPE given an oxidative insult. Exp Eye Res 2014; 119: 111-4.

21. Dinc E, Ayaz L, Kurt AH. Protective Effect of Combined Caffeic Acid Phenethyl Ester and Bevacizumab Against Hydrogen Peroxide-Induced Oxidative Stress in Human RPE Cells Curr Eye Res 2017; 42: 1659-66.

22. Mozaffarieh M, Schoetzau A, Sauter M, Grieshaber M, Orgül S, Golubnitschaja O, Flammer J, et al. Comet assay analysis of single-stranded DNA breaks in circulating leukocytes of glaucoma patients. Mol Vis 2008; 14: 1584-8.

23. Singh NP. The comet assay: Reflections on its development, evolution and applications Mutat Res Rev Mutat Res 2016; 767: 23-30.

24. Mozaffarieh M, Schötzau A, Josifova T, Flammer J. The effect of ranibizumab versus photodynamic therapy on DNA damage in patients with exudative macular degeneration. Mol Vis 2009; 15: 1194-9.

25. Lee E, Oh E, Lee J, Sul D, Lee J. Use of the tail moment of the lymphocytes to evaluate DNA damage in human biomonitoring studies. Toxicol Sci 2004; 81: 121-32.

26. Ashby J, Tinwell H, Lefevre PA, Browne MA. The single cell gel electrophoresis assay for induced DNA damage (comet assay): measurement of tail length and moment Mutagenesis 1995; 10: 85-90.