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Original Research

Providing preclinical prediction of diabetic retinopathy via oxidative stress levels

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Abstract

Background and aims: Although diabetic retinopathy (DR) is a disease that develops due to diabetes mellitus (DM), it also brings problems in the clinic. Depending on the diagnosis and related developments, the role of metabolites that cause stress in the cell and disorders in metabolism should be constantly reviewed. We aim to examine inflammation, oxidative stress levels, and DNA damage in DM and DR patients. **Material and method:** A total of 3 groups of 30 patients with a 5-year history of DM, 30 patients with a 5-year history of DR, and healthy individuals with the same demographic characteristics as both patient groups, between the ages of 45 and 65 years who came to the Bezmialem Vakif University Faculty of Medicine Ophthalmology Outpatient Clinic were included. Total antioxidant status (TAS), total oxidant status (TOS), total thiol (TT), and native thiol (NT) of patients and healthy individuals by photometric methods; inflammation biomarkers interleukin-1 beta (IL-1β), interleukin-6(IL-6), and tumor necrosis factor-alpha (TNF-α) were measured by photometric methods with commercially purchased ELISA kits. **Results:** Oxidative stress and inflammation were statistically increased in both DR and DM patients, and antioxidant levels were statistically decreased in both DR and DM patients ($p<0.01$). IL-1β levels ($p<0.001$), TNF-α, and IL-6 levels ($p<0.01$) were found statistically significant and high. DNA damage was found higher in patients with DR compared to both the control group ($p<0.001$) and the patient group with DM ($p<0.001$). **Conclusions:** Increased oxidative stress and prominent inflammation in DR patients may provide clinical guidance on the pathogenesis, prognosis, and treatment strategies of the disease.

Keywords: diabetes mellitus, diabetic retinopathy, DNA damage, inflammation, oxidative stress.

Background and aims

Diabetes mellitus (DM) is a complex disease in which there are high blood sugar levels over a long time characterized by hyperglycemia, microvascular disease of the eye, and various pathophysiological disorders. These high blood sugar levels cause symptoms of weakness and tiredness, repeated urination, increased hunger, and increased thirst [1]. It is thought that 400 million people worldwide have diabetes, and almost half of these patients are assumed to have diabetic retinopathy (DR). Time must pass for DR to occur, and this time interval occurs in stages [2].

DM has many complications, and the most well-known is DR, which impairs the

function of the eye at low ages. There are studies showing that inflammation damages nerve cells and disrupts the mechanism [3]. With chronic hyperglycemia, progressive retinal dysfunction occurs due to vascular damage such as blood-retinal barrier rupture [4]. The diagnosis of DR is classified as ophthalmoscopic, with the deterioration of tissues occurring in the vessels. DR has two clinical stages: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). NPDR occurs in the early stages of DR. In this stage, the permeability of the vessels and occlusion of the capillaries are observed [5, 6].

The amount of reactive oxygen species in the environment causes the oxidative balance in the body to be disturbed, which means that DNA and lipids are damaged. As a result, normal cellular function is impaired [7]. It is known that hyperglycemia causes oxidative damage to the retina, and this has a vital role in the pathogenesis of DR [8]. Excessive accumulation of ROS can disrupt the tissue around retinal vessels and eventually lead to DR. Various metabolic diseases can cause oxidative damage to the retina. Hyperglycemia also causes four classical metabolic abnormalities in the retina in this way [9, 10]. Due to its structure, the retina contains polyunsaturated fatty acids, and when it is constantly exposed to UV light, retinal pigment epithelial (RPE) cells are damaged. Therefore, it is susceptible to oxidative stress [11].

The relationship between DR pathogenesis and inflammation has become an increasingly important issue with studies. Studies in both animal models and humans show inflammation in most stages of DR (5). In DR, the vessels narrow, and this causes biochemical and metabolic changes. The narrowing of the vessels reduces the normal blood flow rate. Cell degeneration and retinal perfusion occur when capillary pericytes are destroyed. Oxygen demand in tissues can increase the production of proinflammatory cytokines [12].

