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Oxidative stress and antioxidant enzyme imbalance in patients with diabetes mellitus: a cross-sectional study

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ABSTRACT

Background: Diabetes mellitus is a chronic metabolic disorder characterized by persistent hyperglycemia, leading to oxidative and nitrosative stress that contributes to microvascular and macrovascular complications. Alterations in antioxidant defense mechanisms and increased production of reactive oxygen species play a key role in diabetes-associated tissue damage. **Objective:** To evaluate oxidative stress markers, antioxidant enzyme activity, and the expression of insulin signaling- and glucose transporter-related genes in patients with diabetes mellitus compared with healthy controls. **Materials and Methods:** In this cross-sectional study, oxidative stress markers, including superoxide dismutase (SOD), NADPH oxidase activity, nitric oxide metabolites (NOx), and lipid peroxidation (LPO; malondialdehyde levels), were assessed in healthy controls (n = 15) and diabetic patients (n = 10). Gene expression analysis of insulin signaling and glucose transporter genes (IRS1, IRS2, GLUT1-4, and PPARG) was performed using quantitative PCR and normalized using the $2^{-}\Delta\Delta Ct$ method. **Results:** Diabetic patients exhibited significantly reduced SOD activity (3.0 ± 0.3 vs 4.7 ± 0.4, $p < 0.01$) and NADPH oxidase activity (4.3 ± 0.5 vs 6.7 ± 0.6, $p < 0.01$) compared to controls. In contrast, NOx levels (48.0 ± 5.5 vs 23.0 ± 4.0, $p < 0.001$) and lipid peroxidation (3.9 ± 0.5 vs 1.5 ± 0.3, $p < 0.001$) were significantly increased. Gene expression analysis revealed significant downregulation of IRS1, IRS2, and GLUT1-4, while PPARG expression was significantly upregulated in diabetic patients. **Conclusion:** The findings demonstrate that diabetes mellitus is associated with oxidative stress imbalance and impaired antioxidant defence, along with altered insulin signaling gene expression. These changes may contribute to disease progression and complications, highlighting oxidative stress as a potential therapeutic target.

Keywords: Diabetes Mellitus, NADPH oxidase enzymes, Nox, ROS, Superoxide Dismutase (SOD) Assay.

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by elevated blood glucose levels due to deficiencies in insulin secretion, insulin action, or both. International standards have made it obvious how to diagnose and categorize it [1,2]. The global prevalence of diabetes has markedly increased in recent decades, leading to a substantial rise in diabetes-related microvascular and macrovascular complications, such as ischemic heart disease, neuropathy, nephropathy, and retinopathy, which collectively contribute significantly to morbidity and mortality [3,4]. Increasing evidence suggests that oxidative stress is a key factor in the development of diabetes and its consequences. Chronic hyperglycemia leads to the overproduction of reactive oxygen species (ROS), which overwhelm the body's natural antioxidant defense systems and cause damage to cells and molecules [5]. Oxidative stress further contributes to insulin resistance, β-cell dysfunction, endothelial injury, and chronic inflammation associated with diabetes mellitus. Moreover, persistent oxidative imbalance accelerates the progression of diabetic microvascular and macrovascular complications, thereby increasing disease severity and associated morbidity [6].

Hyperglycemia is linked to oxidative stress through several interconnected metabolic pathways, including mitochondrial dysfunction, activation of NADPH oxidase enzymes, glucose autoxidation, and

and the formation of advanced glycation end products (AGEs) [7,8]. Excessive production of reactive oxygen species (ROS) disrupts cellular homeostasis and contributes significantly to the pathogenesis of diabetes mellitus and its associated complications. Oxidative stress has been strongly implicated in the development of insulin resistance, pancreatic β-cell dysfunction, endothelial injury, and cardiovascular abnormalities in both type 1 and type 2 diabetes mellitus [9]. Furthermore, obesity-associated oxidative stress and persistent low-grade inflammation aggravate metabolic dysregulation and accelerate the progression of diabetic microvascular and macrovascular complications [10]. Recent studies have also demonstrated that chronic oxidative imbalance impairs insulin signaling pathways, alters glucose transporter activity, and promotes vascular inflammation, thereby increasing disease severity and long-term morbidity [7-10]. These findings collectively underscore oxidative stress as a common mechanism driving diabetes mellitus and its consequences, highlighting its significance as a possible therapeutic target.

