The relationship between melatonin level and antioxidant enzymes in diabetic patients with and without nephropathy

Asaad Al-Khafaji¹, Seyed M. Mir¹, Esmaeil Damden¹, Fatemeh Mohammadzadeh¹, Maryam Abolghasemi¹ and Mahmoud H. Hadwan²

¹Department of xxxxx, Faculty of Medicine, Golestan University of Medical Sciences, Gorgan, Iran
²Chemistry Department, College of Science, University of Babylon, Hilla City, Babylon Governorate, 51002, Iraq

ABSTRACT

Background and objective: Diabetes is the most common cause of chronic renal disease globally. Diabetic nephropathy (DN) is one of the most serious consequences of type 2 diabetes. Melatonin, a powerful antioxidant that has been shown to alleviate DN, deficiency and a functional relationship between melatonin and insulin have been linked to the etiology of type 2 diabetes mellitus. The purpose of this research is to assess the relationship between melatonin level and antioxidant enzyme activity (catalase, glutathione peroxidase, superoxide dismutase, paraoxonase 1, and glutathione-s-transferase) in diabetic patients with and without nephropathy.

Methods: This case-control study was conducted on 45 healthy control subjects, 45 diabetic patients without nephropathy, and 45 diabetic patients with nephropathy. Serum samples of participants were used to evaluate antioxidant enzyme activities, melatonin levels, and MDA using specific assays.

Results: The results showed that the concentration of melatonin is not affected in diabetic patients without nephropathy, but decreased significantly in diabetic patients with nephropathy when compared with healthy subjects. Antioxidant enzymes activity in sera of diabetic patients with and without nephropathy were significantly lower than that of healthy subject group. The superoxide dismutase enzyme has a specific exception because its activity is elevated, unlike other antioxidant enzymes.

Conclusions: Melatonin decreased significantly in sera of diabetic patients with nephropathy. Diabetic nephropathy affects antioxidant enzymes activity and lipid peroxidation significantly compared with healthy controls.

Keywords: antioxidants, diabetic nephropathy, diabetes mellitus, enzymes, melatonin, oxidative stress

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder divided into two major types: type 1 (T1DM, accounting for approximately 5% of diabetes cases) and type 2 (T2DM, accounting for 90%-95% of DM cases). Diabetes, particularly T2DM, is becoming increasingly prevalent worldwide, a trend linked to rising obesity rates, susceptible minority populations, and an aging demographic against the backdrop of polygenic risk. Complications from diabetes are common in individuals with either T1DM or T2DM, and these can lead to significant morbidity and mortality. The chronic complications of diabetes are generally divided into microvascular and macrovascular, with the former being substantially more prevalent than the latter.

Diabetic nephropathy (DN) is a serious complication of diabetes mellitus (DM) that can lead to chronic kidney failure. Diabetes increases the risk of end-stage renal failure tenfold. According to the International Diabetes Federation (IDF), 40% of individuals with diabetes will develop end-stage renal disease. Moreover, diabetes and hypertension, either individually or combined, contribute to approximately 80% of all end-stage renal failure cases. The earliest sign of DN is microalbuminuria. After a 10 years, roughly 20% of people with microalbuminuria develop nephropathy, and nearly 20% progress to end-stage renal disease. Due to the lack of effective treatments for hyperglycemia, around 20% of patients with T1DM develop end-stage kidney failure within a decade, and 75% do so within less than two decades.

Oxidative stress results from an imbalance between oxidants and antioxidants. This can be triggered by an increase in reactive oxygen species production, a decrease in antioxidant activity, or both. High blood sugar-induced oxidative stress has also been associated with increased endothelial cell death, both in vivo and in vitro. DN is a primary cause of irreversible kidney failure. Numerous prior studies suggest that oxidative stress is a central mechanism involved in the onset and progression of diabetic vascular complications. Increased reactive oxygen species (ROS) generation has been linked to the mitochondrial respiratory chain through oxidative phosphorylation, NAD(P)H oxidase, advanced glycation end products (AGE), polyol pathway defects, and uncoupled nitric oxide synthase (NOS). Overproduction of ROS, which regulates the activation of protein kinase C, nitrogen-activated protein kinases, and various cytokines and transcription factors, leads to increased expression of genes encoding components of the extracellular matrix (ECM), resulting in fibrosis and end-stage renal disease. The activation of the renin-angiotensin system (RAS) exacerbates ROS-induced kidney damage in diabetic nephropathy. Therapies that inhibit ROS generation may potentially mitigate kidney damage associated with DN.

