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Assessment of 17 β -Estradiol Levels, Serum Oxidant–Antioxidant Balance, and Interleukin-8 Expression in Relation to Clinical Findings in Patients with Diabetic Nephropathy

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ABSTRACT:

Background and Objective:

Diabetic nephropathy (DN) is one of the most serious and common chronic microvascular complications of diabetes, representing a major cause of end-stage renal disease (ESRD). It typically begins with albuminuria in the early stages and progresses to a gradual decline in renal function. Both genetic and environmental factors have been implicated in its onset and progression. The present study aimed to investigate the association between 17- β -estradiol levels, pro-oxidant-antioxidant balance (PAB Assay), and interleukin-8 (IL-8) in relation to DN.

Methods:

In this cross-sectional study, 60 patients with DN were evaluated. Fasting blood sugar (FBS) and HbA1C levels were measured using standard biochemical methods at the hospital laboratory and specialized diabetes clinic. Levels of 17 β -estradiol and IL-8 in blood samples were determined using ELISA kits, while IL-8 expression was assessed via qRT-PCR.

Results

The results indicated that the mean 17- β -estradiol level was significantly higher in the patient group compared to the control group (37.95 ± 10.02 vs. 32.27 ± 8.47 pg/ml; $P = 0.001$). A significant difference in pro-oxidant–antioxidant balance (PAB) was also observed between the two groups (patients: 190.65 ± 42.02 HK vs. controls: 164.58 ± 44.48 HK; $P = 0.001$) and IL-8 levels were markedly elevated in DN patients compared

to controls (46.98 ± 15.58 vs. 28.32 ± 7.86 pg/ml; $P = 0.0001$). Also, the results of RT-PCR analysis show that IL8 expression in DN patients were up-regulated compared to the control subjects.

Conclusion

Beta-estradiol may play a protective role against diabetic complications by enhancing antioxidant activity in the kidneys. Furthermore, IL-8 seems to modulate inflammatory and immune responses, potentially contributing to protective mechanisms against diabetes and its associated renal complications.

1. INTRODUCTION

Diabetes mellitus (DM) is a major non-communicable disease of significant social importance that has reached epidemic proportions globally. According to the International Diabetes Federation (IDF), about 537 million people currently live with diabetes, and this figure is expected to increase by more than 50%, reaching approximately 784 million by 2045 [1]. DN is a clinical condition marked by persistent albuminuria and a gradual decline in kidney function, typically associated with a characteristic pattern of glomerular damage. Both T1DM and T2DM are associated with increased risk of macro- and micro-vascular complications. DN develops in approximately 20%–50% of individuals with diabetes and represents the leading cause of end-stage kidney disease (ESKD) in many regions. It accounts for 28% of patients initiating renal replacement therapy in the United Kingdom [2], 44% in the United States, and 38% in Australia [3]. The condition is commonly linked to arterial hypertension and an elevated risk of cardiovascular complications and mortality. Oxidative stress plays a pivotal role in the onset and progression of both DM and chronic kidney disease (CKD) [4]. Persistent hyperglycemia disturbs the equilibrium between reactive oxygen species (ROS) production and the body's antioxidant defense mechanisms, resulting in oxidative stress, accumulation of advanced glycation end products (AGEs), heightened inflammatory activity, and increased cellular apoptosis [5]. Concurrently, it induces inflammation, fibrosis, and tubular interstitial injury within renal tissues, accelerating the decline of kidney function [6–8]. Oxidative stress and systemic inflammation mutually amplify one another, creating a detrimental feedback loop. Therefore, controlling oxidative stress is crucial for individuals with diabetes and is strongly influenced by dietary and lifestyle factors that affect antioxidant and pro-oxidant balance. The antioxidant properties of steroid hormones have been demonstrated in various cell types and tissues [9]. Among them, 17β -estradiol has been shown to protect cells from oxidative stress-induced aging [9–11] and to regulate multiple mechanisms that defend against oxidative damage in mice [9]. Numerous studies have reported that estrogen therapy can alleviate diabetic complications [12], protect against diabetes-related brain injury [12], and restore endothelial function impaired by diabetes [13]. Despite substantial evidence supporting its beneficial effects, the specific role of 17β -estradiol in diabetic rats and its antioxidant potential remains insufficiently understood. Interleukin-8 (IL-8), a chemokine with diverse sources and biological functions [14], plays a key role in mediating leukocyte—particularly neutrophil—chemotaxis and activation [15].

