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Reactive Oxygen Species-Mediated Cardiovascular Disease In Diabetes: A Sex-Stratified Case-Control Study In Punjab, Pakistan

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Abstract

This study investigates sex-based differences in reactive oxygen species (ROS)-mediated cardiovascular disease (CVD) development among diabetic individuals residing in central Punjab, Pakistan. A case-control study was carried out on 200 participants, comprising 100 males and 100 females, each further divided into diabetic and non-diabetic (control) groups. All participants underwent thorough clinical evaluation and detailed medical history recording. Fasting serum samples were analyzed for lipid profile parameters including total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL). Additionally, oxidative stress markers — superoxide dismutase (SOD), reduced glutathione (GSH), malondialdehyde (MDA), catalase (CAT), and nitric oxide (NO) — were quantified. Diabetic females exhibited significantly elevated TC, TG, and LDL levels ($p=0.001$) alongside reduced HDL ($p=0.045$) compared to non-diabetic female controls. Likewise, diabetic males demonstrated increased TC and TG ($p=0.001$) with decreased HDL ($p=0.05$) relative to their non-diabetic counterparts. Inter-group comparison revealed that diabetic females had significantly higher cholesterol, LDL ($p=0.045$), and TG ($p=0.001$) levels than diabetic males. Regarding oxidative stress, diabetic females showed markedly reduced SOD, GSH, CAT, and NO levels ($p<0.001$), along with elevated MDA ($p=0.001$), compared to non-diabetic females. Furthermore, when compared to diabetic males, diabetic females displayed significantly lower SOD ($p=0.001$) and GSH ($p=0.05$) with correspondingly higher MDA levels ($p=0.05$). These findings suggest that women with diabetes are at a greater risk of developing CVD than their male counterparts. This sex-based disparity in oxidative stress and lipid dysregulation may offer valuable insights for the development of more targeted and sex-specific therapeutic strategies.

Introduction

Diabetes mellitus ranks among the most widespread chronic diseases globally. Its likelihood increases progressively with age; while the global prevalence was initially estimated at around 5%, this figure rises to approximately 20% in individuals aged 80 years and older [1,2]. This age-related increase is largely attributable to elevated fasting and postprandial glucose levels, which reflect underlying disruptions in peripheral or hepatic insulin sensitivity as well as impaired function of pancreatic beta cells [3]. Among the various forms of diabetes, type 2 diabetes mellitus (T2DM) is by far the most common, accounting for 85–90% of all diagnosed cases and occurring predominantly in the adult population [2,3].

When examined through a gender lens, T2DM tends to be less prevalent in women than in men during earlier life stages [3,4]. However, this pattern shifts significantly at menopause, after which the risk of developing T2DM in women rises dramatically, from approximately 6% to as high as 20% [4,5]. The pathophysiology of T2DM involves a dual mechanism: peripheral insulin resistance combined with a declining capacity of pancreatic beta cells to secrete adequate insulin. Experimental evidence from in-vivo studies has highlighted the important regulatory role of estradiol in glucose and insulin homeostasis, including its ability to enhance beta cell function, improve insulin sensitivity, reduce obesity, and modulate lipid metabolism [2,5,6].

Dietary patterns rich in fats and refined carbohydrates are known to promote oxidative stress, manifesting as increased lipid peroxidation, protein carbonylation, depletion of antioxidant defenses, and reduced glutathione levels [7,8]. Collectively, these biochemical disturbances contribute to the onset and progression of obesity, cancer, diabetes, and cardiovascular diseases (CVDs). Among CVD-related pathologies, atherosclerosis is particularly significant, as it underlies the majority of cardiac conditions. Both oxidative stress and chronic inflammation are well-established drivers of atherogenesis [6,7,9].

Currently, CVDs, diabetes mellitus, cancer, and respiratory diseases are recognized as the four leading causes of global morbidity and mortality, with CVDs topping the list as the primary cause of death worldwide [8,9]. Oxidative stress is increasingly understood to be a central mediator in CVD development [10,11]. Notably, gender-based disparities in CVD prevalence suggest that oxidative stress may not affect men and women equally, underscoring the need to investigate sex-specific differences in oxidative stress mechanisms and their contribution to cardiovascular risk [10,12].

