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Metabolic and Anti-inflammatory Response to Melatonin Administration in Patients with Diabetic Nephropathy

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Original Paper

Introduction. Data on the effects of melatonin administration on metabolic parameters in patients with diabetic nephropathy (DN) is limited and controversial. This study was performed to analyze the effects of melatonin administration on metabolic status in patients with DN.

Methods. This randomized, double blind, placebo-controlled clinical trial was performed on 60 patients with DN. Patients were randomly assigned into two groups to take either 10 mg/d of melatonin (n = 30) or placebo (n = 30) for 12 weeks. Fasting blood samples were taken at baseline and 12 weeks after intervention to quantify metabolic parameters.

Results. Melatonin administration significantly reduced plasma fasting glucose (β = -10.64 mg/dL; 95% CI: -20.37 to -0.90; $P < .05$), insulin (β = -2.37 μIU/mL, 95% CI: -3.33 to -1.41; $P < .001$), insulin resistance (β = -0.67, 95% CI: -0.98 to -0.35; $P < .001$), significantly increased insulin sensitivity (β = 0.01, 95% CI: 0.006 to 0.01; $P < .05$), and plasma HDL-cholesterol levels (β = 2.75 mg/dL, 95% CI: 0.75 to 4.75; $P < .05$) when compared with the placebo. Melatonin also caused a significant increase in total antioxidant capacity (TAC) (β = 140.45 mmol/L; 95% CI: 80.48 to 200.41; $P < .001$), and glutathione (GSH) levels (β = 50.36 μmol/L, 95% CI: 94.08 to 0.02; $P < .05$) when compared with placebo. Ultimately, melatonin could upregulate gene expression of peroxisome proliferator-activated receptor gamma (PPAR-γ) ($P < .05$) in comparison with placebo.

Conclusion. Results of this study indicated that melatonin administration for 12 weeks in DN patients had beneficial effects on glycemic control, HDL-cholesterol, TAC and GSH levels, and gene expression of PPAR-γ, but did not affect other metabolic parameters.

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INTRODUCTION

Diabetic nephropathy (DN) is one of the most important complications of diabetes mellitus. About 382 million people suffer from diabetes in the world 40% of whom are affected by DN.[1] Mortality has been reported to be higher in patients with DN, nearly twenty to forty times than patients without nephropathy.[1] DN ranges from microalbuminuria to progressive chronic kidney disease (CKD) and is currently considered as the main cause of end-stage renal disease (ESRD) in adults.[2] Several factors such as hyperglycemia, hypertension and genetic variations affect the pathogenesis of the disease.[3-5] In addition, an increase in oxidative damage and

inflammatory factors is directly influenced by chronic hyperglycemia.[6]

As a reaction to darkness, the pineal gland secretes melatonin. This biomarker plays a protective role against inflammation, oxidative stress and metabolic disorders. Clinical studies have reported positive effects of melatonin on blood pressure, insulin metabolism, lipoprotein profiles, biomarkers of oxidative stress and inflammatory markers in different groups of patients.[7-12] However, the results of some studies have suggested caution concerning the administration of melatonin.[13] A number of studies suggested beneficial effects of melatonin on glucose homeostasis, serum lipoproteins, markers of renal function and gene expression related to insulin and lipid metabolism in patients in early stages of DN. Celinski K *et al*.[14] showed that melatonin and tryptophan reduced pro-inflammatory cytokines and improved plasma triglycerides and LDL-cholesterol levels in non-alcoholic fatty liver patients with impaired fat metabolism accompanied by hypertriglyceridemia and hypercholesterolemia. In another study, Maldonado MD *et al*.[15] demonstrated that treatment with melatonin before strenuous exercise improved oxidative stress and lipid metabolism in football players. Kozirog M *et al*.[7] showed that melatonin administration for 2 months significantly improved antioxidative defense [i.e., an increase in catalase activity and a decrease in malondialdehyde (MDA) levels] and reduced LDL-cholesterol levels in patients with metabolic syndrome.