IL-8 is also implicated in the development of several diabetes-related complications, including diabetic retinopathy, DN, CVD, and infections. Notably, changes in serum IL-8 levels have been observed prior to significant declines in renal function among patients with kidney injury [16]. IL-8, as one of the key proinflammatory cytokines involved in the pathogenesis and progression of DN, primarily recruits neutrophils and promotes oxidative stress, thereby increasing vascular permeability and contributing to endothelial damage in the diabetic kidney [17, 18]. Furthermore, the IL-8 gene polymorphism (IL8-rs4073) has been strongly associated with DN in Asian populations [19]. Other studies have reported elevated circulating IL-8 levels in patients with T2DM [17]. Bioinformatic analyses of endothelial precursor cells from T2DM patients have also revealed that IL-8 and CXCL1 are among the most highly expressed genes in diabetic samples [20]. Therefore, the present study aims to investigate the relationship between oxidative/antioxidant balance, serum 17β -estradiol levels—both of which are known to influence the development of DN—and IL-8 gene expression in these patients.

2. MATERIAL AND METHOD

2.1. Sampling

All participants provided informed consent prior to enrollment. Based on urinary albumin excretion rate (UAE), this study included 75 patients with T2DM and macroalbuminuria ($n = 26$, $\text{UAE} \geq 300$ mg/24 h). Exclusion criteria encompassed

individuals with a prior diagnosis of urolithiasis, conditions that could confound proteinuria measurements, recent or ongoing viral hepatitis or liver cirrhosis, a history of clinical cardiovascular disease or acute or chronic infections. Subsequently, 10 mL of venous blood was collected and sent to the laboratory. The serum samples were centrifuged and then transferred to the Research Center of the Faculty of Medicine, Islamic Azad University, Mashhad Branch, for further analysis. The control group consisted of non-diabetic patients hospitalized at the same center, whose non-diabetic status was confirmed through blood glucose testing and verified by an internal medicine specialist or an endocrinologist.

2.2. Biochemical parameters

2.2.1. Serum level of 17 β -Estradiol

Serum 17 beta-estradiol levels in patients was measured using a commercial ELISA kit (ZellBio Company).

2.2.2. Pro-oxidant-antioxidant balance (PAB Assay)

PAB levels were measured in patients' serum samples according to the instructions mentioned in previous studies (11). Standard solutions were prepared by mixing defined ratios of 250 M H_2O_2 (0–100%) and 3 M uric acid, followed by the addition of 10 M NaOH. The TMB cation solution was obtained by dissolving 60 mg of TMB powder in 10 mL of dimethyl sulfoxide (DMSO) and subsequently adding this solution to 20 mL of acetate buffer (0.05 M, pH 4.5). Then, 70 μ L of freshly mixed chloramine T solution (100 mM) was added to the pre-prepared solution, shaken immediately, and incubated for two hours in the dark condition at room temperature (23–27°C).

To prepare horseradish peroxidase solution, 25 units of peroxidase enzyme solution was mixed with 20 mL TMB cation, and the prepared mixture was divided into 1 mL microtubes and stored in -20. TMB working solution was prepared by gently mixing 200 μ L of TMB/DMSO with 10 mL of acetate buffer (0.05 M, pH 5.8). To measure the level of PAB in the serum of patients' samples, 200 μ L of the working solution was added to each well of the ELISA plate, then 10 μ L of patient serum, distilled H_2O (empty well), and standard solutions were added, and gently mixed, also plates were incubated at 37°C for 15 minutes under dark conditions. Then, 50 μ L of 2 N HCl was added to each well as a stop solution, and a wavelength of 450 nm was used to measure PAB, although the reference wavelength was 620 nm or 570 nm. PAB levels were expressed in arbitrary HK (Hamidi-Koliakos) units, corresponding to the percentage of H_2O_2 determined in the standard solutions. Finally, the PAB values of the unknown samples were calculated using the standard curve.