Findings from experimental studies present a complex picture. While some animal-based research has indicated higher oxidative stress in male rats compared to females, human studies have reported elevated oxidative stress biomarkers in young males relative to age-matched females [10,11,13]. Additionally, reactive oxygen species (ROS) production has been found to be greater in men than in women. A growing body of evidence points to superior antioxidant capacity in women, suggesting that females may be inherently more resilient to oxidative damage under normal physiological conditions [2,10,11].

The protective advantage seen in premenopausal women against atherosclerosis appears to diminish significantly following menopause, at which point CVD risk escalates considerably [12,14]. In contrast, men tend to experience a more rapid rise in cardiovascular risk at an earlier age. Understanding the gender-based mechanisms underlying atherosclerosis progression is therefore essential to curb the rising burden of CVD-related mortality [12,15]. Epidemiological data indicate that T2DM disproportionately amplifies cardiovascular risk in women, who face approximately 27% greater stroke risk and a 44% higher relative risk of CVD compared to diabetic men [6,13,16]. This heightened vulnerability in women has been partly linked to greater weight gain and higher body mass index (BMI) associated with diabetes

[6,13]. These observations reinforce the importance of considering sex-specific factors in the clinical management and therapeutic planning for diabetic patients [15,16].

The primary objective of the present study is to investigate how gender modulates oxidative stress responses and their role in the development of cardiovascular risk factors in the context of diabetes mellitus. Given the limited research available in this specific domain, this study seeks to bridge the gap by exploring the relationship between oxidative stress, diabetes, and sex-based differences in cardiovascular disease progression, with the ultimate goal of contributing to the development of more targeted, gender-sensitive therapeutic approaches [10,12,16,17].

Nevertheless, the precise role of gender in modulating oxidative stress-related cardiovascular complications in type 2 diabetes mellitus (T2DM) remains insufficiently understood. To the best of the authors' knowledge, the present study represents the first investigation conducted specifically on Pakistani males and females from central Punjab aimed at characterizing gender-based oxidative stress changes in diabetes mellitus and their relationship to cardiovascular disease progression.

MATERIAL AND METHODS

Place of work

The present study is a case-control study, carried out from the year 2017-2018 in the department of Biochemistry, Minhaj University, Lahore, and approved by the ethical committee of Minhaj University, Lahore.

Subjects

The subjects in the present study included males (n= 100) and females (n=100), were taken written informed consent. Males and females both were categorized into control, n=30, and diabetics n=70, aged between 45 to 60 years. Each individual was subjected to written informed consent. Diabetic men and women who have not any other health complications and free from any type of medications and met the current WHO diagnostic criteria for diabetes i.e fasting plasma glucose level ≥ 7mmol/l (126mg/dl) or 2 hours' plasma glucose ≥ 11mmol/l (200mg/dl) with individuals with HbA1c greater than 6.2% were considered and included in the present study.

Methods

Venous blood sampling was performed obtaining 10ml of blood by venipuncture in a vial containing no additives after overnight 12 hours fast. The serum was extracted from blood samples by centrifuging blood at 3000rpm for 15 minutes. The serum was preserved at --20°C for further analysis of lipid profile (cardiovascular markers) and antioxidants measurement.

Estimation of lipid profile parameters

Cardiovascular markers i-e serum TC, HDL-C, TGs, and LDL-C were determined using commercial assay kit (Randox® kit,Randox Laboratories,Unitted Kingdom). LDL-C =Total cholesterol - Triglycerides - HDL-C

Estimation of superoxide Dismutase Activity

SOD assay refers to the method established by *Kakkar et al*., 1984. 0.5ml serum with 0.5ml trichloroacetic acid [TCA,10%) was centrifuged at 13000 pm for 10minutes. After collecting the supernatant [15µl) into a separate tube, 120µl sodium pyrophosphate buffer [pH 8.3, 0.052M), 12 µl phenazine methosulphate [186µM), and 36µl nitroblue tetrazolium [300µM) was added. To start the reaction, 24ul of NADH solution [780µM) was added and the reaction mixture was allowed to incubate at 37°C for 1.5 minutes. After incubation for 90sec, the reaction was terminated by adding 12ul of glacial acetic acid. To the mixture 400ul of n-butanol

was added and the mixture was stirred vigorously. And, following incubation for ten minutes, the mixture was centrifuged at 2000rpm for 5 minutes at 25°C. The upper butanol layer was taken out and the color intensity of the chromogen extract in the *n*-butanol was measured at 560nm by spectrophotometer (18).