The pineal gland secretes the hormone melatonin, also known as N-acetyl-5-methoxytryptamine. Its physiological expression follows a circadian cycle that is largely controlled by light signals. Melatonin plays a role in several biological processes, including oxidative stress, apoptosis, proliferation, energy metabolism, damage repair, and...
The role of melatonin in antioxidative defense systems was initially suggested when it was demonstrated that melatonin effectively scavenges the extremely hazardous hydroxyl radical (•OH).\(^8\) This finding has been repeatedly confirmed by in vitro and in vivo experiments. Melatonin's direct ability to neutralize free radicals extends beyond •OH. It is reported that melatonin also neutralizes singlet oxygen (1O2), a high-energy version of O2 with significant molecular toxicity. In addition, melatonin eliminates peroxynitrite (ONOO\(^-\)), the byproduct of superoxide anion (O2\(^-\)) and nitric oxide radical (NO\(^*\)) interaction.\(^8\)

The impact of melatonin on renal ischemia/reperfusion (I/R) was evaluated in diabetic rats subjected to renal I/R injury.\(^9\) Melatonin treatment was observed to prevent renal damage caused by oxidative stress and cell apoptosis in these diabetic rats. Additionally, the combined treatment of melatonin and Losartan Potassium in rats with DN improved levels of glutathione (GSH), superoxide dismutase (SOD), malondialdehyde (MDA), and nitric oxide (NO), thereby mitigating the adverse effects of diabetes on these oxidative stress markers (reference is needed here, please).

The current study aimed at assessing the relationship between melatonin levels, lipid peroxidation marker (MDA), and antioxidant enzymes' activities (such as SOD, catalase or CAT, glutathione peroxidase or GPx, glutathione-s-transferase or GST, and paraoxonase 1 or PON1) in diabetic patients with/without nephropathy. Melatonin, known for its potent antioxidant properties, is of interest due to its role in relation to antioxidant enzymes. It helps scavenge free radicals and other ROS, which could otherwise damage cells and induce oxidative stress. In addition, the cell's defense against oxidative damage relies on antioxidant enzymes including GPx, CAT, and SOD. The mechanisms through which melatonin exerts its protective effects can be better understood by exploring the connection between melatonin and antioxidant enzymes.

**MATERIALS AND METHODS**

**Population, study design, and sample size**

In the current case-control research, 135 participants were involved, comprising 45 diabetes patients without nephropathy, 45 diabetic patients with nephropathy, and 45 healthy individuals as controls. Group 1 (G1) represents healthy participants; group 2 (G2) represents diabetic patients without nephropathy; and group 3 (G3) represents diabetic patients with nephropathy.

This study was approved by the Golestan University of Medical Sciences ethical committee (approval code: IR.GOUMS.REC.1401.372)
**Inclusion criteria**

The inclusion criteria encompassed individuals with clinically diagnosed T2DM, with or without nephropathy, provided the duration of nephropathy was at least one year. Diabetic patients were selected based on fasting blood sugar (FBS) levels above 126 mg/dl and HbA1c levels above 6.0%. Those with FBS levels above 126 mg/dl, HbA1c levels above 6.0%, and elevated serum creatinine and urea levels were identified as diabetic patients with nephropathy. The diagnosis of T2DM, along with the duration of diabetes and diabetic nephropathy, was confirmed by an endocrinologist. Control subjects were healthy adult volunteers with FBS levels below 100 mg/dl and HbA1c levels below 6.0%. Every participant provided written informed consent. Healthy individuals who met any of the exclusion criteria were disqualified from the study. Participants were initially interviewed to gather their medical histories.

**Exclusion criteria**

Exclusion criteria for both the case and control groups included a history of cigarette smoking, heart failure, hyper- or hypothyroidism, malignancy, and liver disease.