2.2.3. Evaluation of IL-8 Gene Expression by RT-PCR

Primers for the IL-8 gene were designed using Genrunner software. Their accuracy and specificity were confirmed through analysis with the NCBI BLAST tool. Real-time PCR was carried out on a CORBETT system using SYBR Green dye. The relative expression of the IL-8 gene was determined by normalizing its expression levels to that of a housekeeping gene (e.g., GAPDH).

2.2.3.1. RNA Extraction and cDNA Synthesis

Total RNA was extracted using RNX Plus reagent (CinnaGen, Iran) and stored at -80°C until cDNA synthesis. cDNA was then synthesized according to the manufacturer's instructions in the DyNAmo™ cDNA Synthesis Kit protocol.

2.2.3.2. RT-PCR Analysis

The GAPDH gene served as an internal control. The synthesized cDNA was diluted at a 1:4 ratio and used as the template for qRT-PCR reactions prepared with SYBR Green Master Mix. Each reaction had a final volume of 20 μ L, consisting of 10 μ L PCR Master Mix, 1–2 μ L cDNA template, 1 μ L each of forward and reverse primers, 0.8 μ L of specific probe (10 pM), and 6 μ L of nuclease-free water. Ct (cycle threshold) values were determined and analyzed using dedicated software. All reactions were performed in duplicate on the Rotor-Gene 6000 system (Corbett, Australia). The thermal cycling conditions were as follows: Initial denaturation (Hold step): 95°C for 10 minutes, PCR cycling (40 cycles): Denaturation: 95°C for 15 seconds, Annealing/Extension: 60°C for 60 seconds. The primer sequences used in this study were presented in Table 1. The relative expression of IL8 was determined with the comparative cycle threshold (C_q) ($2^{-\Delta\Delta C_q}$) method.

Table 1. Forward and reverse primers for IL-8 and GAPDH

Gene	Sense Primer	Antisense Primer
IL-8	5'- TCAGTGCAAGAACTCTGT -3'	-
GAPDH	5- GCTTCGGCAGCACATATACTAAAAT - 3	5- CGCTTCACGAATTTGCGTGTTCAT - 3

2.3. Association analysis

Data analysis was performed using the Student's t-test and analysis of variance (ANOVA), while nominal-scale data were analyzed using the Chi-square test. Logistic regression was used to examine the relationship between variables. The software used in this study was SPSS v.28 and the significance level was considered to be less than 5%.

3. RESULTS AND DISCUSSION

3.1. Results

Table 2 presents the demographic characteristics and biochemical parameters of DN patients and control subjects. Among the DN patients, 52.0% (n = 39/75) were male and 48.0% (n = 36/75) were female. The mean age of DN patients was 58.56 ± 16.81 years, whereas that of the control group was 51.14 ± 12.80 years. The mean body mass index (BMI) of DN patients was 27.90 ± 2.11 kg/m², compared with 28.05 ± 3.61 kg/m² in the control group. The mean serum 17- β -estradiol concentration was 37.95 ± 10.02 pg/ml in DN patients and 32.27 ± 8.47 pg/ml in controls, demonstrating a statistically significant difference between the two groups (P = 0.001). Similarly, the mean PAB level was significantly higher in DN patients (190.65 ± 42.02) than in controls (164.58 ± 44.48 HK, P = 0.001). Furthermore, the serum IL-8 concentration was markedly elevated in DN patients compared with control subjects (46.98 ± 15.58 pg/ml versus 28.32 ± 7.86 pg/ml). In the evaluation of IL8 gene expression, 75 DN samples were evaluated. Based on the results, there was significant difference in the relative expression ($2^{-\Delta\Delta C_q}$) between patient and control groups so that IL8 expression in DN patients were up-regulated in relation to individuals from the control subjects.