Considering the pathogenesis of DN which is associated with increased biomarkers of inflammation and oxidative stress and since there is evidence that melatonin has anti-inflammatory and antioxidant effects, we hypothesized that melatonin intake might help patients with diabetes and DN. To our knowledge, so far there was no study evaluating the effects of melatonin on metabolic profiles of patients in early stages of DN. Therefore, this trial was performed to analyze the effects of melatonin administration on glycemic control, serum lipoproteins, biomarkers of inflammation and oxidative stress in these patients.

MATERIALS AND METHODS

Trial Design and Participants

This randomized, double blind, placebo-controlled trial, registered in the Iranian registry of clinical trials (No: IRCT20150606022562N5) was performed by Kashan University of Medical Sciences (KAUMS) at the Internal Clinic in Kashan, Iran from December 2018 to March 2019. This study was performed on patients with DN, aged 40 to 85 years old, glomerular filtration rate 15 to 89 mL/minute/1.73m^2, moderate blood pressure (systolic: 140 to 160 mmHg and diastolic: 80 to 100 mmHg), no specific cardiovascular disease, cancer, inflammatory diseases, autoimmune, and hyper- or hypo-thyroidism, without urinary tract infection or other factors of proteinuria. We defined DN as renal disease in patients with diabetes who had proteinuria, with or without elevation of serum creatinine levels.[16] Exclusion criteria were included: special illness that leads to hospitalization, high blood pressure (systolic and diastolic pressure above 160 mmHg and 100 mmHg, respectively), unwillingness to cooperate, taking fluvoxamine and any antioxidant supplement, working at night shifts, smoking and alcohol consumption, breastfeeding and pregnancy. This trial was performed according to the principals of the Declaration of Helsinki and the ethics committee at KAUMS approved the study protocol. A written informed consent was obtained from all participants enrolled in the study.

Study Design

Participants were randomly allocated into two groups to intake either melatonin capsules (2 × 5 mg/d) (n = 30) or placebo (n = 30) one hour before bedtime for 12 weeks. Melatonin and placebo capsules were produced in the same shape and package by Zahravi Pharmaceutical Company (Tabriz, Iran) and Barij Essence Pharmaceutical Company (Kashan, Iran), respectively. However, melatonin and its placebo were provided by two different companies, both had similar packaging and patients and researcher were not aware of the content of the package until the end of study. Adherence to melatonin and placebo was determined by counting the tablet containers, which the patients had to return after the study. Moreover, participants received a daily reminder message on their cell phones to take their supplements. All participants completed 3-day dietary records at weeks 1, 7, and 12 of the trial. To calculate participants' nutrient intake, using these 3-day food records, we applied Nutritionist IV software (First Databank, San Bruno, CA) adopted for the Iranian food pattern.

Outcomes

Primary outcomes were homeostasis model of assessment-estimated insulin resistance (HOMA-IR) and insulin levels. Secondary outcomes were serum lipoproteins concentrations, and biomarkers of inflammation and oxidative stress. At baseline and end-of-trial, 15 mL of fasting blood samples were obtained from each patient at Kashan reference laboratory. Commercial kits were used to determine fasting plasma glucose (FPG) and serum lipoproteins concentrations (Pars Azmun, Tehran, Iran). All inter- and intra-assay coefficient variances (CVs) for FPG, serum lipoproteins, blood urea nitrogen and creatinine were less than 5%. Serum insulin levels were quantified by ELISA kit (Monobind, California, USA) with inter- and intra-assay CVs of below 6%. To determine the HOMA-IR and the quantitative insulin sensitivity check index (QUICKI) scores, suggested formulas were used.[17] Plasma total nitrite concentrations were measured using Griess method.[18] Plasma total antioxidant capacity (TAC) concentrations were measured using the method of ferric reduction antioxidant power developed by Benzie and Strain.[19] Total glutathione (GSH) and MDA levels were measured using Beutler's method [20] and thiobarbituric acid reactive substances by spectrophotometric test, respectively [21] with CVs less than 5%.