**Biochemical analysis**

Samples for this project were sourced from a previous project approved by the Golestan University of Medical Sciences, with registration number IR.GOUMS.REC.1401.158. In that study, 5 mL of venous blood was collected from participants who were referred to Sayad Shirazi Hospital (Golestan, Iran) after obtaining informed consent. Subsequently, serum samples were isolated by centrifugation at 2500 rpm for 15 minutes and stored at -80 °C until analysis. The activity of the antioxidant enzymes (SOD, CAT, GPx, GST, and PON), along with malondialdehyde (MDA) as a marker of lipid peroxidation, and melatonin levels in diabetic patients with and without nephropathy, and the control group were then determined.

The GST assay is based on measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced GSH. Enzyme activity is indicated by measuring the substrate GSH binding rate with dinitrophenyl in unit time. The residual GSH reacts with disulfide double nitro benzoic acid (DTNB) to form yellow glucosinolate nitro benzoic acid anion (TNB), the concentration of which is determined to calculate GSH reduction. Hence, the activity of GST was indirectly calculated by measuring the optical density (OD) value at 412 nm.10

The GPx activity was measured by monitoring the decrease in reduced GSH concentration using Ellman's reagent [5,5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB)].11

The SOD activity was determined using a direct protocol based on the enzyme’s ability to slow the autoxidation of pyrogallol in the presence of EDTA at pH 8.2 by 50%. This method is rooted in the competition between radical dismutation by SOD and pyrogallol.
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autoxidation by O$_2^{•−}$. $^{12}$

The CAT activity was determined by following the method of Hadwan and Ali. $^{13}$ Ten microliters of the sample were added to each well, followed by the addition of 140 $\mu$L of 3% H$_2$O$_2$. The reaction was then stopped by ammonium metavanadate after 10 minutes in a 96-well plate at room temperature in the dark, and the absorbance was measured at a wavelength of 450 nm.

The concentration of hydrogen ions produced during thiolactone hydrolysis to homocysteine in the test solution, comprising 5 mM substrate, 1 mM CaCl$_2$, 0.0005% bovine serum albumin, 0.004% phenol red, and 20 $\mu$L of serum in 5 mM HEPES buffer (pH 7.0), was measured. Absorbance was recorded for 4 minutes at 412 nm and expressed in min$^{-1}$ mL$^{-1}$. PON1 activity toward homocysteine thiolactone was determined using a standard curve created by titrating the enzymatic reagent with various concentrations of HCl. $^{14}$

Thiobarbituric acid reactive substances (TBARS) were used to assess lipid peroxidation in sera. The TBARS assay provides a simple, reproducible, and standardized method for evaluating lipid peroxidation in serum. The MDA-thiobarbituric acid (MDA-TBA) adduct formed by the reaction of MDA with 1,3-Diethyl-2-thiobarbituric acid (DETBA) at high temperatures (90-100$^{°}$C) under acidic conditions is detected colorimetrically at 530-540 nm or fluorometrically at 515 nm excitation and 555 nm emission. When evaluated fluorometrically, this reaction exhibits significantly greater sensitivity. $^{15}$

**Data collection**

Data on urea, creatinine, FBS, and HbA$_1$c were obtained from the previous study (Can you please provide citation for the previous study). The serum level of melatonin was measured using an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Cat No. of General Melatonin: #ZB-1101C-H9648). Serum antioxidant enzymes and malondialdehyde (MDA) levels were measured manually using specific chemical reagents, as stated in previous section.

**Statistical analysis**

Data analysis was performed using SPSS version 23 statistical software (SPSS IBM Corporation, USA). All values were expressed as the mean ± standard deviation (SD). The Shapiro-Wilk test was used to evaluate the distribution of quantitative data. The comparison of biochemical variables between groups was assessed using the Kruskal-Wallis test (pairwise comparison). Correlation analysis of variables was conducted using the Spearman correlation coefficient test. A $p$-value of <0.05 was considered statistically significant.
RESULTS

Participants were divided into three groups (45 DM patients with diabetic nephropathy, 45 DM patients without nephropathy, and 45 healthy individuals serving as controls). Biochemical and clinical characteristics are presented in Figure 1. And, Table 1 shows the enzymatic antioxidant activities and lipid peroxidation levels. The results revealed an increase in HbA1c levels in diabetic patients with and without nephropathy as compared to the healthy control group. Furthermore, urea and creatinine levels were found to be significantly higher in diabetes individuals with nephropathy than those without.