Table 2. Demographic information of HT patient and control subject

Parameter	DN patients	Control	P-Value
Men	39 from 75 (52.0%)	22 from 50 (44.0%)	-
Women	36 from 75 (48.0%)	28 from 50 (56.0%)	-
Age (years)	58.56 ± 16.81	51.14 ± 12.80	0.009
BMI (kg/m ²)	27.90 ± 2.11	28.05 ± 3.61	0.785
17beta-estradiol (pg/ml)	37.95 ± 10.02	32.27 ± 8.47	0.001
PAB (HK)	190.65 ± 42.02	164.58 ± 44.48	0.001
IL-8 (pg/ml)	46.98 ± 15.58	28.32 ± 7.86	0.0001

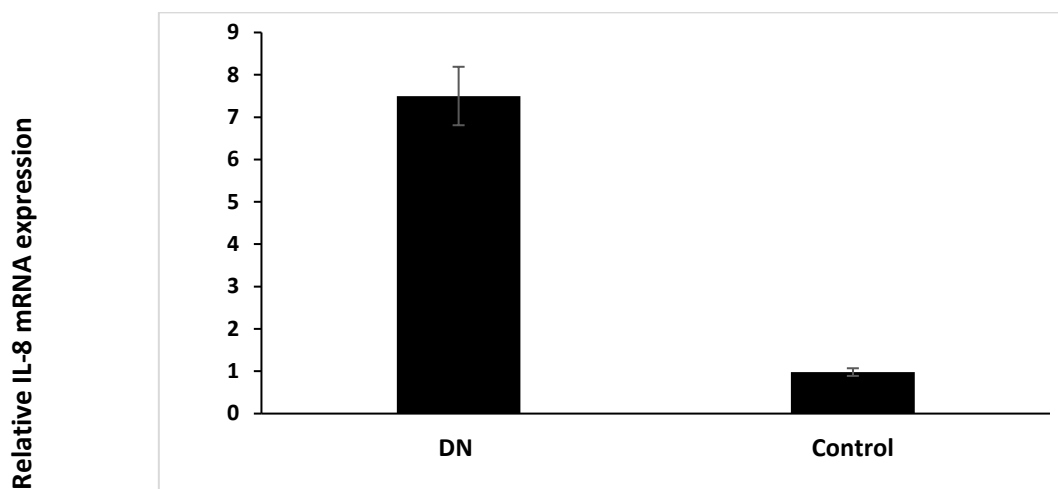


Fig.1. Relative IL8 mRNA expression in DN patients compared to control subjects.

The analysis of the relationship between clinical indices and serum 17- β -estradiol revealed a significant inverse correlation between 17- β -estradiol levels and HbA1c ($P=0.004$, $r=-0.253$) as well as insulin ($P=0.008$, $r=-0.234$) in the total study population (including both control subjects and DN patients). In contrast, a significant positive correlation was observed between 17- β -estradiol and creatinine ($P=0.002$, $r=0.348$), as well as IL-8 ($P=0.041$, $r=0.237$), among DN patients. The analysis of clinical indices in relation to PAB levels demonstrated a negative correlation between PAB and FBS ($P=0.018$, $r=-0.212$), HbA1c ($P=0.001$, $r=-0.297$), insulin ($P=0.004$, $r=-0.258$), and creatinine ($P=0.000$, $r=-0.351$) in the total study population. A similar inverse association between PAB and creatinine ($P=0.000$, $r=-0.395$) was also observed among DN patients. Finally, the analysis of clinical indices in relation to IL-8 revealed a positive correlation between IL-8 and FBS ($P=0.000$, $r=0.431$), HbA1c ($P=0.000$, $r=0.586$), insulin ($P=0.008$, $r=0.237$), and creatinine ($P=0.000$, $r=0.400$) in the total study population. A similar positive association between IL-8 and HbA1c ($P=0.002$, $r=0.345$) and serum 17- β -estradiol ($P=0.041$, $r=0.237$) were also observed among DN patients. The relationships between 17- β -estradiol and the PAB and IL-8 parameters in DN patients are presented in Figs. 2 and 3. The results indicated no significant correlation between 17- β -estradiol and PAB ($r = -0.042$, $P = 0.716$). However, a significant positive correlation was observed between 17- β -estradiol and IL-8 ($r = 0.236$, $P = 0.041$).

