Araştırma Makalesi / Research Article

The investigation of oxidative stress parameters in patients with lipoid proteinosis

Lipoid proteinozis hastalarında oksidatif stres parametrelerinin araştırılması

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Abstract

Background: This study aims to investigate the role of oxidative stress in pathogenesis of Lipoid Proteinosis (LP) by measuring the parameters of serum oxidative stress such as total antioxidant status (TAS), total oxidant status (TOS), advanced oxidation protein products (AOPP), oxidative stress index (OSI), ferric reducing antioxidant power (FRAP), lipid hydroperoxide (LOOH) and total free sulfhydryl groups (Total-SH).
Methods: Fourteen patients with LP diagnosed clinically, radiologically and histopathologically and 14 sex-, age- and body mass index- matched healthy volunteers were included in this prospective case-control study. Serum TAS, TOS, AOPP, FRAP, LOOH and Total-SH were measured spectrophotometrically and OSI were calculated.
Results: Cases with LP had significantly lower serum levels of TAS, FRAP and Total –SH (p< 0.001, for each) and higher levels of OSI, AOPP and LOOH (p= 0.001, p= 0.018, and p= 0.047, respectively). Serum TOS levels were higher in the LP group when compared to that of the control group, but it was not statistically significant (p= 0.081). ROC analysis demonstrated that TAS, FRAP, Total-SH and OSI levels independently predicted LP with strong sensitivity and specificity.
Conclusion: Our results demonstrate that decreased levels of antioxidant parameters and increased levels of oxidant parameters might be used as an ancillary laboratory tests in the diagnosis of LP disease.

Keywords: Lipoid Proteinosis; Oxidative Stress; Antioxidant

Öz.

Amaç: Bu çalışmadaki amacımız, total antioksidan seviye (TAS), ferrik iyon indirgeyici antioksidan güç (FRAP) ve toplam serbest sülfhidril grupları (Total-SH) gibi antioksidan parametreler ile total oksidan seviye (TOS), ileri oksidasyon protein ürünleri (AOPP), oksidatif stres indeksi (OSI) ve lipid hidroperoksit (LOOH) gibi oksidasyon parametrelerini ölçerek Lipoid Proteinozlu (LP) hastalarda oksidatif stresin rolünü araştırmaktır.
Materyal ve Metod: Bu prospektif vaka kontrol çalışmasına klinik, radyolojik ve histopatolojik olarak tanı konulan 14 LP hastası ve cinsiyet, yaş ve vücut kitle indeksi benzer 14 sağlıklı gönüllü katılımcı dahil edildi. Serum TAS, TOS, AOPP, FRAP, LOOH ve Total-SH seviyeleri spektrofotometrik olarak ölçüldü ve OSI değeri hesaplandı.
Bulgular: LP'li hastalarda serum TAS, FRAP ve Total –SH (p< 0,001; her biri için) seviyeleri daha yüksek bulunurken OSI, AOPP ve LOOH düzeyleri daha düşük (sırasıyla, p= 0,001, p= 0,018 ve p= 0,047) bulundu. LP hastalarında serum TOS seviyeleri kontrol grubuna göre daha yüksekti, ancak bu yükseklik istatistiksel olarak anlamlı değildi (p= 0,081). Yapılan ROC analizinde TAS, FRAP, Total-SH ve OSI seviyelerinin LP hastalığını güçlü bir duyarlılık ve yüksek bir özgüllük ile öngördüğünü gösterdi.
Sonuç: LP hastalığının tanısında, antioksidan parametrelerindeki azalma ve oksidan parametrelerindeki artmanın yardımcı laboratuvar testleri olarak kullanılabileceğini göstermektedir.

Anahtar Kelimeler: Lipoid Proteinozis; Oksidatif Stres; Antioksidan

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Introduction

Lipoid proteinosis (LP) is a rare autosomal recessive genodermatosis characterized by accumulation of amorphous hyaline substance in the skin and mucous membranes (1). This disease, which affects both sexes equally and whose incidence and prevalence is not known, develops as a result of pathogenic mutations in the extracellular matrix protein 1 (ECM1) gene in the first chromosome (2). This gene plays an important role in wound healing by regulating angiogenesis, epidermal differentiation, dermal collagen and proteoglycans binding (1,3). The ECM1 protein also binds to the matrix metalloproteinase-9 (MMP9) enzyme, which plays an important role in immune cell function (4). Mutations in the ECM1 gene prevent this protein from interacting properly with MMP9, thus contributing to the pathological changes that develop in LP (4).