MATERIAL AND METHODS

Study Subjects

This cross-sectional observational study included blood samples obtained from diabetic and non-diabetic individuals after informed consent. Subjects were divided into two groups: healthy controls (n = 15) and patients with diabetes mellitus (n = 10). The study was conducted in accordance with institutional ethical guidelines.

Ethics

The study protocol was reviewed and approved by the Institutional Review Board, and written informed consent was obtained from all participants before enrollment (Approval No.: IMS/IEC/November/2025/74).

Sample Collection and Processing

Venous blood samples were collected into heparinized tubes and processed on the same day. Samples were centrifuged at 2500 × g for 5 min. Plasma was separated and stored for lipid peroxidation (LPO) and nitric oxide metabolites (NOx) assays. The cellular pellet was resuspended in ice-cold ultra-pure water and subjected to ethanol–chloroform extraction (62.5:37.5, v/v) to remove hemoglobin. The aqueous layer was collected and stored at -70°C until further analysis.

Inclusion Criteria

Participants included in the present study were individuals diagnosed with Type 2 Diabetes Mellitus (T2DM) in accordance with the diagnostic criteria established by the American Diabetes Association. Eligible subjects were within the age group of 30 to 65 years, representing the adult population commonly affected by T2DM. Both male and female participants were included to ensure a balanced representation of gender and to enhance the generalizability of the findings. Additionally, only those individuals who were willing to participate in the study and provided written informed consent were enrolled, ensuring adherence to ethical standards and research guidelines.

Exclusion Criteria

Participants with conditions affecting oxidative stress were excluded, including chronic kidney disease, liver disorders, and cardiovascular disease. Individuals with acute infections, recent antioxidant supplementation, smoking, alcohol use, pregnancy, or medications altering oxidative balance were also excluded to ensure accuracy and reliability of biochemical parameters in the study.

Superoxide Dismutase (SOD) Activity

SOD activity was measured spectrophotometrically at 560 nm. The reaction mixture containing phosphate buffer, phenazine methosulfate (PMS), and nitro blue tetrazolium (NBT) was incubated with the sample. The reaction was initiated by adding NADH and terminated using acetic acid and butanol. Absorbance was recorded at 560 nm.

NADPH Oxidase Activity

NADPH oxidase activity was measured in blood-derived samples using a modified spectrophotometric method based on cytochrome c reduction. The reaction mixture consisted of phosphate buffer saline (pH 7.2), $MgCl_2$, cytochrome c, and sodium azide. The reaction was initiated by NADPH, and absorbance was measured at 550 nm. Enzyme activity was calculated using the extinction coefficient.

Nitric Oxide Metabolites (NOx) Assay

Nitrite levels were estimated using the Griess reaction. The sample was mixed with sulfanilamide and N-naphthyl ethylenediamine in phosphoric acid and incubated at 37 °C for 30 min. Absorbance was measured at 540 nm. Nitrite concentration was calculated using a sodium nitrite standard curve.

Lipid Peroxidation (LPO) Assay

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) levels using the thiobarbituric acid (TBA) method. Samples were treated with SDS, acetic acid, and TBA, then heated in a boiling water bath. After cooling and centrifugation, absorbance was measured at 532 nm. Results were expressed as nmol MDA/mg protein [12].

Gene Expression Analysis by qPCR

Total RNA was extracted using TRI reagent. cDNA was synthesized and amplified using SYBR Green chemistry. Gene expression of IRS1, IRS2, GLUT1- 4, and PPARG was analyzed. GAPDH was used as an internal control. Relative expression levels were calculated using the $2^{-}\Delta\Delta Ct$ method.

qPCR

Gene-specific primers were designed based on NCBI reference sequences [Table 1]. Primer specificity was confirmed using melt curve analysis, demonstrating a single peak for each amplicon. Amplification efficiencies ranged between 90-110%. All qPCR reactions were performed in triplicate (technical replicates) with at least three independent biological samples per group. Primer specificity was confirmed by melt curve analysis, showing a single peak for each gene. GAPDH was used as the internal control based on its stable expression across samples; however, we acknowledge that the inclusion of multiple housekeeping genes could further improve normalization accuracy.