In DM, cytochrome c influx from the mitochondrial membranes to the cytosol is seen because of the deterioration in the retinal mitochondrial structure. Therefore, acceleration due to the apoptotic process takes place. In addition, mitochondrial DNAs (mtDNA) are damaged, and the rate of self-replication of mitochondria decreases. The transcription of the proteins produced by mtDNA is disrupted, so the electron transport chain is disrupted, and free radicals continue to spread [13]. Increased production of proinflammatory mediators also plays an essential role in the pathogenesis of DR. In addition, it significantly triggers the development and progression of DR through inflammation mechanisms. Biochemical and pathological retinal abnormalities reveal the relationship between DM and inflammation [14].

Today, developments in optical coherence tomography (OCT) provide information about the morphological formations that occur in the retinal structure. In retinal diseases such as DR, it shows the relationship between morphological changes and the retina, which can be considered clinically important biomarkers. OCT angiography is a method that provides information about the retinal vascular system by observing the movement of blood in the vessel. OCT is a device that provides reliable information about the retinal nerve fiber layer (RNFL) [15–17]. New treatment modalities are still being explored to reduce the impact and progression of DR. We aim to correlate oxidative stress, inflammation, and DNA damage with optical coherence tomography (OCT) in patients diagnosed with DR; to examine the levels of inflammation and oxidative stress in DM and DR patients.

Material and method

Measurement of total thiol (TT), native thiol (NT), and disulfide (DIS) levels

Total thiol (TT – Rel Assay Diagnostics, RL0178, Mega Tip, Turkey), native thiol (NT – Rel Assay Diagnostics, RL0185, Mega Tip, Turkey), and disulfide (DIS) levels were measured with the method developed by Erel [18]. This method is based on the reduction of proteins, which transform into a reversible disulfide form under oxidative conditions, to sulfhydryl groups, and again disulfide bonds to thiol groups. The TT and NT content of the samples were measured with the

modified Ellman reagent. NT levels were subtracted from TT levels, and the amount of DIS bond was calculated by dividing the results by two.

Measurement of total antioxidant status (TAS) and total oxidant status (TOS)

TOS and TAS were measured by colorimetric methods developed by Erel [19]. TAS results are expressed in mmol Trolox Eq./L, and TOS results in μmol H_2O_2 Eq./L units. Oxidative stress index (OSI) was calculated as the ratio of TOS level to TAS level.

Measurement of inflammatory biomarkers

Serum interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α) concentrations were measured with specific commercial ELISA kits according to the manufacturer's instructions (IL-1β: E0143Hu, IL-6: E0090Hu and TNF-α: E0082Hu – Bioassay Technology Laboratory, China). Concentrations were determined with a spectrophotometric plate reader (Varioskan Flash Multimode Reader, Thermo, Waltham, USA) at 450 nm wavelength.

DNA damage measurement

Leukocyte DNA damage was analyzed by the alkaline single-cell gel electrophoresis (Comet Assay) method [20]. About 6 μl of thawed whole blood was mixed with low melting temperature agarose (0.7%) (Sigma-Aldrich – A9414). It was then embedded on slides covered with agarose gel (1%) (Sigma-Aldrich – A4718) at the normal melting temperature, covered with a coverslip, and allowed to solidify in a cold environment. After the gel solidified, coverslips were removed from the slide, and cells were lysed by keeping the slides in a lysis buffer for at least 4 hours. It was then subjected to electrophoresis (300 mA) for 20 minutes in an alkaline buffer (pH: 13). After electrophoresis, cells stained with ethidium bromide (5 mg/ml) (Sigma-Aldrich – E7637) were examined by fluorescence microscopy (Excitation: 546 nm, Emission: 20 nm). DNA tail percentages were analyzed by counting an average of 50 cells using Comet Assay analysis program IV (Perceptive Instruments, Suffolk, UK).

Study design and patients

Patients between the ages of 45–65 years in the Ophthalmology Department of Bezmialem Vakif University Faculty of Medicine were included in the study. The working groups are as follows: 5-year history of DR, 5 years of DM history and no diagnosis of retinopathy, and healthy volunteers with the same demographics as patients with DM and DR. Thirty individuals from each group were included, and blood was taken from the participants into a biochemistry gel tube and a lithium heparin tube. Blood samples were separated into serum and leukocytes. Visual acuity, eye pressure, slit-lamp examinations of anterior and posterior segments, and OCT were evaluated. Ethical approval was obtained from the Clinical Research Ethics Committee of Bezmialem Vakif University, numbered 71306642-0.505.04.