Free radicals and reactive oxygen species (ROS) are constantly produced in small amounts during resting period by cellular metabolic processes (5). However, overproduction of ROS or inadequacy of antioxidant defense system induces oxidative stress (6). Weakening of the antioxidant defense system during oxidative stress results in increased ROS production, which in turn results in lipid, protein, and DNA damage in cells (7). Therefore, oxidative stress plays an important role in the induction and progression of many diseases such as atherosclerosis, cancer, autoimmune diseases, insulin resistance, neurodegenerative diseases, and metabolic syndrome (8,9).

To the best of our knowledge, there are no studies measuring the levels of oxidative stress parameters in LP patients. This study aims to investigate the role of oxidative stress in pathogenesis of LP by measuring the parameters of serum oxidative stress such as total antioxidant status (TAS), total oxidant status (TOS), advanced oxidation protein products (AOPP), oxidative stress index (OSI), ferric reducing antioxidant power (FRAP), lipid hydroperoxide (LOOH) and total free sulfhydryl groups (Total-SH).

Materials and Methods

Study Population

Fourteen patients (mean age= 15.07 ± 7.21 years) with LP diagnosed clinically, radiologically and histopathologically and 14 sex-, age- and body mass index (BMI)-matched healthy volunteers (mean age= 15.43 ± 8.55 years) were included in this prospective case-control study. The study was approved by the Local Ethics Research Committee of Harran University and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. All subjects provided written informed consent prior to participation in the study. Patients with acute or chronic systemic and infectious diseases, regular drug or vitamin consumption, antioxidant substances use, or cigarettes or alcohol consumption were excluded from the study.

Blood Sampling

Venous blood samples were taken in the morning after at least eight hours of overnight fasting. Collected samples were centrifuged at 1500 rpm for 10 minutes and separated serum samples were stored at -86 ^{o}C until analysis. Subsequently, oxidative stress parameters of all participants were studied at the same time and with the same serum samples.

Measurement of oxidative stress parameters

Serum oxidative stress parameters, including total antioxidant status (TAS), total oxidant status (TOS), oxidative stress index (OSI), advanced oxidation protein products (AOPP), ferric reducing antioxidant power (FRAP) assay, lipid hydroperoxide (LOOH) and total free sulfhydryl groups (Total-SH) were measured as described in our previous studies (10–12).

1) Measurement of the TAS

The determination of TOS was performed using a microplate reader (Varioskan Lux, Thermo Scientific, USA) according to the method of Erel (13). In this method, hydroxyl radical, which is the most potent radical, is produced via Fenton reaction. In the classical Fenton reaction, the hydroxyl radical is produced by mixing of ferrous ion solution and hydrogen peroxide solution. In the assay, ferrous ion solution, which is present in the reagent-1, is mixed with hydrogen peroxide, which is present in the reagent-2. The sequential process produced radicals such as brown-colored dianisidinyl radical cation, produced by the hydroxyl radical. In this assay, antioxidative effect of the sample against the potent free-radical reactions, which is initiated by the produced hydroxyl radical, is measured. The assay had excellent precision values of less than 3%. The results are expressed as mmol Trolox equivalent/L.

2) Measurement of the TOS

The determination of TOS was performed using a microplate reader (Varioskan Lux, Thermo Scientific, USA) according to the method of Erel (14). Briefly, oxidants present in the sample oxidize the ferrous ion-*o*-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide, and the results are expressed as µmol H_2O_2 equivalent/L.

3) Calculation of the OSI

For calculation of OSI, an important indicator of oxidative stress, firstly TOS and TAS units were calculated. Oxidative stress index was calculated according to the following formula: OSI (arbitrary units) = TOS (μmol H_2O_2 equivalent/L)/TAS (μmol Trolox equivalent/L) × 100 (11).