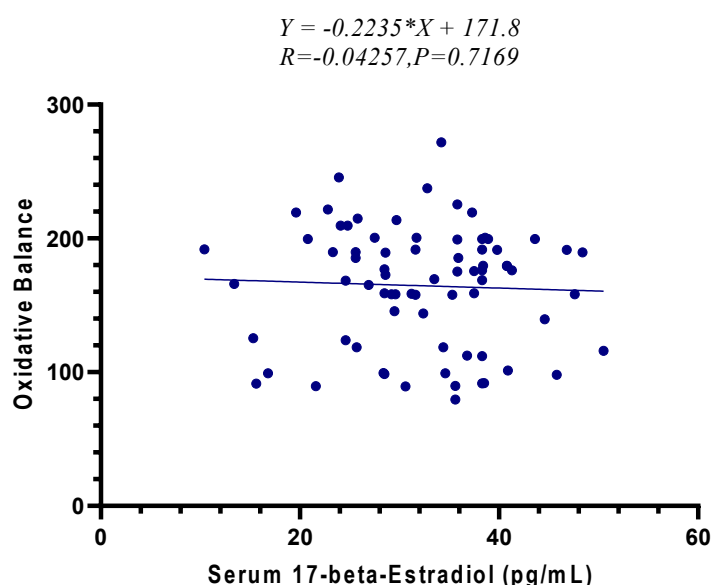


Fig.2. Correlation chart between serum 17-beta estradiol and PAB in DN patients.

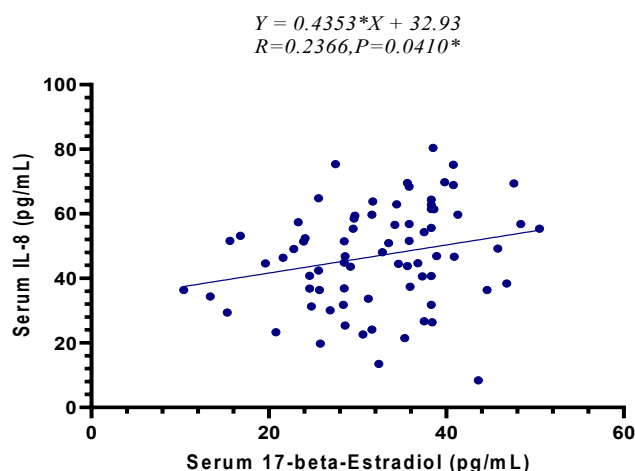
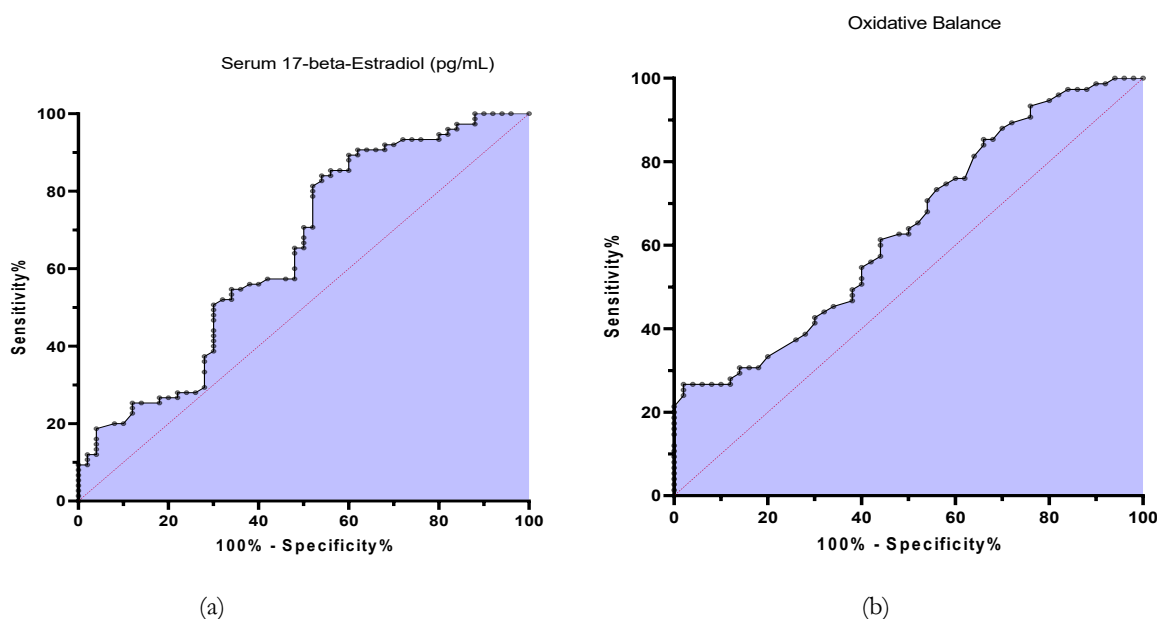
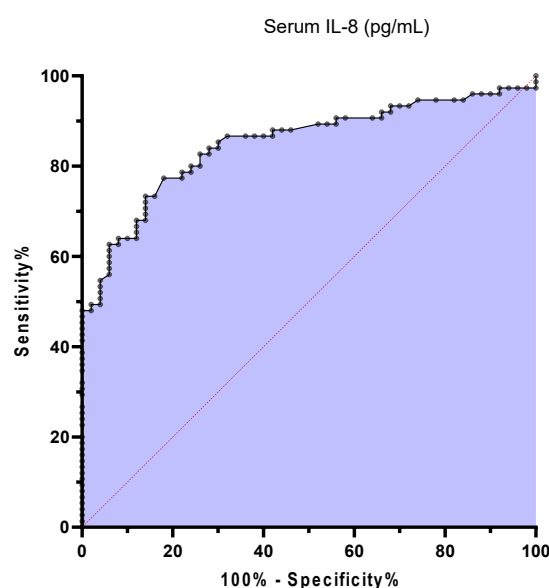