The results shown in Table 1 demonstrated that melatonin concentration remained unaffected in patients with diabetes, irrespective of the presence or absence of nephropathy. Urea, creatinine, and HbA1c concentrations were significantly higher in diabetes patients with nephropathy compared to those without nephropathy.

The activity of antioxidant enzymes (CAT, GPx, GST, and PON) in diabetic patients, both with and without nephropathy, significantly decreased compared to the control group, as shown in Figure 1. In contrast, SOD activity was considerably higher in diabetic patients, regardless of the presence of nephropathy, compared to the healthy subject group.

The results of the correlation analysis between melatonin concentration, antioxidant enzymes, MDA, and clinical laboratory variables in all participants are presented in Table 2. The findings revealed a significant negative correlation between melatonin and urea (p = 0.033), creatinine (p = 0.001), FBS (p = 0.013), HbA1c (p = 0.008), and a significant positive correlation between melatonin and GPx (p = 0.03) in all participants.

DISCUSSION

The present study investigated the relationship between serum melatonin concentration and antioxidant enzymes in diabetic patients with and without nephropathy. The findings show elevated HbA1c levels in diabetic patients with and without nephropathy, in comparison to the healthy control group (see Figure 1). Additionally, urea and creatinine levels were found to be significantly higher in diabetes patients with nephropathy than those without. The DN is a major complication that can lead to kidney failure and necessitate a kidney transplant. It is clinically characterized by either microalbuminuria (80-150 mg/L or above) or proteinuria (0.5 g/24 h). This stage can be described using various terms, such as clinical nephropathy, overt nephropathy, microalbuminuria, or proteinuria. DN occurs in 40% of diabetic individuals, even when high glucose levels are maintained over prolonged periods.

The data provided in Table 1 suggest that melatonin concentration is not significantly altered in diabetic individuals, regardless of nephropathy. This might be related to the body’s capacity to produce the absorbed levels of melatonin on a continual basis. Previous studies have demonstrated the potential of melatonin in treating DN in both humans and laboratory animals. While these studies provide diverging interpretations regard-
Table 1. The pairwise comparison of enzymatic antioxidant activities and lipid peroxidation among diabetic patients with and without nephropathy, and control group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls (G1, n=45)</th>
<th>DM with nephropathy (G2, n=45)</th>
<th>DM without nephropathy (G3, n=45)</th>
<th>Comparison</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, n(%)</td>
<td>Male 24(53.3) Female 21(46.7)</td>
<td>Male 33(37.3) Female 12(26.7)</td>
<td>Male 28(62.2) Female 17(37.8)</td>
<td>Mean±SD</td>
<td>Mean Rank</td>
</tr>
<tr>
<td>GPx (U/L)</td>
<td>117.66±25.24</td>
<td>90.78±34.68</td>
<td>57.66±22.63</td>
<td>32.70</td>
<td>G1 G2 0.001</td>
</tr>
<tr>
<td>GST (U/L)</td>
<td>87.87±18.88</td>
<td>42.36±13.76</td>
<td>58.82±16.84</td>
<td>62.76</td>
<td>G1 G2 0.0001</td>
</tr>
<tr>
<td>CAT (Katal/L)</td>
<td>44.62±19.38</td>
<td>21.31±9.72</td>
<td>30±16.34</td>
<td>62.08</td>
<td>G1 G2 0.0001</td>
</tr>
<tr>
<td>SOD (U/L)</td>
<td>59.44±40.26</td>
<td>80.60±13.80</td>
<td>74.73±17.60</td>
<td>74.62</td>
<td>G1 G2 0.0001</td>
</tr>
<tr>
<td>PON1 (U/L)</td>
<td>67.56±16.99</td>
<td>51±11.19</td>
<td>54.67±18.10</td>
<td>59.78</td>
<td>G1 G2 0.0001</td>
</tr>
<tr>
<td>MDA (μmol/L)</td>
<td>1.02±0.25</td>
<td>1.44±0.17</td>
<td>1.28±0.21</td>
<td>71.72</td>
<td>G1 G2 0.0001</td>
</tr>
</tbody>
</table>

DM= diabetes mellitus; GPx= glutathione peroxidase; GST= glutathione-S-transferase; MDA= malondialdehyde; PON1= Paraoxonase 1; SOD= superoxide dismutase.
Figure 1  Biochemical and clinical characteristics of diabetic patients with and without nephropathy, and the control group. The asterisk (*) represents significance compared to the healthy donors, and the double asterisks (**) denote significance when compared to the diabetic patients’ group.