Isolation of Lymphocytes, RNA Extraction and cDNA Synthesis

Lymphocytes were isolated using 50% percoll solution (Sigma-Aldrich, Dorset, UK) gradient by centrifugation for 20 min and 3000 rpm at 4 °C.[22] Total RNA was extracted based on acid guanidinium-phenol-chloroform procedure using RNX™-plus reagent (Cinnacolon, Tehran, Iran) according to the manufacturer's instructions. RNAs was treated with DNAase I (Fermentas, Lithuania) for the elimination of any genomic DNA contamination.[22]

Real-time PCR Analysis

Appropriate primers for peroxisome proliferator-activated receptor gamma (PPAR-γ), low-density lipoprotein receptor (LDLR), interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), and glyceraldehyde-3 phosphate dehydrogenase-as an internal control-were designed (Table 1). Quantitative Real-time PCR was performed by the LightCycler® 96 sequence detection systems (Roche Diagnostics, Rotkreuz, Switzerland) using 4 µl of 5 × EVA GREEN I master mix (Salise Biodyne, Japan), 10 ng cDNA, 200 nM of each forward and reverse primers in final volume of 20 µl.[23,24] Relative transcription values were calculated by the Pffafi's method.[23,24] Reference gene in this method is often a housekeeping gene.

Sample Size Calculation

To calculate the sample size, we used a randomized clinical trial sample size calculation formula where type one (α) and type two errors (β) were 0.05 and 0.20 (power = 80%), respectively. According to the previously published trial,[25] we used 2.20 as the SD and 1.75 as the change in mean (d) of HOMA-IR score as the primary outcome. Therefore, based on the formula, we needed 25 participants in each group; after allowing for 20%

Table 1. Specific Primers Used for Real-time Quantitative PCR

Gene	Primer	Product Size (bp)	Annealing Temperature (°C)
GAPDH	F: AAGCTCATTTCCTGGTATGACAACG	126	61.3
	R: TCTTCCTCTTGTGCTCTTGCTGG		
PPAR-γ	F: ATGACAGACCTCAGACAGATTG	210	54
	R: AATGTTGGCAGTGGCTCAG		
LDLR	F: ACTTACGGACAGACAGACAG	223	57
	R: GGCCACACATCCCATGATTC		
IL-1	F: GCTTCTCTCTGGTCCTTGG	174	56
	R: AGGGCAGGGTAGAGAAGAG		
TNF-α	F: GTCAACCTCCTCTCTGCCAT R: CCAAAGTAGACCTGCCCAGA	188	52
TGF-β	F: TTGAGACTTTTCCGTTGCCG R: CGAGGTCTGGGGAAAAGTCT	227	56

Abbreviations: GAPDH, glyceraldehyde-3-Phosphate dehydrogenase; IL-1, interleukin-1; LDLR, low-density lipoprotein receptor; PPAR-γ, peroxisome proliferator-activated receptor gamma; TNF-α, tumor necrosis factor alpha; TGF-β, transforming growth factor beta.

dropouts in each group, the final sample size was considered to be 30 cases in each group.

Randomization

Computer-generated random numbers were used for randomization. The researchers and patients were not aware of randomization details until the final analyses were completed. Enrolment of participants, randomization, and allocating them to treatment or placebo groups were performed by trained staff at the dialysis clinic.

Statistical Methods

The Kolmogorov-Smirnov test was used to determine if the data was normally distributed. To determine the differences in anthropometric measures and dietary intakes between two groups, the independent-samples *t*-test was used. Multiple linear regression models were used to evaluate treatment impacts on study outcomes after adjusting for baseline values. The effect sizes were presented as the mean differences with 95% confidence intervals. Pearson chi-square test was applied for comparison of categorical variables. *P* values < .05 were considered as significant. SPSS version 18 was used for statistical analyses.

RESULTS

In the melatonin group, 8 patients and in the placebo group, 6 patients were excluded because of personal reasons (Figure 1). Finally, 46 participants [melatonin (n = 22) and placebo (n = 24)] completed the trial. Mean age, baseline and end-of-trial weight and BMI of study participants were not statistically different between two groups (Table 2).