Estimation of Reduced Glutathione

Glutathione estimation employed the method of (Beutler et al.,1963). 0.5 ml TCA (10%) was added to 0.5ml serum and centrifuged for ten minutes. To the 40µl of supernatant, 150µl of disodium phosphate buffer (0.03M, pH=7.4) was added. Then, 25µl of 0.001M DTNB (5,5'-dithiobis (2-nitrobenzoic acid)/ Ellman's Reagent, freshly prepared) solution was added to the reaction mixture. The reduction of DTNB with GSH produced a yellow complex that was measured by a spectrophotometer at 412nm (19) .

Estimation of Catalase

Catalase was estimated according to the method of Sinha *et al.,* 1972. 360µl phosphate buffer (1mM) was added in 40 µl serum and allowed to centrifuge at 13000 rpm for 10 minutes at room temperature and allowed to stand for 5 minutes. 25µl of supernatant mixed with 180µl of phosphate buffer. To the reaction mixture, 75 µl of 0.2M hydrogen peroxide (freshly prepared) was added to initiate the reaction. Then 360µl solution of potassium dichromate and acetic acid was added to the reaction composite and was incubated for ten minutes in boiling water. Then allowed to stand for cooling and optical density was taken at 530nm (20).

Estimation of Malondialdehyde

Estimation of malondialdehyde refers to the method of Ohkawa *et al.,* 1979. 40µl serum was mixed with 360 µl phosphate buffer. The mixture was centrifuged at 13000 rpm.for 10minutes at room temperature. 15µl of supernatant was mixed with 15µl SDS, 96ul TBA, 96ul acetic acid, and 18ul refined water. It was kept at 90°C for 60 minutes. After that 60ul distilled water and 300ul n-butanol pyridine mixture was also included. It was stirred violently and centrifuged at 4000 rpm at 25°C. The upper butanol –pyridine layer was separated and absorbance was taken at 532nm (21).

Determination of Endothelial Nitric oxide synthase

This estimation is based on the conversion of L-arginine to L-citrulline and nitric oxide by nitric oxide synthase and then Nitric oxide was measured according to the method of Cortas, et al.,1990. For this, 100µl of blood was added into an incubation mixture containing 50 µl [50mM Trishydro chloric acid, pH 7.4), 80µl 5mM NADPH, 30µl 100mM arginine, and 20mM 50µl calcium `chloride. The reaction mixture was kept in a shaking water bath for 20 minutes. Afterward, nitric oxide was then measured by adding 500µl of NaOH [55mmol/L) and 400µl of 75mmol/L ZnSO4 to the reaction mixture. Following that, the reaction mixture was centrifuged at 3000rpm for 15 minutes. To the supernatant, ethylenediamine was added and the color intensity was measured at 540nm. (22)

STATISTICAL ANALYSIS

Statistical analysis was performed by SPSS using two way-ANOVA.$P<0.05$ was considered statistically significant

RESULTS

Serum lipid profile in diabetic and non-diabetic males and females

Table 1 shows lipid profile of diabetic males and females individuals. Statistical analysis of data by two way ANOVA showed insignificant effects of gender on total cholesterol and LDL-C $F= 3.084$ ($p = 0.084$) and $F= 1.502$ ($p = 0.223$) respectively.