Statistical analysis

All data analyzes were performed using SPSS version 25.0 (IBM, Armonk, NY, USA). Numerical data were expressed as mean ± standard deviation (SD), a p-value of <0.05 was considered statistically significant. TT, NT, TAS, and TOS levels in serum were statistically compared in two groups using Mann-Whitney U-test, Chi-square test, or Student t-test.

Limitations

Completing the groups with 30 volunteers each, selecting the DR patient group without making any distinction between "proliferative or non-proliferative", and not including the treatment periods of the patient groups are seen as limitations in our study.

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Results

Comparisons of the patient (DR and DM) and control groups in terms of demographic characteristics, duration of diabetes, and HbA_{1c} data are shown in Table 1.

It is seen that TAS is significantly reduced in patients with DR compared to the healthy control group, and TAS is significantly reduced in patients with DM compared to the healthy control group ($p<0.01$, $p<0.05$, respectively). Likewise, TAS was found significantly reduced in patients with DR compared to DM ($p<0.01$) (Figure 1A).

TOS was found significantly higher in patients with DR compared to both the control group and the patient group with DM ($p<0.01$). TOS was also found significantly higher in patients with DM compared to the healthy control group ($p<0.05$) (Figure 1B).

OSI was found significantly higher in patients with DR compared to both the healthy control group and the patient group with DM ($p<0.01$). OSI was found significantly higher in patients with DM compared to the control group ($p<0.05$) (Figure 1C).

TT was found statistically lower in the DR patient group compared to both the

Table 1: Age, duration of diabetes, and HbA_{1c} data of healthy control groups, diabetes mellitus (DM) and diabetic retinopathy (DR) patient groups.

	Control mean ± SD	DM mean ± SD	DR mean ± SD
Age (Years)	53.17±5.12	54.43±7.21	56.17±4.39
Duration of diabetes (Years)	–	5.49±0.37	5.13±0.09
HbA1c (%)	5.16±0.28	7.88±1.12	7.13±1.28

SD = Standart deviation.