Based on the 3-day dietary records obtained

Table 2. General Characteristics of Study Participants[1]

	Placebo Group (n = 24)	Melatonin Group (n = 22)	*P*[2]
Gender (%)			
Male	14 (58.3)	13 (59.1)	> .05†
Female	10 (41.7)	9 (40.9)	
Age, y	64.3 ± 7.7	66.9 ± 6.9	> .05
Height, cm	165.1 ± 7.1	164.4 ± 10.0	> .05
Weight at Study Baseline, kg	75.6 ± 11.3	77.4 ± 13.0	> .05
Weight at End-of-trial, kg	75.5 ± 11.5	77.4 ± 12.9	> .05
BMI at Study Baseline, kg/m^2	27.8 ± 4.2	28.8 ± 5.3	> .05
BMI at End-of-trial, kg/m^2	27.7 ± 4.2	28.7 ± 5.2	> .05

[1]Data are means ± SDs.
[2]Obtained from independent *t*-test.
†Obtained from pearson chi-square test.

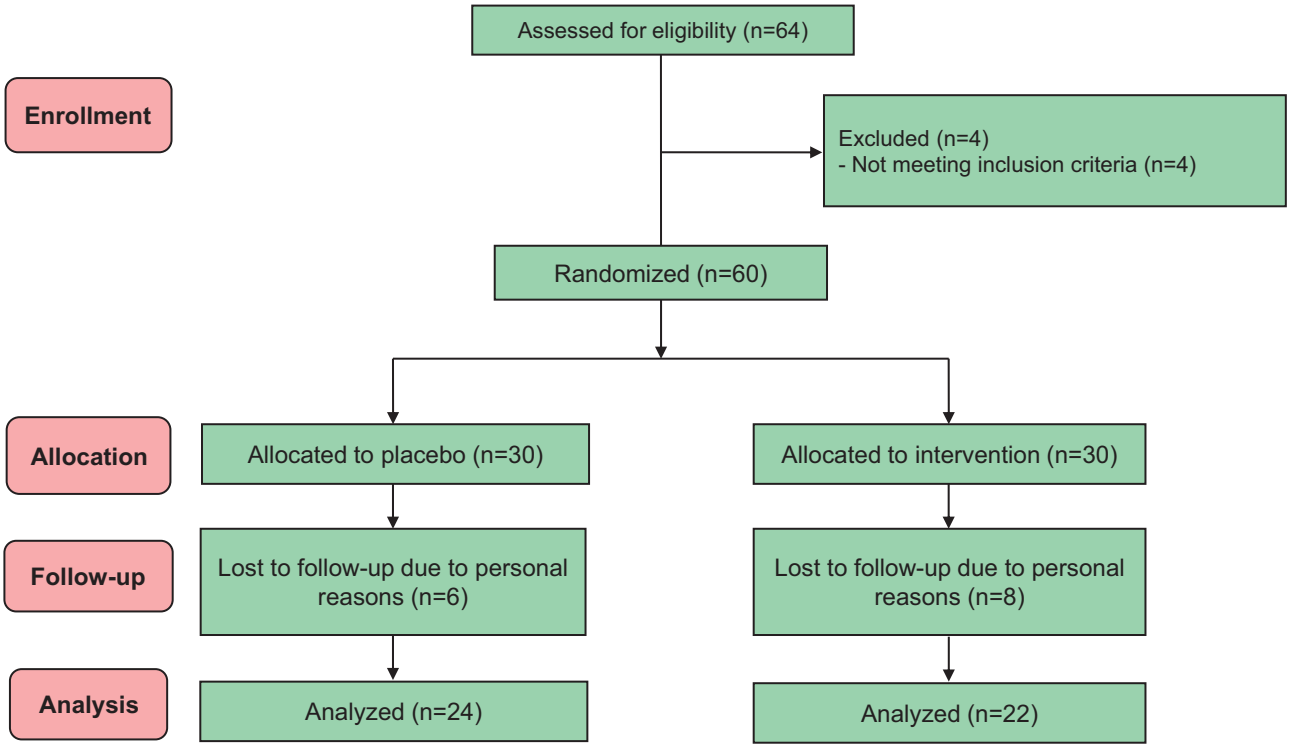

Figure 1. Summary of patient flow diagram.