Fig.3. Correlation chart between serum 17-beta estradiol and IL-8 in DN patients.

The receiver operating characteristic (ROC) curve analysis (Fig. 4a) for the serum 17 β -estradiol assay in patients with DN compared with the control group revealed a weak diagnostic performance, as indicated by the area under the curve (AUC = 0.6457, SD = 0.05166, $P < 0.001$). The Youden index, which identifies the threshold that maximizes the combined sensitivity and specificity, was used to determine the optimal cut-off point. Based on this analysis, the 17 β -estradiol assay demonstrated a sensitivity of 84% and a specificity of 46% for detecting nephropathy. The optimal cut-off value for serum 17 β -estradiol in relation to nephropathy was established at >39.15 pg/mL. The results of the ROC curve analysis (Fig. 4b) for serum PAB levels in DN patients compared to the control group showed that the diagnostic performance of this biomarker with respect to nephropathy was weak (AUC = 0.6384, SD = 0.04997, $P < 0.008$). The Youden index, which identifies the point with the highest combined sensitivity and specificity, was used to determine the optimal cut-off value. Based on this analysis, the PAB test demonstrated a sensitivity of 26% and a specificity of 98%, with an optimal cut-off value of >127.4 HK. Similarly, the ROC curve analysis (Fig. 4c) for serum IL-8 levels in DN patients versus controls indicated a satisfactory diagnostic performance (AUC = 0.8504, SD = 0.03426, $P < 0.0001$). Using the Youden index, the optimal diagnostic threshold was determined, yielding a sensitivity of 73% and a specificity of 86% for the serum IL-8 assay, with an optimal cut-off value of >36.75 pg/mL.





(c)

Fig. 4. ROC curves for assessing the diagnostic performance of 17β-Estradiol (a), PAB (b) and serum IL-8 levels (c) in DN patients.