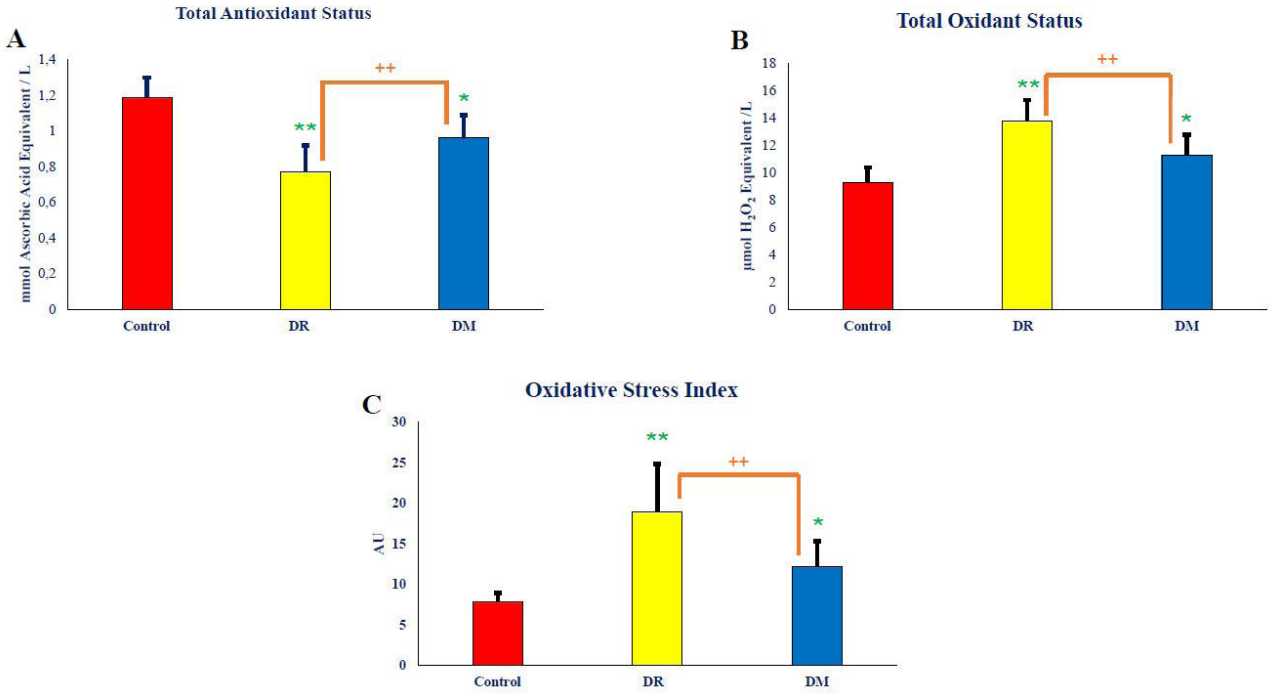

Figure 1: Oxidative stress biomarkers in diabetes mellitus (DM) and diabetic retinopathy (DR) patients and healthy controls. (A) Total antioxidant status (TAS). (B) Total oxidant status (TOS) (C) Oxidative stress index (OSI). (*p<0.05; **p<0.01, ***p<0.001). Differences in DM and DR compared to control. (+p<0.05; ++p<0.01, +++ p<0.001). Differences between DM and DR patients group. These values were considered statistically significant.

healthy control and DM patient groups (p<0.05) (Figure 2A). NT in patients with DR was found significantly lower than both the healthy control group and the patient group with DM (p<0.01, p<0.001, respectively). NT was found lower in patients with DM compared to the healthy control group (p<0.05) (Figure 2B). DIS levels in patients with DR were found high in both the control group and patients with DM (p<0.01). DIS levels were found significantly higher in patients with DM compared to the control group (p<0.05) (Figure 2C).

IL-1β levels were found higher in patients with DR compared to both healthy controls and patients with DM (p<0.001, p<0.01, respectively). IL-1β levels were found significantly higher in patients with DM compared to the healthy control group (p<0.05) (Figure 3A).

IL-6 levels in patients with DR were found statistically higher than both in patients with DM and the healthy control group (p<0.01). IL-6 levels were also found higher in patients with DM compared to the healthy control group (p<0.05) (Figure 3B).

TNF-α values in patients with DR were found higher than in both healthy controls and patients with DM (p<0.01, p<0.05, respectively). TNF-α values were found higher in patients with DM compared to the healthy control group (p<0.05) (Figure 3C).

DNA damage was found statistically significant and higher in patients with DR compared to both healthy controls and patients with DM (p<0.001). Likewise, DNA damage was found higher in DM patients compared to the healthy control group (p<0.001) (Figure 4).

Visual acuity in patients with DR was found lower than in both healthy controls and patients with DM, but there was no statistically significant difference (Figure 5A). Although macular thickness was higher in patients with DR compared to both the control and DM patient groups, it was not significant (Figure 5B). Choroidal thickness was found statistically significant and higher in patients with DR compared to both the control and DM patient groups (p<0.01) (Figure 5C). Retinal nerve fiber layer (RNFL) levels in patients with DR were found significantly higher than both in patients with DM and the healthy control group (p<0.01) (Figure 5D).