4) Measurement of the AOPP

The determination of AOPP was performed using a microplate reader (Varioskan Lux, Thermo Scientific, USA) according to the method of Witko-Sarsat et al (15). Briefly, 200 μL of serum diluted 1:5 in phosphate buffered saline (PBS) was placed in each well of a 96-well microtiter plate. Then, 10 μL of 1.16 mol/L potassium iodide were added to each well, followed by 20 μL of acetic acid. Finally, the absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 200 μL of PBS, 10 μL of potassium iodide and 20 μL of acetic acid. Calibration curve was prepared using chloramine-T at concentrations of 0 to 100 μmol/L. AOPP concentrations were expressed in μmol/L of chloramine-T equivalents.

5) Measurement of the FRAP

The determination of serum FRAP levels were performed using a microplate reader (Varioskan Lux, Thermo Scientific, USA) according to the method of Benzie and Strain (16). The method measures the ability of antioxidants contained in a sample to reduce ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions. This reduction at a low pH causes a colored ferrous-tripyridyltriazine complex that absorbs light at λ = 593 nm. The results were expressed as μmol/L.

6) Measurement of the LOOH

The determination of serum LOOH levels by the ferrous ion oxidation-xylenol orange (FOX2) assay were performed using a microplate reader (Varioskan Lux, Thermo Scientific, USA) (17). The principle of the assay depends on the oxidation of ferrous ion to ferric ion through various oxidants, and the produced ferric ion is measured with xylenol orange. LOOHs are reduced by triphenyl phosphine (TPP), which is a specific reductant for lipids. The difference between with and without TPP pretreatment gives LOOH levels. The results were expressed as μmol/L.

7) Measurement of the Total -SH Groups

The determination of serum total -SH levels were performed using a microplate reader (Varioskan Lux, Thermo Scientific, USA) according to the method of Ellman (18) as modified by Hu et al (19). Briefly, 200 μL of buffer containing 0.1 M Tris, 10 mM EDTA, pH 8.2, and 10 μL serum was added to each well of a 96-well microtiter plate, followed by 10 μL of 10 mM 5,5-dithiobis 2-nitrobenzoic acid (DTNB) in methanol. Blanks were run for each sample as a test, but without DTNB. Following incubation for 15 min at room temperature, sample absorbance was read at 412 nm. Sample and reagent blanks were subtracted. The concentration of −SH groups was calculated using reduced glutathione as the free −SH group standard and the results were expressed as mmol/L.

Statistical Analysis

Statistical analyses were conducted with SPSS for Windows version 23.0 software (IBM SPSS Inc, Chicago, IL, USA). The Shapiro Wilk test was employed to assess the normal distribution of data. Numerical variables with normal distribution were presented as mean ± standard deviation. Categorical variables were stated as number (n) and percentage (%). Comparison of two-sample numerical variables was conducted using the Unpaired Student's t-test. The Chi-square test was also used to compare categorical variables. Receiver operating characteristic (ROC) analysis was used to evaluate the diagnostic performance of oxidative stress parameters. The confidence interval (CI) was accepted as 95% throughout the analyses. A two-tailed *p* value of <0.05 was considered statistically significant.

Results

Fourteen patients with acute LP and 14 healthy controls were enrolled in the study. The demographic characteristics of subjects are shown in Table 1. No statistically significant differences were found between cases with LP and control group in terms of age, gender and body mass index (p= 0.946, p= 0.663, p= 0.246, respectively).

Table 2 and Figure 2 described the oxidative stress parameters for LP and control groups. Cases with LP had significantly lower serum levels of TAS, FRAP and Total –SH (p< 0.001, for each) and higher levels of OSI, AOPP and LOOH (p= 0.001, p= 0.018, and p= 0.047, respectively). Serum TOS levels were higher in the LP group when compared to that of the control group, but it was not statistically significant (p= 0.081).