However, HDL-C and TGs showed significant gender effects F= 11.6 (p= 0.001) and F= 8.156 (p= 0.005). However, diabetes/disease condition showed significant effects (p=0.000) on total cholesterol (F= 42.843), HDL-C (F= 50.65), LDL-C (F= 20.41) and on TGs (37.041). On the other hand, gender x disease interaction (GxD) showed significant effect on HDL-C (F= 13.01, p= 0.000). However insignificant effects were observed on total cholesterol (F= 0.142, p= 0.624), LDL (F= 1.24, p= 0.268) and TGs (F= 0.392, p= 0.533).

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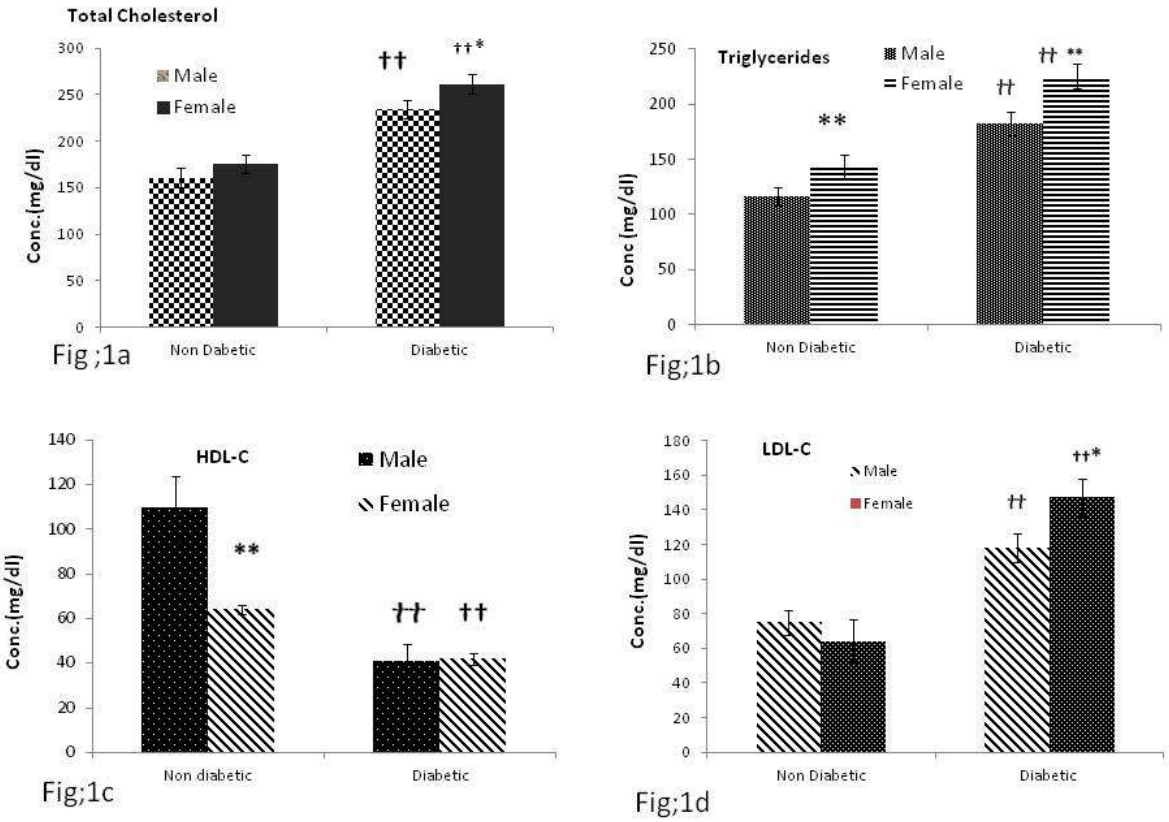

Figure 1: Evaluation of lipid profile in Diabetic and non Diabetic male and female subjects. (A) Total Cholesterol (TC), (B) Triglycerides (TGs), (C) High-density lipoprotein cholesterol (HDL-C), (D) low-density lipoprotein- cholesterol (LDL-C). The results are expressed as the mean ± SEM. The results were analyzed by two-way ANOVA followed by LSD (least significant difference) test. The P-value < 0.05 was considered to be statistically significant. The significance of the difference is indicated by *P <0.05, **p<0.01, ***p<0.001 when non-diabetic and diabetic males were compared with respective diabetic and nondiabetic females. And †p<0.05,

††p<0.01, †††p<0.001 when the diabetic group was compared with respective non- diabetic controls.