during the trial, we found no significant difference in dietary macro- and micronutrient intakes (data not shown). Melatonin administration significantly reduced FPG (β = -10.64 mg/dL, 95% CI: -20.37 to -0.90; $P < 0.05$), insulin (β = -2.37 μIU/mL, 95% CI: -3.33 to -1.41; $P < .001$), HOMA-IR (β = -0.67, 95% CI: -0.98 to -0.35; $P < .001$), significantly increased QUICKI (β = 0.01, 95% CI: 0.006 to 0.01; $P < .05$), and HDL-cholesterol levels (β = 2.75 mg/dL, 95% CI: 0.75 to 4.75; $P < .05$) when compared with the placebo (Table 3). Melatonin intake also caused a significant increase in TAC (β = 140.45 mmol/L, 95% CI: 80.48 to 200.41; $P < .001$) and GSH levels (β = 50.36 μmol/L, 95% CI: 94.08 to 0.02; $P < .05$) in comparison with placebo. Melatonin intake did not affect other metabolic parameters.

Melatonin upregulated gene expression of PPAR-γ ($P < .05$) when compared with the placebo in peripheral blood mononuclear cells of patients with DN, but did not affect gene expression of LDLR, IL-1, TNF-α, and TGF-β (Figure 2 and 3).

DISCUSSION

We evaluated the potential beneficial effects of melatonin administration on glycemic control and markers of cardio-metabolic risk in patients with DN. We found that melatonin administration during 12 weeks had beneficial effects on glycemic control, HDL-cholesterol, TAC and GSH levels, and gene expression of PPAR-γ, but did not affect other metabolic parameters in DN patients.

Effects on Glycemic Control and Serum Lipoproteins

Clinical and experimental studies have shown a causal association between changed insulin signaling and the progress of kidney disease with metabolic and non-metabolic origin.[26] In addition to controlling glycaemia and renal function, the management of dyslipidemia and other cardiovascular disease risk factors is necessary.[27,28] For instance, DN is found to be associated with higher levels of plasma triglycerides and lower levels of HDL-cholesterol even among the patients with good control of LDL-cholesterol.[29] This study showed that melatonin administration during 12 weeks to patients with DN resulted in a significant reduction in FPG, insulin and HOMA-IR score, and a significant elevation in QUICKI, HDL-cholesterol levels and gene expression of PPAR-γ when compared with the placebo, but did not

Table 3. The Effect of Melatonin Administration on Metabolic Status in Patients with Diabetic Nephropathy

Variables	Placebo Group (n = 24)		Melatonin Group (n = 22)		Difference in Outcome Measures Between Melatonin and Placebo Treatment Groups[1]	
	Baseline	Week 12	Baseline	Week 12	β (95% CI)	*P*[2]
FPG, mg/dL	128.2 ± 30.7	127.1 ± 28.9	138.0 ± 26.1	124.1 ± 25.7	-10.64 (-20.37 to -0.90)	< .05
Insulin, μIU/mL	12.9 ± 3.6	13.4 ± 3.7	13.4 ± 3.5	11.5 ± 3.3	-2.37 (-3.33 to -1.41)	< .001
HOMA-IR	4.0 ± 1.4	4.1 ± 1.3	4.1 ± 1.4	3.5 ± 1.3	-0.67 (-0.98 to -0.35)	< .001
QUICKI	0.31 ± 0.01	0.31 ± 0.01	0.30 ± 0.01	0.32 ± 0.01	0.01 (0.006 to 0.01)	< .05
Triglycerides, mg/dL	179.1 ± 70.6	183.8 ± 68.4	184.9 ± 72.3	175.9 ± 73.3	-13.00 (-30.59 to 4.59)	> .05
VLDL-cholesterol, mg/dL	35.8 ± 14.1	36.7 ± 13.7	36.9 ± 14.4	35.2 ± 14.6	-2.60 (-6.11 to 0.91)	> .05
Total cholesterol, mg/dL	148.3 ± 30.8	147.7 ± 38.2	161.4 ± 38.5	157.0 ± 41.6	-3.81 (-16.14 to 8.52)	> .05
LDL-cholesterol, mg/dL	72.3 ± 29.6	70.7 ± 36.5	79.2 ± 27.6	74.2 ± 31.7	-3.14 (-15.26 to 8.97)	> .05
HDL-cholesterol, mg/dL	40.1 ± 5.7	40.3 ± 6.4	45.3 ± 9.0	47.6 ± 8.1	2.75 (0.75 to 4.75)	< .05
Total-/HDL-cholesterol ratio	3.7 ± 1.0	3.7 ± 1.1	3.6 ± 0.9	3.3 ± 0.8	-0.30 (-0.63 to 0.02)	> .05
Total nitrite, μmol/L	43.7 ± 6.3	43.7 ± 5.8	42.6 ± 4.4	41.9 ± 5.1	-0.91 (-3.15 to 1.33)	> .05
TAC, mmol/L	699.5 ± 141.3	703.8 ± 172.9	602.2 ± 165.6	774.0 ± 108.1	140.45 (80.48 to 200.41)	< .001
GSH, μmol/L	386.7 ± 85.9	372.4 ± 96.3	329.8 ± 38.1	376.4 ± 74.2	50.36 (6.65 to 94.08)	< .05
MDA, μmol/L	1.9 ± 0.4	2.0 ± 0.5	1.8 ± 0.3	1.9 ± 0.3	-0.05 (-0.23 to 0.13)	> .05
BUN, mg/dL	19.1 ± 5.2	21.1 ± 6.1	23.8 ± 9.1	25.4 ± 13.3	-1.15 (2.58, 4.90)	> .05
Creatinine, mg/dL	1.3 ± 0.4	1.3 ± 0.4	1.6 ± 0.7	1.4 ± 0.5	-0.10 (0.04, -0.24)	> .05