In our study, OCT values, TOS, OSI, DIS, IL-1β, IL-6, TNF-α, and DNA damage were

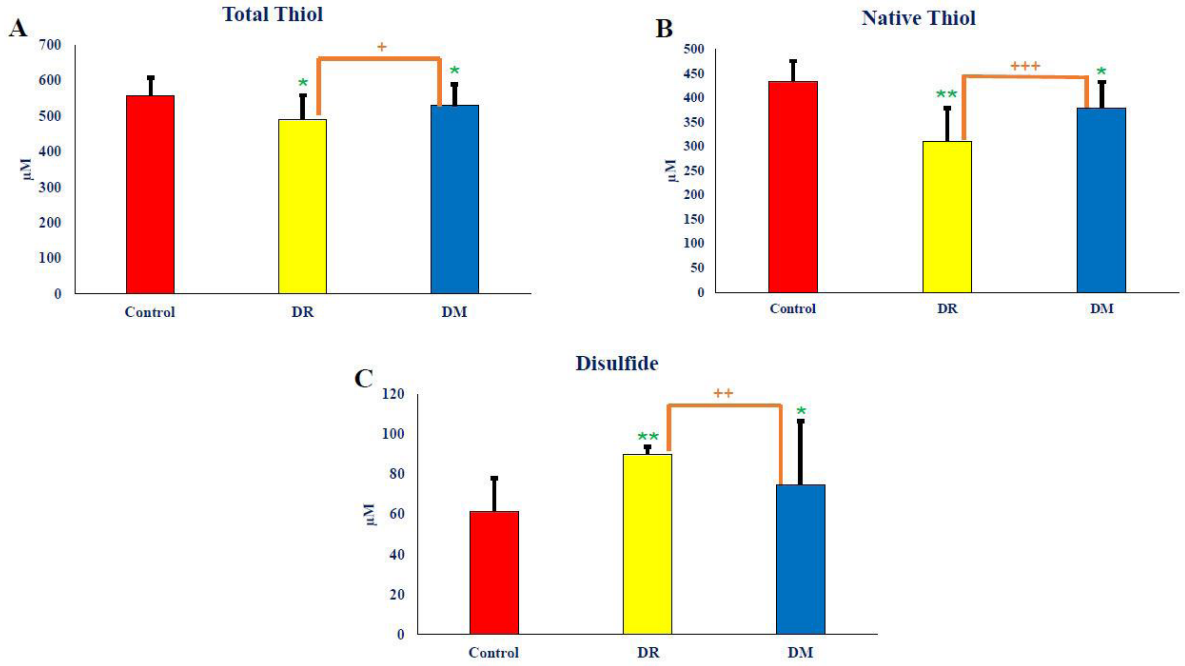

Figure 2: Thiol homeostasis biomarkers in diabetes mellitus (DM) and diabetic retinopathy (DR) patients and healthy controls. (A) Total thiol (TT). (B) Native thiol (NT). (C) Disulfide (DIS). (*p<0.05; **p<0.01, ***p<0.001). Differences in DM and DR compared to control. (+p<0.05; ++p<0.01, +++p<0.001). Differences between DM and DR patients group. These values were considered statistically significant.

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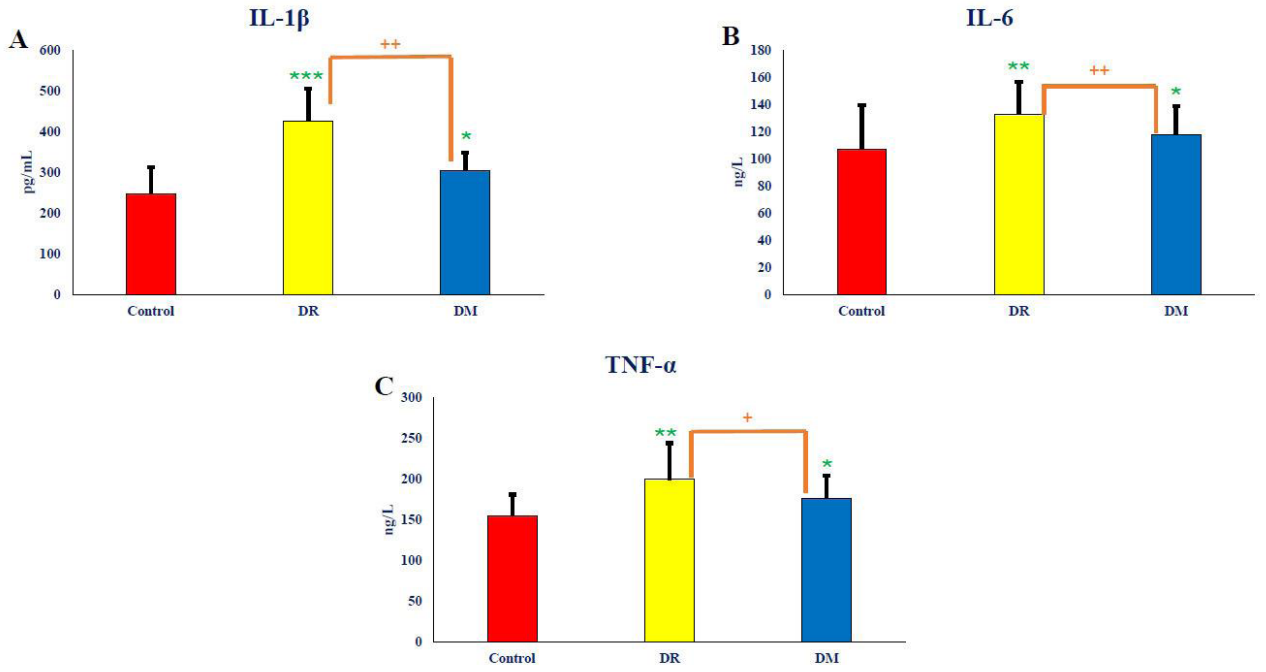