Serum oxidative stress markers in diabetic and non-diabetic males and females

Data analyzed by two way ANOVA showed significant effects of gender on SOD (F= 63.86, p=0.000), GSH (F= 234.7, p= 0.000), MDA (F= 106.487, p=0.000) NOS (F= 4.280, p= 0.041, however, insignificant effect on catalase (F= 0.188, p= 0.666) was observed. Effects of disease were shown to be significant on SOD (F=105.909, p=0.000), GSH (F= 552.46, p=0.000), MDA (F= 21.19, p=0.000), catalase (F= 902.7,p=0.000) and NOS (166.72, p=0.000). Effects of interaction between GXD (gender disease) showed significant effects on SOD (F= 5.02 , p= 0.027) and MDA (F= 15.75 ,p= 0.000) and non significant effects were observed in GSH, catalase and NOS (F= 0.733 , p= 0.394), (F= 0.188 ,p= 0.665) and F= 0.127, p= 0.722) respectively

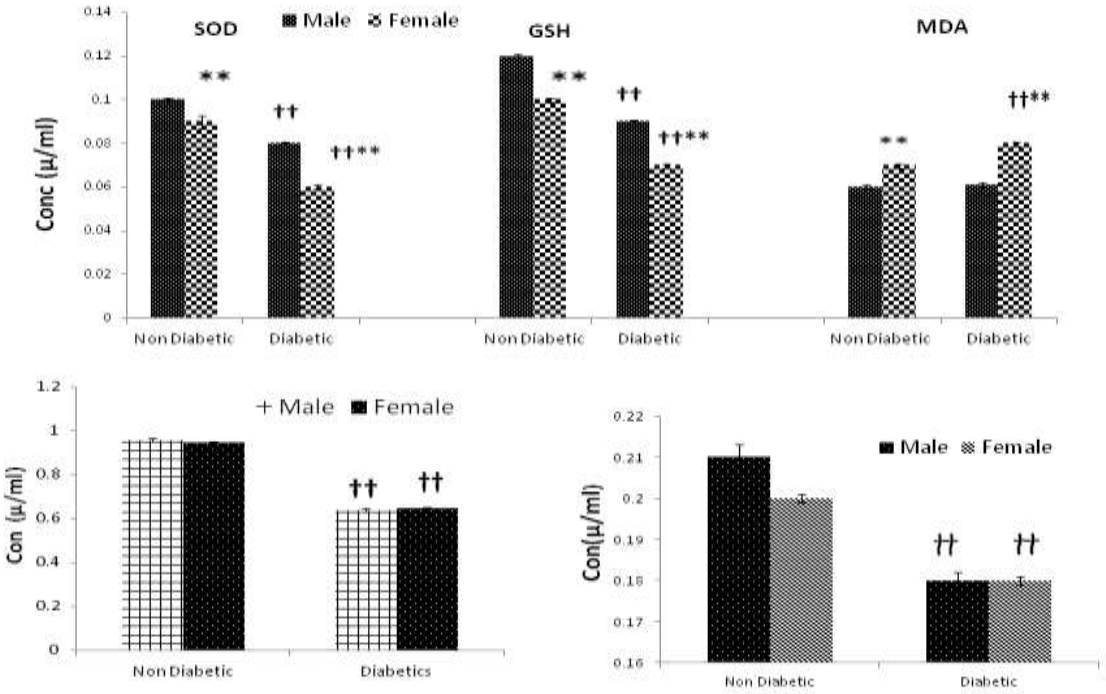

Figure 2: Evaluation of oxidative stress in diabetic and non-diabetic male and female subjects. (A) SOD, (B) GSH, (C) MDA, (D) CAT, (E) NOS. The results are expressed as the mean ± SEM. The results were analyzed by two-way ANOVA followed by LSD (least significant difference) test. The P-value < 0.05 was considered to be statistically significant. The significance of the difference is indicated by *P <0.05, **p<0.01, ***p<0.001 when non-diabetic and diabetic males were compared with respective diabetic and non-diabetic females. And †p<0.05, ††p<0.01, †††p<0.001 when the diabetic group was compared with respective non- diabetic controls