Data are mean ± SDs.
[1]"Outcome measures" refers to the change in values of measures of interest between baseline and week 12. β [difference in the mean outcomes measures between treatment groups (melatonin group = 1 and placebo group = 0)].
[2]Obtained from multiple regression model (adjusted for baseline values of each biochemical variables).
Abbreviations: BUN, blood urea nitrogen; FPG, fasting plasma glucose; GSH, total glutathione; HOMA-IR, homeostasis model of assessment-estimated insulin resistance; MDA, malondialdehyde; QUICKI, quantitative insulin sensitivity check index; TAC, total antioxidant capacity.

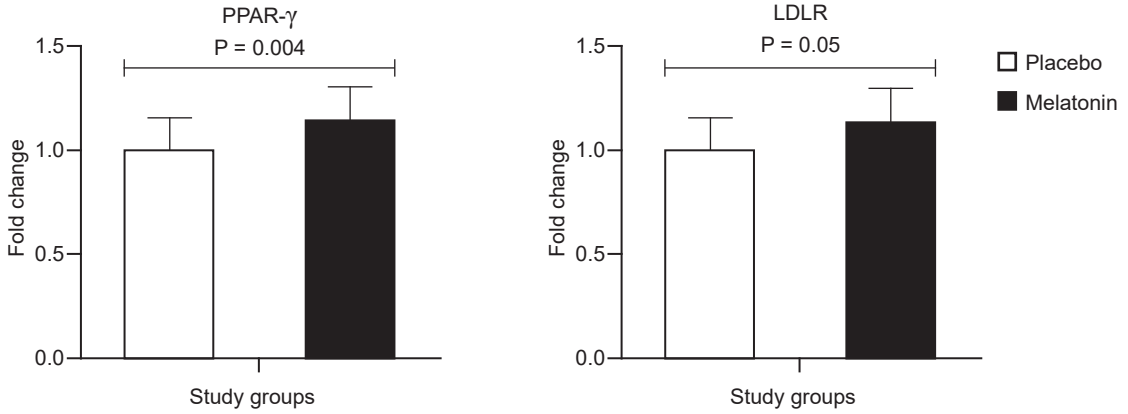

Figure 2. Effect of 12-week administration with melatonin or placebo on expression ratio of PPAR-γ and LDLR gene in PBMC of patients with DN (Abbreviations: LDLR, low-density lipoprotein receptor; DN, diabetic nephropathy; PPAR-γ, peroxisome proliferator-activated receptor gamma; PBMC, peripheral blood mononuclear cells).