Primer sequences for all target genes (IRS1, IRS2, GLUT1-4, and PPARG) are provided in Table 1, along with corresponding accession numbers. Amplification efficiencies for all primer pairs were determined using standard curve analysis and were within the acceptable range of 90-110%, with correlation coefficients (R^2) > 0.99. Melt curve analysis was performed at the end of each amplification run, demonstrating a single, specific peak for each target gene, confirming the absence of nonspecific amplification and primer-dimer formation. All reactions were carried out in technical triplicate, and experiments included at least three independent biological replicates per group. Gene expression levels were normalized using GAPDH as the reference gene and calculated using the $2^{-}\Delta\Delta Ct$ method.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Comparisons between diabetic patients and controls were performed using the unpaired Student's t-test. A p-value < 0.05 was considered statistically significant.

RESULTS

A total of 25 participants were included in the study, comprising 10 patients with type 2 diabetes mellitus and 15 healthy controls. The demographic and clinical characteristics of the study population are presented in [Table 2]. The mean age of the participants was comparable between the two groups (56.3 ± 5.7 years in diabetic patients vs. 56.5 ± 7.4 years in controls; p = 0.94). Similarly, no significant difference was observed in gender distribution between the groups (p = 0.51). However, body mass index (BMI) was significantly higher in diabetic patients compared to controls (35.3 ± 6.2 kg/m² vs. 23.5 ± 2.8 kg/m²; p < 0.001). As expected, fasting blood glucose levels and HbA1c values were markedly elevated in the diabetic group (p < 0.001 for both). In addition, the prevalence of smoking was significantly higher among diabetic patients (70% vs. 20%; p = 0.02). Although alcohol consumption did not differ significantly between the groups (p = 0.42), hypertension was substantially more prevalent in diabetic individuals (90% vs. 13.3%; p < 0.001).

Oxidative stress markers were significantly altered in diabetic patients compared to healthy controls. Superoxide dismutase (SOD) activity was significantly decreased in diabetic individuals (3.0 ± 0.3 vs 4.7 ± 0.4, p < 0.01). Similarly, NADPH oxidase activity was reduced in diabetic patients (4.3 ± 0.5 vs 6.7 ± 0.6, p < 0.01).

Table 1: Primer sequences used for qPCR analysis

Gene	Accession Number	Forward Primer (5′→3′)	Reverse Primer (5′→3′)
IRS1 (Insulin receptor substrate 1)	AAB27175.1	CAAGACCATCAGCTTCGTGA	GATGGTCTCGTGCATGTTCT
IRS2 (Insulin receptor substrate 2)	NM_003749.3	CAACAACAACAACCACAGCG	CTTGTTGATGTTCAGGCAGC
PPARG	NG_011749.1	TTTACACAATGCTGGCCTCC	GCCAAGTCGCTGTCATCTAA
GLUT1 (SLC2A1)	NP_006507.2	CTCTGTGGGCCTTTTCGTTA	TACACACCGATGATGAAGCG
GLUT2 (SLC2A2)	BC060041.1	TGTCCAGAAAGCCCCAGATA	CTTTCTGCTCACTCGATGCT
GLUT3 (SLC2A3)	BC039196.1	TGGTTATTGGCCTCTTCTGC	AGCTCTTCAGACCCAAGGAT
GLUT4 (SLC2A4)	M91463.1	CTGGACGAGCAACTTCATCA	CTCAAGTTCTGTGCTGGGTT