Table 2. The correlation analysis between melatonin and other parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Spearman's rho</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>-0.184</td>
<td>0.033</td>
</tr>
<tr>
<td>Creatinine</td>
<td>-0.276</td>
<td>0.001</td>
</tr>
<tr>
<td>FBS</td>
<td>-0.214</td>
<td>0.013</td>
</tr>
<tr>
<td>HbA1c</td>
<td>-0.227</td>
<td>0.008</td>
</tr>
<tr>
<td>GPx</td>
<td>0.187</td>
<td>0.030</td>
</tr>
<tr>
<td>GST</td>
<td>0.143</td>
<td>0.098</td>
</tr>
<tr>
<td>CAT</td>
<td>-0.057</td>
<td>0.514</td>
</tr>
<tr>
<td>SOD</td>
<td>-0.109</td>
<td>0.207</td>
</tr>
<tr>
<td>PON1</td>
<td>-0.046</td>
<td>0.600</td>
</tr>
<tr>
<td>MDA</td>
<td>0.004</td>
<td>0.966</td>
</tr>
</tbody>
</table>
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In exploring melatonin’s influence on blood glucose concentrations, they consistently acknowledge melatonin’s beneficial impact on antioxidant levels and oxidative stress.

Earlier research by Ertik et al (2022) identified a significant increase in plasma glucose levels in diabetic rats and those treated with melatonin, compared to control rats. However, no significant difference was observed between diabetic rats and diabetic rats treated with melatonin. In this context, the study’s findings were consistent with those of other studies. In contrast, several studies reported that melatonin significantly and effectively regulated hyperglycemia.

The activities of antioxidant enzymes in the sera of diabetic patients, with and without nephropathy, were significantly lower compared to those in the healthy control group. An exception was noted in the case of SOD enzyme, where its activity increased, contrary to the pattern seen in other antioxidant enzymes. Goodarzi et al. (2010) demonstrated that excessive generation of ROS in diabetes correlates with a reduction in antioxidant defenses. This can take the form of a decrease in the concentration of a specific antioxidant molecule or an overall decline in the antioxidant status of the cell.

This study observed that GPx activity was significantly reduced in diabetic groups, with and without nephropathy, compared to the healthy control group. Diabetes and associated metabolic disorders, leading to elevated ROS concentrations in mitochondria-rich ß-cells, can negatively impact GPx activity. Hyperglycemia induces an increase in superoxide generation, producing superoxide radical (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) via superoxide dismutase activity. ROS, such as H$_2$O$_2$ and O$_2^-$, inhibit GPx activity. As GSH serves as a substrate for GPx, a decrease in GSH primarily impacts enzyme activity and the rate of peroxide detoxification. Organic hydroperoxides, the main substrate for GPx, contribute to diabetes complications and can modify cell membranes when present in excessive amounts.

Various studies have reported decreased blood selenium levels in diabetic patients, with serum selenium concentration found to correlate with GPx. Each GPx subunit contains an active-site selenium atom. Selenium deficiency has been linked to increased oxidative stress and the development of DN. Previous studies have also indicated an inverse relationship between microalbuminuria, and selenium and GPx plasma concentrations in diabetic patients. Individuals with varying degrees of chronic kidney disease have benefited from selenium supplementation in terms of increased cellular GPx activity. In line with previous studies, our current research identified reduced GPx activity in T2DM patients, both with and without nephropathy.

Diabetic patients with nephropathy exhibited significantly lower CAT activity compared to healthy subjects. This deficiency could be attributed to glycation-induced enzyme inhibition, leading to decreased CAT activity, as seen in patients with nephropathy. It’s worth noting that reduced catalase activity may result in conditions like methemoglobinaemia and hemolytic anemia, potentially linked to a glucose-6-phosphate dehydrogenase deficit or other unidentified factors. Moreover, in combination with redox-active metal ions, dimin-
ished CAT activity may harm heme proteins, trigger cell death, and generate highly harmful hydroxyl radicals.\(^{30}\)