DISCUSSION

A substantial body of evidence has confirmed that oxidative stress is closely linked to hyperlipidemia and hyperglycemia, both of which are hallmark features of diabetes mellitus that contribute to its wide-ranging complications (23, 24). The findings of the current study revealed a significant elevation in oxidative stress markers among both diabetic males and females in comparison to their non-diabetic counterparts. This was

particularly evident in diabetic females, who demonstrated a pronounced reduction in superoxide dismutase (SOD) and reduced glutathione (GSH) levels. These observations are consistent with earlier reports indicating that decreased SOD activity reflects compensatory responses to ROS-mediated oxidative burden in individuals under chronic oxidative stress (25,26). Structural alterations in SOD, arising from intermolecular and intramolecular protein cross-linking, may lead to the accumulation of hydrogen peroxide (H_2O_2), thereby promoting lipid peroxidation, atherosclerosis, and ultimately cardiovascular complications (27). Recent research has further demonstrated that SOD is highly susceptible to glycation in hyperglycemic conditions, and glycation-induced structural impairments in SOD activity are now considered a direct contributor to oxidative stress progression in T2DM (28).

Furthermore, diabetic females in the present study exhibited significantly lower SOD activity and GSH concentrations compared to their male diabetic counterparts. These findings are consistent with research conducted on Arab populations in Kuwait, where higher ROS levels were documented in females than in males. A recent cross-sectional study by Naa Yeng et al. (2026) similarly identified sex-based disparities in oxidative stress markers among Ghanaian T2DM patients, with notable differences in MDA and glutathione peroxidase (GSH-Px) levels across sexes and complication types (29). Other investigations have found no gender-dependent relationship in SOD2 mRNA expression, indicating that the relationship between sex and SOD activity remains complex and organ-specific, with no universal consensus established to date (30).

In parallel with the oxidative stress findings, the present study documented elevated total cholesterol (TC) levels in both diabetic males and females relative to non-diabetic controls, with diabetic females showing a comparatively greater increase in TC than their male counterparts. These results are in line with previously published studies suggesting that elevated circulating lipids and protein oxidation products serve as reliable indicators of poorly managed T2DM and its associated complications (31).

The observed increase in triglyceride (TG) levels in both control and diabetic females, compared to their respective male controls, further supports the notion of a stronger association between oxidative stress and lipid dysregulation in females. These findings collectively suggest that women with diabetes face a substantially higher risk of cardiovascular complications, driven in part by greater lipid oxidation (32,33). This is consistent with the ADA Standards of Care in Diabetes (2025/2026), which recognize low HDL-C and elevated TG as the most prevalent dyslipidemic pattern in T2DM and a major driver of cardiovascular risk (34).

Interestingly, some clinical and preclinical studies have reported contrasting findings, noting higher oxidative stress in men than in women. One study found that vascular cells derived from male subjects exhibited greater ROS production compared to those obtained from females (35). These apparent contradictions may partly reflect the influence of immune system differences between sexes, particularly given growing evidence that women are disproportionately affected by autoimmune and inflammatory conditions, which may independently contribute to atherogenic processes.

The current study found that LDL cholesterol was significantly elevated in diabetic females compared to diabetic males. Additionally, both LDL and HDL-C demonstrated significant changes an increase and decrease, respectively, in diabetic males and females relative to their non-diabetic controls. These dyslipidemic patterns are consistent with established literature linking insulin resistance, hypertriglyceridemia, and reduced HDL-C to increased coronary heart disease risk in T2DM (36,37).

A study conducted on the Asian Pakistani population previously reported higher LDL-C and lower HDL-C in women compared to men, with abdominal obesity identified as a key contributor to cardiac risk. Similarly, research on urban Indian

populations found that HDL-C and TC levels were elevated in premenopausal women relative to age-matched men, while elevated TG and lipoprotein levels were identified as predominant cardiovascular risk factors in women (38,39). The 2024 ESC Guidelines for cardiovascular disease management in diabetes have further reinforced that LDL-C remains the primary modifiable lipid risk factor in T2DM and underscore the need for sex-sensitive treatment approaches (40).