Table 2: Demographic and clinical characteristics of study participants

Variable	Total (n = 25)	Diabetes Patients (n = 10)	Controls (n = 15)	p-value
Age (years) (Mean ± SD)	56.4 ± 6.8	56.3 ± 5.7	56.5 ± 7.4	0.94
Gender				0.51
Male, n (%)	13 (52.0%)	6 (60.0%)	7 (46.7%)	
Female, n (%)	12 (48.0%)	4 (40.0%)	8 (53.3%)	
Body Mass Index (BMI) (kg/m²)	28.2 ± 7.6	35.3 ± 6.2	23.5 ± 2.8	<0.001
Duration of Diabetes (years)	—	5.6 ± 2.9	—	—
Fasting Blood Glucose (mg/dL)	122.0 ± 48.5	171.6 ± 39.0	88.9 ± 14.7	<0.001
HbA1c (%)	6.74 ± 2.60	9.73 ± 2.30	4.75 ± 0.50	<0.001
Smoking Status				0.02
Smokers, n (%)	10 (40.0%)	7 (70.0%)	3 (20.0%)	
Non-smokers, n (%)	15 (60.0%)	3 (30.0%)	12 (80.0%)	
Alcohol Consumption				0.42
Yes, n (%)	10 (40.0%)	5 (50.0%)	5 (33.3%)	
No, n (%)	15 (60.0%)	5 (50.0%)	10 (66.7%)	
Hypertension				<0.001
Yes, n (%)	11 (44.0%)	9 (90.0%)	2 (13.3%)	
No, n (%)	14 (56.0%)	1 (10.0%)	13 (86.7%)	

Values are expressed as mean ± standard deviation (SD) for continuous variables and number (percentage) for categorical variables. Statistical significance was assessed using *Student's t-test* for continuous variables and *Chi-square test* for categorical variables. A p-value < 0.05 was considered statistically significant.

Table 3: Oxidative stress markers and antioxidant enzyme activities in study groups

Parameter	Control (n = 15) Mean ± SD	Diabetes Mellitus (n = 10) Mean ± SD	p-value
SOD activity (U/mg protein)	4.7 ± 0.4	3.0 ± 0.3	< 0.01
NADPH oxidase (U/mL)	6.7 ± 0.6	4.3 ± 0.5	< 0.01
NOx levels (µmol/mL)	23.0 ± 4.0	48.0 ± 5.5	< 0.001
Lipid peroxidation (MDA, nmol/mg protein)	1.5 ± 0.3	3.9 ± 0.5	< 0.001