Figure 3: Inflammation biomarkers in diabetes mellitus (DM) and diabetic retinopathy (DP) patients and healthy controls. (A) Interleukin-1 beta. (B) Interleukin-6. (C) Tumor necrosis factor-alpha. (*p<0.05; **p<0.01, ***p<0.001). Differences in DM and DR compared to control. (+p<0.05; ++p<0.01, +++p<0.001). Differences between DM and DR patients group. These values were considered statistically significant.

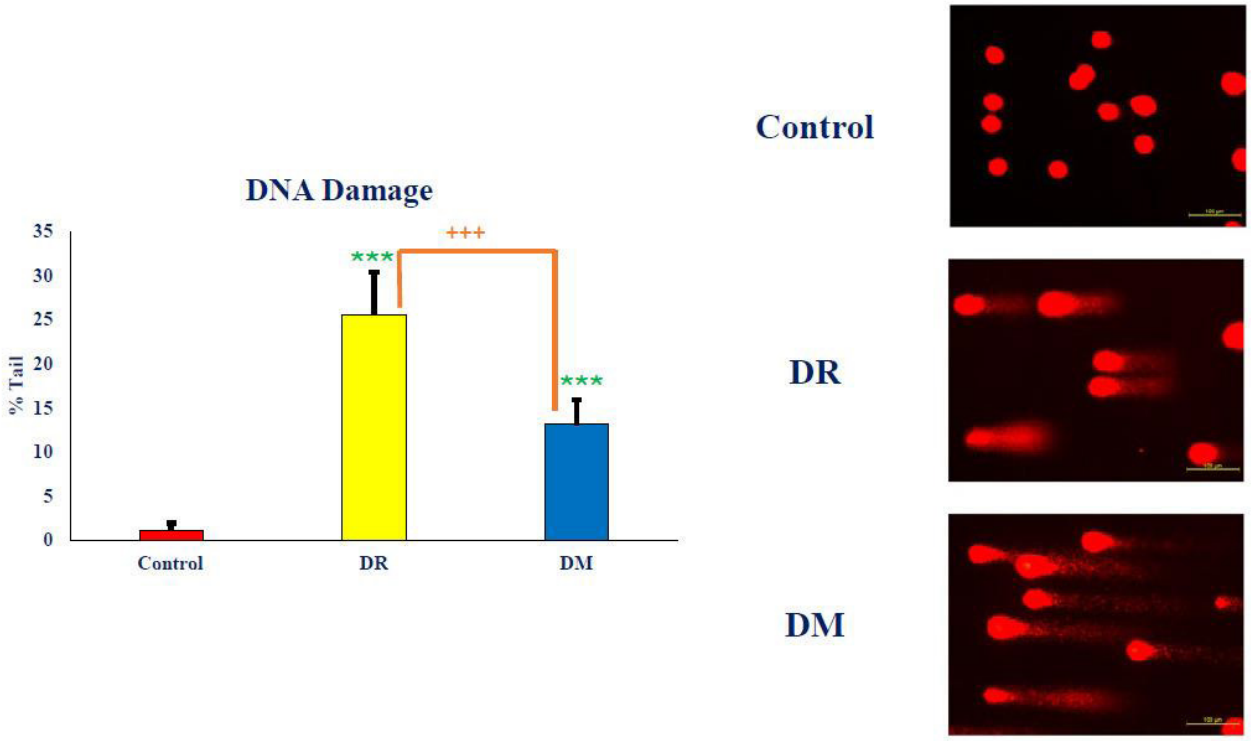

Figure 4: The effect of diabetes mellitus (DM) and diabetic retinopathy (DR) patients on DNA damage. Percentage and image of DNA in the tail in DM and DR patients and healthy control group. (*p<0.05; **p<0.01, ***p<0.001). Differences in DM and DR compared to control. (+p<0.05; ++p<0.01, +++p<0.001). Differences between DM and DR patients group. These values were considered statistically significant.

statistically positively correlated, while TAS, TT, and NT levels were negatively correlated (Table 2).