ROC analysis was performed to determine the cut-off values of antioxidant parameters and oxidant parameters to predict LP patients. The cut-off levels were determined, and the areas under curve (AUC) were calculated as is demonstrated in Table 3 and Figure 1. The cut-off value of antioxidant parameters including TAS, FRAP and Total-SH were determined as 1.31 mmol Trolox Eqv. /L (sensitivity: 79%, specificity: 93%, area under the curve (AUC): 0.857, p= 0.001), 951 μmol/L (sensitivity: 86%, specificity: 100%, AUC: 0.949, p< 0.001) and 0.204 mmol/L (sensitivity: 86%, specificity: 100%, AUC: 0.954, p< 0.001), respectively. Additionally, the cut-off value of oxidant parameters including TOS, OSI, AOPP and LOOH were determined as 14.2 μmol H_2O_2 Eqv. /L (sensitivity: 50%, specificity: 93%, AUC: 0.610, p= 0.323), 0.93 arbitrary unit (sensitivity: 86%, specificity: 71%, AUC: 0.857, p< 0.001), 129 μmol/L (sensitivity: 71%, specificity:

64%, AUC: 0.740, *p*= 0.031) and 1.015 µmol/L (sensitivity: 64%, specificity: 64%, AUC: 0.663, *p*= 0.141), respectively.

Table 1. Demographic parameters from the LP patients and control group.

Variable	Control group (*n*= 14)	LP group (*n*= 14)	*p*-Values
Age (years)	15.42 ± 8.54	15.07 ± 7.20	*=0.946*
BMI (kg/m^2)	21.13 ± 1.28	21.63 ± 1.70	*=0.246*
Gender (male), n (%)	3 (21.4)	4 (28.6)	*=0.663*

Parameters were communicated as mean ± standard deviation.

Table 2. Oxidative stress parameters between LP patients and control group.

Variable	Control group (*n*= 14)	LP group (*n*= 14)	*p*-Value
TOS (µmol H_2O_2 Eqv. /L)	12.73 ± 1.47	14.73 ± 3.85	*=0.081*
TAS (mmol Trolox Eqv. /L)	1.45 ± 0.25	1.12 ± 0.17	*<0.001*
OSI (Arbitrary Unit)	0.90 ± 0.17	1.34 ± 0.39	*=0.001*
AOPP (µmol/L)	121.8 ± 26.4	165.3 ± 58.7	*=0.018*
FRAP (µmol/L)	1197.9 ± 293.8	735.4 ± 112.1	*<0.001*
Total –SH (mmol/L)	0.23 ± 0.03	0.17 ± 0.02	*<0.001*
LOOH (µmol/L)	0.99 ± 0.13	1.15 ± 0.26	*=0.047*

Parameters were communicated as mean ± standard deviation.

Discussion

Lipoid proteinosis is a rare genodermatosis. Although its clinical findings vary, it usually begins with hoarseness in neonatal period. Skin and mucosal lesions usually occur in the first two years of life (2). Approximately half of the patients have epilepsy or neuropsychiatric findings (20). Loss of function mutations in the ECM1 gene causes infiltration with hyaline-like material in the skin, mucosa and internal organs (21). Histopathological examination shows diffuse dermal accumulation of the hyaline material, thickening of the basement membrane and epidermal hyperkeratosis (22). These findings show the strong effect of ECM1 gene on both epidermal and dermal physiology (20).

Matrix metalloproteinases (MMPs) belong to the family of zinc-dependent endopeptidases that are responsible for both pathophysiological and physiological restructuring of tissues (23). MMP-9 directly destroys extracellular matrix proteins and stimulates cytokines and chemokines to regulate tissue restructuring (24). Depending on the non-functional ECM1 gene, upregulation of MMP9 develops and it contributes to the processes leading to the development of epilepsy and neuropsychiatric diseases, which are non-skin signs of LP (4,20). Studies show that there is a relationship between increased oxidative stress and MMP-9 expression (25). Oxidative stress is mainly an imbalance between the production of free radicals and the ability of the body to counteract and detoxify its harmful effects by antioxidants (6). Oxidative stress has been shown to play a role in the etiopathogenesis of many systemic diseases, including malignancies (6,26,27). However, as far as we know, oxidative stress parameters have not been studied in LP disease yet.