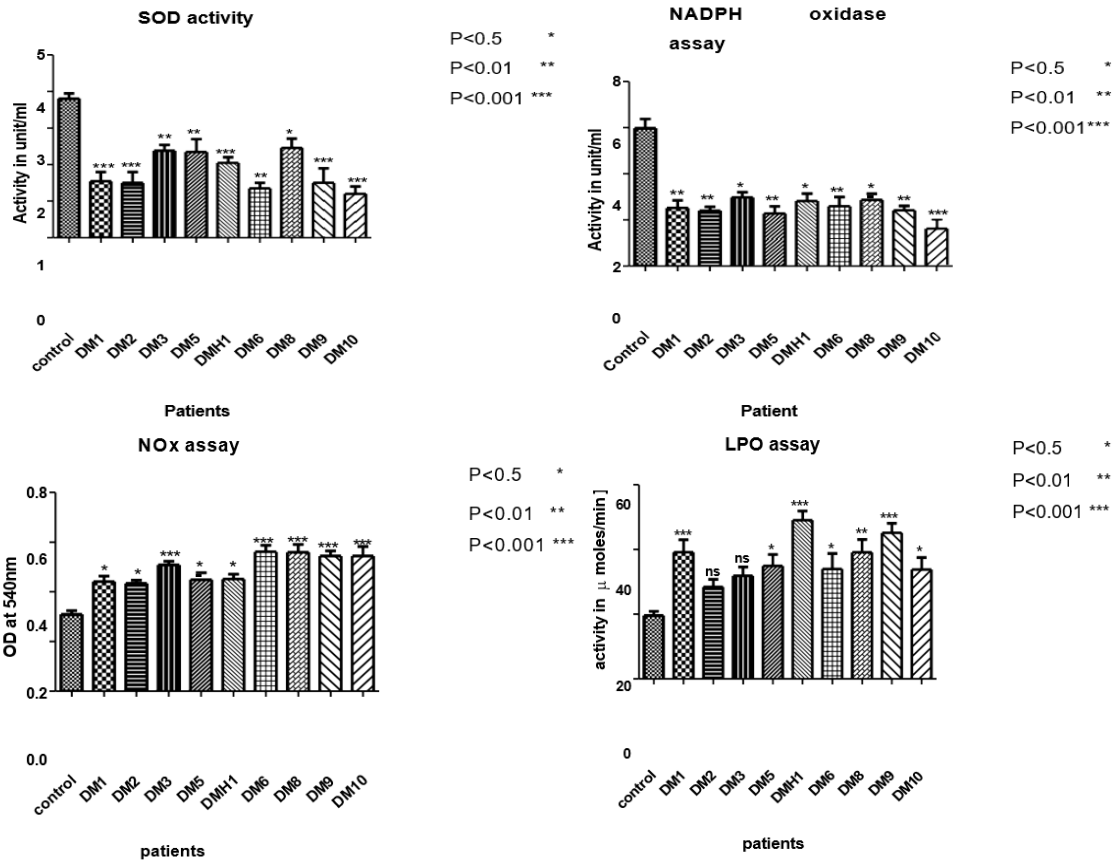

Figure 1: Comparison of oxidative stress markers (SOD, LPO, NOx, and NADPH oxidase activity) between healthy controls (n = 15) and patients with diabetes mellitus (n = 10). Data are expressed as mean ± SD; $p < 0.05$ is considered significant.

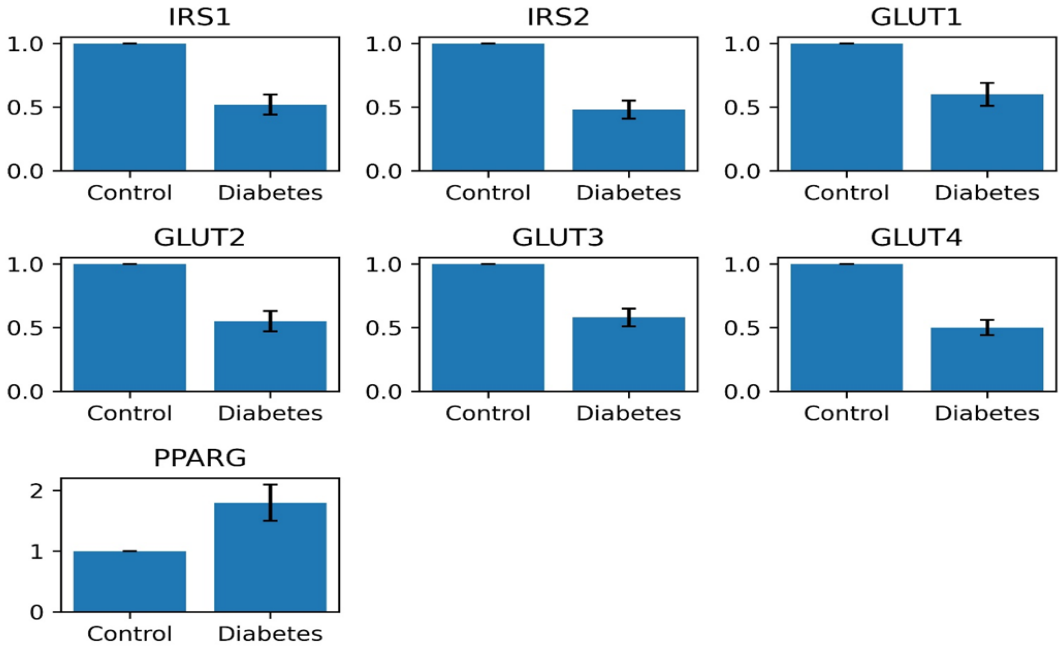

Figure 2: Relative mRNA expression of IRS1, IRS2, GLUT1–4, and PPARG in diabetic patients compared to controls. Expression normalized using the $2^{-}\Delta\Delta Ct$ method. Data are mean ± SD

Oxidative stress markers were significantly altered in diabetic patients compared to healthy controls. Superoxide dismutase (SOD) activity was significantly decreased in diabetic individuals (3.0 ± 0.3 vs 4.7 ± 0.4, $p < 0.01$). Similarly, NADPH oxidase activity was reduced in diabetic patients (4.3 ± 0.5 vs 6.7 ± 0.6, $p < 0.01$).