Discussion

Radicals that cause oxidative stress cause damage to the retina and vessels, which creates a favorable environment for the gradual formation of DR [21–24]. The fatty acid content in the retina is quite high, and it consumes oxygen at high rates for the metabolic functioning to be at normal levels. The high use of oxygen indicates that the retina is sensitive to oxidative stress [25]. In the study of Sanz-González et al. low total antioxidant status was found in the plasma of patients

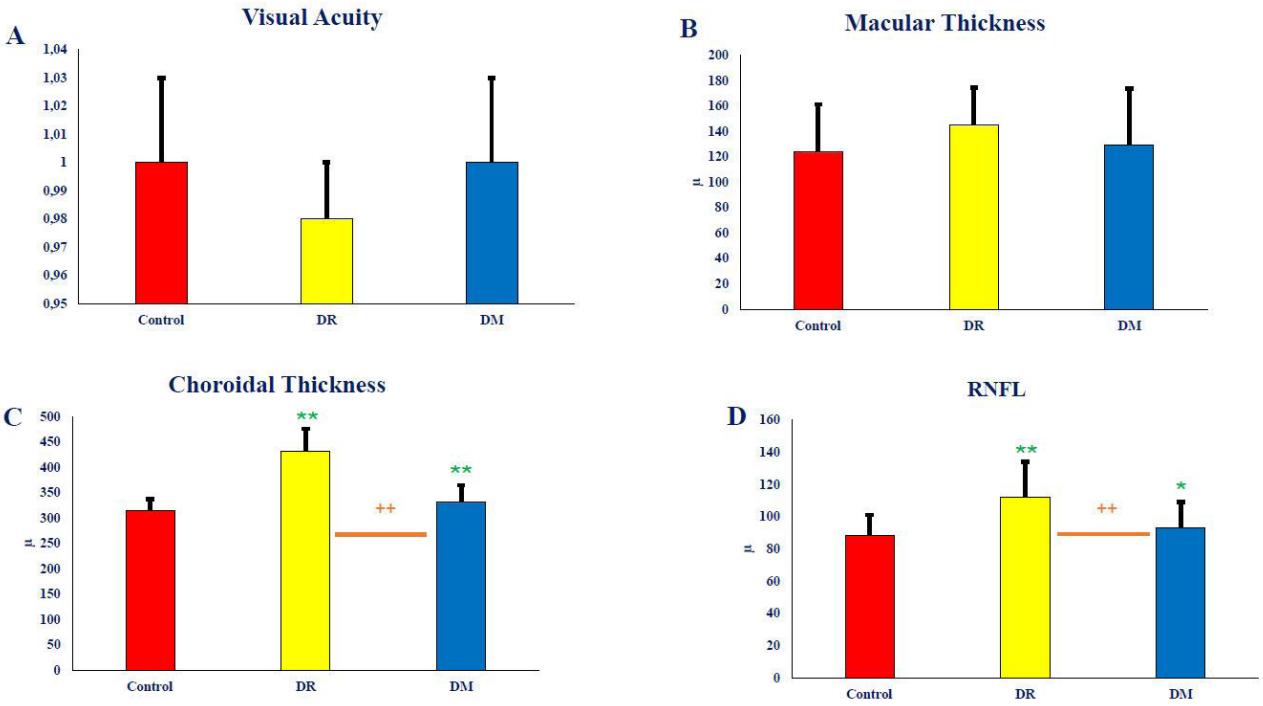

Figure 5: Parameters viewed with Optical Coherence Tomography (OCT). (*p<0.05; **p<0.01, ***p<0.001). Differences in DM and DR compared to control. (+p<0.05; ++p<0.01, +++p<0.001). Differences between DM and DR patients group. These values were considered statistically significant. RNFL= Retinal Nerve Fibril Layer.

Table 2: Correlation of oxidative stress, inflammation, and DNA damage in diabetic retinopathy (DR).