3.2. DISCUSSION

17β-Estradiol is a well-established steroid hormone that modulates the expression of numerous genes and is critically involved in various physiological and pathological processes in both women and men [21], including the regulation of glucose homeostasis and insulin sensitivity [22, 23]. Several studies have demonstrated that 17β-Estradiol exerts anti-apoptotic, anti-inflammatory, and insulinotropic effects on pancreatic β-cells [24]. Findings from human studies on the effects of estrogens in diabetes remain inconsistent. Several clinical investigations have reported that postmenopausal estrogen therapy is associated with a reduced risk of diabetes [25, 26]. However, other studies suggest that estrogen use in postmenopausal women may contribute to increased insulin resistance and impaired glucose tolerance [27, 28]. In 2010, Yamabe et al. investigated the potential beneficial effects of 17β-estradiol on hyperglycemia and pancreatic islet β-cell function in streptozotocin (STZ)-induced diabetic rats. The animals were administered 17β-estradiol orally at a dose of 500 μg/kg body weight per day to assess its influence on hyperglycemia and insulin secretion. After 10 days of treatment, 17β-estradiol markedly reduced plasma glucose levels, increased plasma insulin concentrations, and improved glucose tolerance by enhancing the insulin response to oral glucose intake. These improvements were accompanied by increases in islet number and volume, β-cell proliferation rate, and insulin secretion. However, these protective effects were largely absent in STZ-treated rats with more severe islet damage and hyperglycemia. Overall, the study concluded that 17β-estradiol promotes the regeneration of damaged pancreatic islets by stimulating β-cell proliferation in diabetic rats, leading to improved glucose tolerance and reduced plasma glucose levels. These findings suggest that oral administration of 17β-estradiol could potentially benefit diabetic patients experiencing accelerated loss of islet β-cells [29]. In 2004, Shibata et al. reported that 17β-estradiol protects the kidneys from post-ischemic injury in male rats. The study found that this protective effect was significantly diminished when the animals were pretreated with NG-nitro-L-arginine methyl ester (L-NAME), a non-selective nitric oxide synthase (NOS) inhibitor. Additionally, the researchers observed that renal NO₂⁻/NO₃⁻ (NOx) levels decreased markedly during ischemia and reperfusion but rapidly returned to baseline in 17β-estradiol-treated rats compared with vehicle-treated controls. These findings suggest that 17β-estradiol exerts a protective effect against post-ischemic acute renal failure by enhancing nitric oxide (NO) production [30]. Similarly, Satake et al. (2008) demonstrated that 17β-estradiol prevents ischemia/reperfusion (I/R)-induced renal injury by activating the PI3K/Akt signaling pathway, which leads to an increase in phosphorylated eNOS expression [31]. IL-8 has been established as a critical factor in inducing CKD [32].

IL-8 is a pro-inflammatory polypeptide belonging to the CXC chemokine superfamily, characterized by two cysteine residues separated by a single amino acid at the first three positions [33]. It is produced by a variety of cell types, including adipocytes, monocytes/macrophages, T lymphocytes, and endothelial and epidermal cells [33]. Studies have shown that IL-8 levels are elevated not only in adults with CKD [34], but also in children with pre-dialysis CKD compared to healthy controls [35, 36]. Moreover, IL-8 has emerged as an independent risk predictor for CKD and its associated vascular complications. For example, in patients with T2DM, abnormally high IL-8 levels were associated with a 1.41-fold increased risk of proteinuria [37]. In fact, T2DM is an inflammatory disease and inflammatory cytokines are involved in its pathogenesis. Serum level of IL-8 in T2DM is significantly higher than that in healthy individuals [38]. Furthermore, in patients undergoing hemodialysis, proteomic analyses revealed that elevated IL-8 levels predicted an all-cause mortality risk ratio of up to 1.17, and a cardiovascular-specific mortality risk ratio of up to 1.34 [39].

4. CONCLUSION

DN is a serious microvascular complication of diabetes and a leading cause of chronic kidney failure as well as diabetes-related mortality. Although its pathogenesis is not fully understood, it is known to involve multiple factors, including hemodynamic alterations, genetic predisposition, inflammatory mediators, impaired glucose metabolism, and cytokine dysregulation. This research aimed to investigate the association between 17- β -estradiol levels, PAB, and IL-8 in DN patients. The results revealed that patients with DN had markedly increased circulating levels of IL-8 and 17 β -estradiol, indicative of an enhanced inflammatory milieu. These findings suggest that IL-8 and 17 β -estradiol may serve as potential biomarkers for inflammation-related risk stratification in individuals with T2D, warranting further investigation.

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