In contrast, nitric oxide metabolites (NOx) were significantly elevated in the diabetic group (48.0 ± 5.5 vs 23.0 ± 4.0, $p < 0.001$). Lipid peroxidation levels, measured as malondialdehyde (MDA), were also markedly increased (3.9 ± 0.5 vs 1.5 ± 0.3, $p < 0.001$).

Enzymatic activity in diabetic patients showed significant variation among the examined groups. SOD and NADPH oxidase activity decreased significantly in diabetic mellitus patients in comparison to healthy patients, while the NOx and LPO activity increased significantly in diabetic patients in comparison to normal healthy patients [Table 3 and Figure 1].

qPCR results: we quantified the mRNA expression levels of the genes IRS1, IRS2, GLUT1, GLUT2, GLUT3, GLUT4, and PPARG in the three groups, i.e., healthy, patients. We observed a decline in the expression of IRS1, IRS2, GLUT1, GLUT2, GLUT3, and GLUT4 in patients; we found a significant increase in mRNA expression compared to the healthy group. mRNA expression level of PPARG was found to be increased in diabetic patients, and after treatment, the mRNA expression of PPARG was reduced as compared to the healthy group [Figure 2].

DISCUSSION

The present study demonstrates a significant imbalance between oxidative stress and antioxidant The present study demonstrates a significant imbalance between oxidative stress and antioxidant defense mechanisms in patients with diabetes mellitus, emphasizing the central role of redox dysregulation in the pathogenesis and progression of the disease. Diabetic individuals exhibited significantly reduced activity of important antioxidant enzymes, particularly superoxide dismutase (SOD), along with elevated levels of nitric oxide metabolites (NOx) and lipid peroxidation (LPO), indicating enhanced oxidative and nitrosative stress. These observations are in agreement with previous studies reporting that chronic hyperglycemia induces excessive production of reactive oxygen species (ROS), leading to cellular injury, mitochondrial dysfunction, inflammation, and metabolic abnormalities associated with diabetic complications [13-16]. Persistent oxidative stress contributes to tissue damage and promotes the progression of both microvascular and macrovascular complications commonly observed in diabetes mellitus.

Superoxide dismutase is a major enzymatic antioxidant defense system responsible for the detoxification of superoxide radicals and the maintenance of cellular redox homeostasis. The reduced SOD activity observed in diabetic patients in the present study suggests impaired antioxidant defense capacity and increased vulnerability to oxidative damage. Earlier studies demonstrated that prolonged hyperglycemia promotes glycation and oxidative modification of antioxidant enzymes, thereby reducing their biological activity and accelerating ROS accumulation [17-19]. Excessive ROS generation further damages cellular proteins, lipids, nucleic acids, and mitochondrial membranes, ultimately aggravating insulin resistance and β-cell dysfunction [20,21]. Mitochondrial dysfunction caused by chronic hyperglycemia has been recognized as one of the major sources of oxidative stress in diabetes mellitus, contributing significantly to altered cellular metabolism and energy imbalance [14,16].

NADPH oxidase is widely recognized as an important enzymatic source of ROS in diabetes and related vascular disorders. Although previous investigations have shown increased tissue-specific NADPH oxidase activity in diabetic complications, the present study observed reduced enzyme activity in peripheral blood samples. This discrepancy may reflect adaptive downregulation, disease-stage-dependent alterations, or exhaustion of antioxidant defense systems following prolonged oxidative stress exposure. Furthermore, circulating enzyme activity may not directly correlate with tissue-specific expression patterns in vascular or renal tissues [18,20]. The dynamic regulation of ROS-generating pathways in diabetes highlights the complexity of oxidative stress mechanisms and suggests that enzyme activity may vary according to disease duration, metabolic status, and sample type analyzed.