The present study also observed a significant rise in malondialdehyde (MDA) levels in diabetic females compared to non-diabetic females, indicating heightened lipid peroxidation in this group. Comparable findings have been reported in studies involving diabetic female patients with complications, particularly those associated with acute myocardial infarction, where MDA concentrations were notably elevated relative to male patients(41).

A recent Pakistani study conducted at Mardan Medical Complex (2023–2024) further validated the utility of serum MDA as a predictive biomarker for coronary artery disease severity, demonstrating that elevated MDA correlates with greater CAD progression in patients with comorbid diabetes (42). The increase in lipid peroxidation observed in the context of diabetes-associated obesity is believed to stem from mitochondrial dysfunction and impaired electron transport chain activity, both of which are considered primary drivers of cardiovascular complications in T2DM (23,24).

Moreover, the female endocrine system operates through a dynamic and oscillating hormonal profile, which gives rise to inherent sex-based differences in fat distribution, lipolytic activity, and metabolic redox pathways. Age-related changes in glutathione and MDA concentrations in women are partly attributable to menopause, which is characterized by declining estrogen levels alongside rising gonadotropins and other hormonal shifts(43). Notably, estrogen exhibits antioxidant properties at high physiological concentrations but transitions to pro-oxidant behavior at lower concentrations through its catechol metabolite pathway(44.45). These hormonal dynamics may largely account for the elevated cardiovascular risk observed in postmenopausal diabetic women, and may also explain the development of compensatory metabolic feedback mechanisms in response to fluctuating oxidative conditions.

With respect to enzymatic antioxidants, the gene expression of SOD and glutathione peroxidase has been proposed to be under estrogen-dependent regulation, whereas catalase expression appears to be independent of sex hormones (46). Estrogen replacement therapy has been shown to restore blood GSH levels and upregulate MnSOD and GPx mRNA expression following menopausal decline, underscoring the hormonal dependency of antioxidant gene regulation in women (47).

The current study found a reduction in catalase activity in both diabetic males and females compared to non-diabetic controls; however, the difference between diabetic males and females did not reach statistical significance. These findings align with previously published data by Barp et al. and a study reporting no significant change in catalase activity in the brain, heart, and lungs, but elevated activity in the kidneys of female subjects.

The present study further demonstrated a significant reduction in nitric oxide synthase (NOS) activity in both diabetic males and females compared to non-diabetic controls. This observation is of considerable clinical relevance, as experimental evidence indicates that an intact vascular endothelium suppresses atherosclerotic processes under normal physiological conditions (48). NOS expression is known to be upregulated by lipopolysaccharides and pro-inflammatory cytokines, leading to excessive NO production that contributes to endothelial dysfunction (49). Additionally, ROS-mediated oxidation of tetrahydrobiopterin (BH_4), an essential cofactor for NOS, results in eNOS uncoupling, causing the enzyme to generate superoxide radicals rather than NO, thereby further aggravating endothelial

dysfunction. A 2025 review published in Frontiers in Medicine confirmed that peroxynitrite-mediated oxidation of BH_4 to its inactive form establishes a self-reinforcing cycle of superoxide production and endothelial compromise in T2DM (50). It is important to acknowledge that while diabetic women in the Pakistani population appear to be at significantly greater risk of cardiovascular complications than men, premenopausal estrogen levels and other endocrine factors may confer a degree of cardiovascular protection during earlier life stages. Additionally, sex-specific differences in immune system regulation may independently contribute to the development and progression of diabetes-induced cardiac complications.

CONCLUSION

The present study acknowledges its limitations in not fully capturing the role of hormonal fluctuations and immune-mediated pathways in this process. Nevertheless, it represents a meaningful first step toward elucidating gender-specific, ROS-mediated pathophysiological changes in diabetes mellitus and their implications for cardiovascular risk in the Pakistani population. The findings also hold potential for informing the development of more precise, sex-targeted pharmacological interventions.

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