TOS, AOPP and LOOH are important parameters that show the levels of oxidants (11,14,15,17). AOPP is mainly formed by chlorinated oxidants (hypochlorous acid and chloramines) resulting from myeloperoxidase activity (28). Because of their sensitivity, stability, comfort and cost of detection, the role of AOPP in predicting the severity of oxidative stress and prognosis of the disease has become increasingly important (29). TOS is a colorimetric and automated method for measuring serum total oxidation status (14). LOOH is the first byproducts of oxidized lipids and its quantification can be a useful marker. LOOH is generally intermediates of peroxidation reactions that last longer than any free radical precursor, which makes possible a probable intermembrane translocation within cells, between cells, or between lipoproteins and cells (9). In our study, oxidant parameters such as TOS, AOPP and LOOH were higher in LP patients compared to the control group. Our study also showed that these increased serum oxidant parameters are independent predictors of LP disease.

TAS, FRAP and Total-SH are the parameters that indicate the antioxidant levels in serum (13,16,18). TAS is highly sensitive to determine the total antioxidant effects of compounds such as bilirubin, uric acid, vitamin C, polyphenols and proteins. Although these antioxidant compounds can be measured separately, TAS measurement is more advantageous in terms of time, cost and labor (30). FRAP analysis measures the ability of the antioxidant in the sample to inhibit the oxidative effects of the reactive species for the reaction. Unlike other tests that measure total antioxidant power, the FRAP test is fast, simple, effective and inexpensive. FRAP analysis does not require expensive equipment or high degree expertise to control reaction conditions (31). Thiols are organic compounds containing a sulfhydryl group (-SH) (18). Plasma thiols remove free radicals physiologically and act as antioxidants by various mechanisms. It has been determined that exposure of the proteins to oxidative stress causes a decrease in thiol groups and functional defects (26). Determination of plasma total thiol level and thiol / disulfide homeostasis is a good reflection of excessive free radical formation in many diseases such as diabetes mellitus, chronic renal failure, cardiovascular diseases

Table 3. The receiver operating characteristic (ROC) analyses of oxidative stress parameters for predicting LP disease.

Variable	Area Under Curve	Cut-off levels	Sensitivity (%)	Specificity (%)	*p*-Value
TOS (µmol H_2O_2 Eqv. /L)	0.610	14.2	50	93	*=0.323*
TAS (mmol Trolox Eqv. /L)	0.857	1.31	79	93	*=0.001*
OSI (Arbitrary Unit)	0.857	0.93	86	71	*<0.001*
AOPP (µmol/L)	0.740	129	71	64	*=0.031*
FRAP (µmol/L)	0.949	951	86	100	*<0.001*
Total –SH (mmol/L)	0.954	0.204	86	100	*<0.001*
LOOH (µmol/L)	0.663	1.015	64	64	*=0.141*

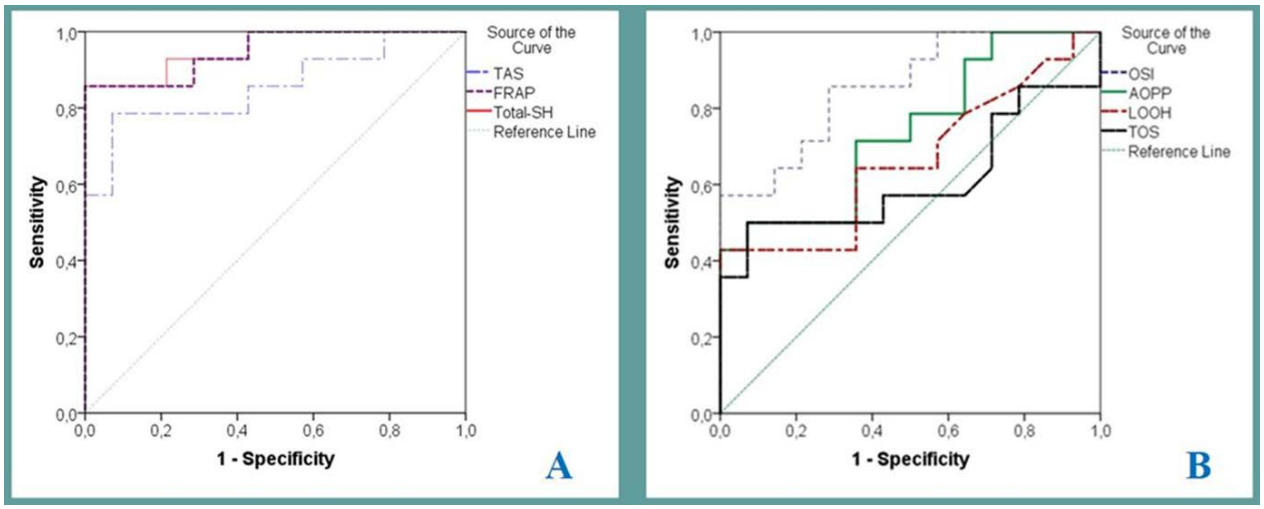

Figure 1. The receiver operating characteristic (ROC) curve of antioxidant (A) and oxidant (B) parameters for predicting LP disease