The significant increase in nitric oxide metabolites observed in diabetic patients indicates enhanced nitrosative stress and endothelial dysfunction. Increased nitric oxide production, particularly through inducible nitric oxide synthase (iNOS), can react with superoxide radicals to generate peroxynitrite, a highly reactive oxidant capable of damaging proteins, lipids, DNA, and cellular membranes [21,22]. Endothelial dysfunction resulting from oxidative and nitrosative stress plays a major role in the development of diabetic vascular complications, including atherosclerosis, hypertension, nephropathy, and cardiovascular disease [23,24]. Elevated lipid peroxidation levels observed in the present study further support the presence of oxidative membrane damage in diabetic individuals. Lipid peroxidation products such as malondialdehyde (MDA) are important indicators of oxidative injury and have been strongly associated with the severity and progression of diabetes-related complications [22,27].

In addition to oxidative stress biomarkers, disturbances in insulin signaling and glucose metabolism also contribute significantly to diabetes progression. Oxidative stress interferes with insulin receptor signaling pathways and glucose transporter activity, leading to impaired glucose uptake and insulin resistance [24,25]. ROS-mediated cellular injury has also been implicated in pancreatic β-cell dysfunction, resulting in reduced insulin secretion and worsening hyperglycemia. Furthermore, activation of inflammatory pathways under oxidative stress conditions contributes to chronic low-grade inflammation and metabolic dysregulation in diabetic patients [28,29]. Recent studies have highlighted the role of transcriptional regulators such as PPARγ in modulating glucose metabolism, lipid homeostasis, and inflammatory responses in diabetes mellitus [26]. Altered expression of these regulatory molecules may represent compensatory responses aimed at improving insulin sensitivity and reducing metabolic stress.

The findings of the present study are supported by accumulating evidence demonstrating the involvement of oxidative stress, endothelial dysfunction, mitochondrial abnormalities, and antioxidant enzyme dysregulation in diabetes mellitus [27-30]. Increased ROS generation not only damages cellular structures but also activates stress-sensitive signaling pathways that promote inflammation, apoptosis, fibrosis, and vascular injury. Such molecular alterations contribute significantly to the development of diabetic nephropathy, neuropathy, retinopathy, and cardiovascular disease. Recent investigations have also emphasized the importance of antioxidant defense systems in preventing oxidative injury and maintaining metabolic homeostasis in diabetic conditions [30].

Despite these findings, the present study has several limitations that should be acknowledged. The relatively small sample size may limit statistical power and reduce the generalizability of the observations. Larger multicenter studies with adequate power calculations are required to validate these findings and establish stronger clinical associations [31]. Additionally, although GAPDH was used as the housekeeping gene for qPCR normalization, previous reports recommend the use of multiple reference genes to improve the reliability, reproducibility, and accuracy of gene expression analysis [32,33]. Recent methodological guidelines also emphasize the importance of rigorous normalization strategies, technical validation, and experimental standardization in oxidative stress-related molecular studies [34-36].

Overall, the present study highlights the critical role of oxidative stress and impaired antioxidant defense mechanisms in the

pathophysiology of diabetes mellitus. Persistent ROS generation, endothelial dysfunction, mitochondrial abnormalities, and altered insulin signaling collectively contribute to metabolic dysregulation and diabetic complications [37-42]. Therapeutic strategies aimed at restoring redox balance, enhancing antioxidant capacity, and reducing oxidative stress–mediated cellular injury may therefore provide promising approaches for the prevention and management of diabetes mellitus and its associated complications. Further research is warranted to understand the molecular pathways underlying oxidative stress better and to develop targeted antioxidant therapies for improving diabetic outcomes.

CONCLUSION

Diabetes mellitus is characterized by persistent hyperglycemia leading to oxidative and nitrosative stress through multiple metabolic pathways. The present study demonstrates a significant imbalance between pro-oxidant and antioxidant defense systems, evidenced by altered SOD, NADPH oxidase, NOx, and lipid peroxidation levels. These changes contribute to redox imbalance, endothelial dysfunction, insulin resistance, and β-cell impairment, thereby promoting the development of diabetic complications. Early identification of oxidative stress and strategies targeting redox balance may help reduce disease progression. Further studies with larger sample sizes are required to validate these findings.

Conflict of interest

The authors declared no conflict of interest.

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