The SOD activity was significantly higher in diabetic patients, both with and without nephropathy, compared to healthy subjects. This indicates that the oxidative stress induced by high glucose levels could increase superoxide radical generation in diabetic individuals, a finding supported by existing literature.\(^ {31},^{32}\) SOD, a specialized enzyme, transforms the harmful superoxide radical (\(\text{O}_2^{•−}\)) into a less damaging compound, hydrogen peroxide (\(\text{H}_2\text{O}_2\)). Although \(\text{H}_2\text{O}_2\) is still harmful to biological tissues, SOD acts as the cell’s first defense line against the destructive effects of oxygen radicals. The widespread distribution of SOD in the body enables it to immediately neutralize superoxide radicals, thus protecting cells from oxidative damage.

For the GST’s activity, it was notably lower in diabetic patients, regardless of the presence of nephropathy, compared to the healthy group. GST, an endogenous antioxidant, might safeguard cells from oxidative stress. One possible reason for GST depletion is its role in conjugating with the glutathione molecule to neutralize ROS,\(^ {33}\) acting as a peroxidase.\(^ {34}\) Furthermore, since GSH is a substrate of the GST enzyme, a reduction in glutathione levels could lead to decreased GST activity.

Few studies have examined the development of PON1’s three activities (PON lactonase, PON arylesterase, and PON paraoxonase), which operate in states of oxidative stress utilizing the body’s antioxidant resources.\(^ {33},^{35}\) In diabetic patients, with or without nephropathy, PON1 activity was significantly lower than in the healthy group. This aligns with previous research showing reduced PON1 activity in renal failure patients, linking it to DN’s progression.\(^ {36},^{37}\) Further, PON1 is believed to play a crucial role in maintaining renal homeostasis, being a circulating hydrolase agent transported by HDL and involved in homocysteine metabolism.\(^ {38}\) Thus, disruptions in this enzyme activity can jeopardize renal function by affecting essential metabolic pathways for kidney homeostasis.\(^ {33}\)

This study found that MDA levels were significantly higher in diabetic patients, with or without nephropathy, than in the healthy group, indicating heightened oxidative stress in these individuals. This corroborates previous research linking lipid peroxidation and antioxidant activity in diabetics, and suggests increased oxidative stress in diabetic kidneys.\(^ {39}\) Peroxyl radicals can extract hydrogen atoms from lipids, propagating further hydroperoxides. ROS and the free radical pathway stimulate nucleic acid, protein, and lipid oxidation. Lipid peroxidation, a significant radical process affecting various unsaturated fatty acids, serves as an indicator of excessive oxidative stress and ensuing cytotoxicity. Albuminuria, a sign of DN, has been associated with substantially higher levels of lipid peroxides in renal proximal tubes, urine, and serum in numerous studies.\(^ {39}\)

This study didn’t find significant differences in antioxidant levels or melatonin concentration between males and females within the same group.

This study has limitations including small sample size, variability in patient characteristics, and potential confounding factors such as chronic conditions, medication usage,
and diet. These factors might have influenced the measurements, complicating the task of understanding DM impact on the variables. Nonetheless, the study contributes to our understanding of antioxidants and melatonin in diabetes, paving the way for further, more comprehensive research.

CONCLUSIONS

The current study found that patients with diabetes and nephropathy exhibited lower melatonin levels. The activities of antioxidant enzymes, including GPx, GST, CAT, and PON1, were diminished in diabetic patients, both with and without nephropathy, in comparison to the control group. Moreover, GPx and GST activities were found to be lower in diabetic patients with nephropathy than in those without. Both diabetic groups reveal increased SOD activity and MDA levels compared to the control group. Interestingly, the MDA level was higher in subjects without nephropathy compared to those with nephropathy.

LIST OF ABBREVIATIONS

Needed please....................

ACKNOWLEDGEMENTS

Add it, if any, please......................

DECLARATIONS

Authors’ contributions

All authors have equally contributed to the research, manuscript preparation, and data analysis. The authors have read, reviewed, and approved the final draft of the manuscript.

Conflict of interest

None.

Data availability

The data that support the findings of this study is available from the corresponding author, upon reasonable request.
Ethical approvals

The samples of this project were obtained from a previous project that was approved by Golestan University of Medical Sciences with registration number IR.GOUMS.REC.1401.158.

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