	Visual acuity right-left (mean)	Macular thickness right-left (mean)	Choroidal thickness right-left (mean)	OCT(RNFL) right-left (mean)
TAS	ns	ns	r= -0.132, p=0.445	r= -0.442, p=0.119
TOS	ns	ns	r= 0.335, p=0.512	r= 0.264, p=0.555
OSI	ns	ns	r= 0,861, p=0,521	r= 0.813, p=0.639
TT	ns	ns	r= -0.223, p=0.229	r= -0.274, p=0.164
NT	ns	ns	r= -0.3162, p=0.215	r= -0.373, p=0.367
DIS	ns	ns	r= 0.437, p=0.318	r= 0.225, p=0.465
IL-1β	ns	ns	r= 0.563, p=0.476	r= 0.313, p=0.299
IL-6	ns	ns	r= 0.443, p=0.427	r= 0.264, p=0.647
TNF-α	ns	ns	r= 0.542, p=0.215	r= 0.334, p=0.368
DNA damage	ns	ns	r=0.852, p=0.165	r= 0.852, p=0.498

OCT = Optical Coherence Tomography; RNFL = Retinal Nerve Fibril Layer; TAS = Total Antioxidant Status; TOS = Total Oxidant Status; OSI = Oxidative Stress Index; TT = Total Thiol; NT = Native Thiol; DIS = Disulfide; IL-1β = Interleukin-1 beta; IL-6 = Interleukin-6; TNF-α = Tumor Necrosis Factor-alpha.

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with DR diagnosed with type II diabetes [26]. In our results, total antioxidant status in patients with DR was found statistically significant and lower compared to both patients with DM and the healthy control group.

Excessive accumulation of ROS can disrupt the tissue around retinal vessels and eventually lead to DR [9]. In the study of Kirboga et al. it was shown that the serum total oxidant status was higher in the DR patient group compared to the control group [27]. In our results, TOS and OSI were found statistically higher in both DM and DR patients compared to the healthy control group. This shows that DM is exposed to more reactive oxygen species in the transition phase to DR.

Previous studies have evaluated the degree of oxidative damage to proteins and thiol levels as a marker of antioxidant status in patients with DR. In the study of Baskol et al. TT levels in patients with DR were found lower than both in patients without DR and in the healthy control group. In another study, Gulpamuk et al. showed that NT and TT levels in patients with DR decreased compared to the control group, and DIS levels were found higher than the healthy control group [28, 29]. The results of our study were correlated with the studies performed, TT and NT levels in patients with DR were lower than both in patients with DM and the control group; DIS levels were found statistically significant and higher than both the DM and healthy control groups.

Many mechanisms suggesting inflammation in the retina have been demonstrated in DR due to DM. Some proinflammatory cytokines are involved in insulin resistance. During hyperglycemia, cytokines such as TNF-α and IL-6 can be produced [12]. In the study of Drumond et al. they found that IL-1β levels were higher in patients diagnosed with DR compared to healthy controls, while there was no change in IL-6 levels [30]. Melo et al. on the other hand, reported that they could not find a link between proinflammatory cytokines (IL-6 and TNF-α) between patients with proliferative DR and type I diabetes patients [31]. In other studies, IL-1β, IL-6, and TNF-α levels were found significantly higher in patients with DR compared to the healthy control group [32, 33]. In our study, the levels of proinflammatory cytokines (IL-6, IL-1β, and TNF-α) in patients with DR were statistically higher and significant compared to both DM and healthy control groups.

The longer the duration of diabetes, the more likely the mitochondria are damaged. Cell death occurs with the increase of ROS in the development of DR [34]. In their study on DNA damage, Song et al. showed that patients diagnosed with diabetes have high levels of DNA damage [35]. This study was correlated with our study, and DNA damage was found significantly higher and statistically significant in patients with DR compared to both DM and healthy control groups. This suggests that in the case of hyperglycemia, oxidative stress predominates in the cell and causes DNA damage.

In this study, we aimed to evaluate the existing oxidative stress, inflammation, and DNA damage in patients with DR and DM. When the literature is examined, since TOC, TAC, OSI, TT, NT, DIS, IL-1β, IL-6, TNF-α, and DNA damage are examined separately, our study is a comprehensive study that evaluates all these parameters together. In addition, our study is important because it is the first study to evaluate both DM and DR simultaneously in terms of oxidative stress and inflammation.

Conclusions

To sum up, inflammation and oxidative stress were statistically increased in patients with DR compared to DM, while antioxidant levels were statistically decreased. Therefore, DNA damage levels were found higher in patients with DR. The importance of early diagnosis before the development of DR in DM patients and the necessity of prophylactic treatment are considered.

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Conflict of Interest

The authors declare no conflict of interest.

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