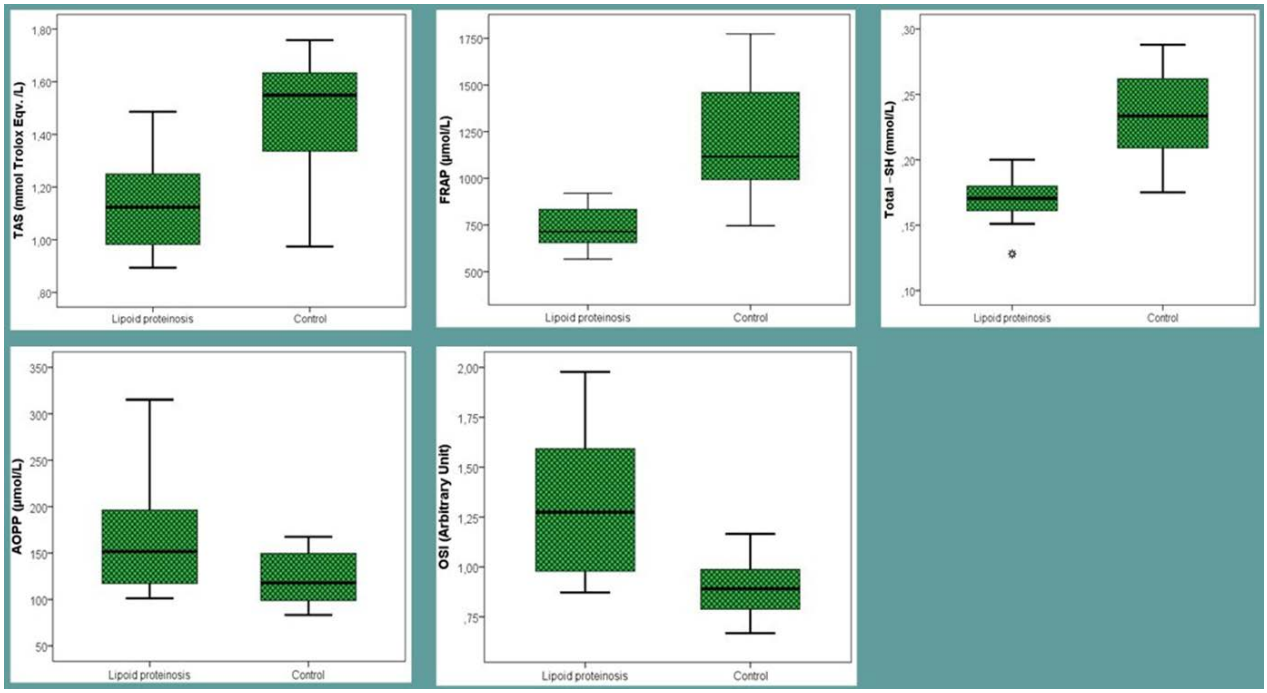

Figure 2. The comparison of serum oxidative stress parameters between LP patients and control group

and cancer (26,32). In our study, levels of antioxidant parameters such as TAS, FRAP and Total-SH were found lower in LP patients compared to the control group. Our study also has indicated that TAS, FRAP and Total-SH are independent predictors of LP. Additionally, ROC analysis demonstrated that OSI, an important indicator of oxidative stress, has a cut-off value of 0.93 or higher and can predict LP with 86% sensitivity and 71% specificity.

In conclusion; this study is the first investigation of oxidative stress in LP and suggests that increased levels of oxidant parameters and decreased levels of antioxidants parameters may play a role in the pathogenesis of LP. We also concluded that the evaluation of changes in the oxidative stress parameters may be useful in the diagnosis of LP. However, comprehensive studies are needed to clarify these results.

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