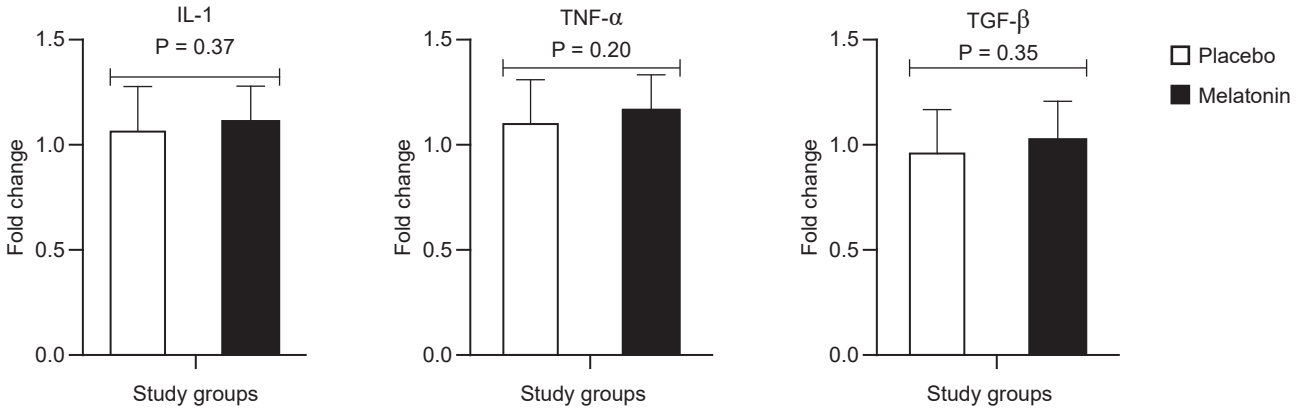

Figure 3. Effect of 12-week administration with melatonin or placebo on expression ratio of IL-1, TNF-α and TGF-β gene in PBMC of patients with DN (Abbreviations: IL-1, interleukin-1; DN, diabetic nephropathy; PBMC, peripheral blood mononuclear cells; TNF-α, tumor necrosis factor alpha; TGF-β, transforming growth factor beta).

influence other lipoproteins and LDLR expression. In a meta-analysis, melatonin administration significantly reduced FPG and increased QUICKI, but had no significant influence on insulin levels, HOMA-IR score or HbA1c levels.[30] We also showed that melatonin significantly reduced FPG, insulin concentrations, HOMA-IR score, and total cholesterol/HDL-cholesterol ratio, and increased QUICKI and serum HDL-cholesterol, but had no significant effect on other metabolic parameters in T2DM patients with coronary heart disease.[25] In a study by Garfinkel D *et al.*,[31] HbA1c levels were significantly decreased after 5 months of treatment with melatonin in diabetic patients with insomnia. In nonalcoholic steatohepatitis patients, HOMA-IR score was significantly reduced after treatment with melatonin.[10] However, in another study one-year treatment with melatonin had no significant effect on insulin or markers of glucose homeostasis.[32] In patients with schizophrenia melatonin improved hypertriglyceridemia, but did not change plasma cholesterol, FPG and insulin concentrations.[33] In a similar study, administration of melatonin along with olanzapine and lithium carbonate significantly inhibited the elevation of plasma cholesterol levels.[34] Diabetic kidney disease and cardiovascular disease (CVD) are associated with poor glycemic control and dyslipidemia.[35-38] It has been shown that melatonin has insulin-inhibiting effect via both receptors subtype MT1 and MT2 as well as the cGMP, cAMP and IP3 signaling pathways.[39, 40] Melatonin also stimulates glycogen synthesis and increases glucose uptake and/or insulin sensitivity to lower plasma glucose through a PKC/Akt/GSK3b dependent pathway.[41] On the other hand, melatonin enhances glucose transport to skeletal muscle cells by IRS-1/PI3-kinase pathway.[42]

Effects on Biomarkers of Inflammation and Oxidative Stress

We found that taking melatonin during 12 weeks by patients with DN was associated with a significant increase in GSH and TAC concentrations when compared with the placebo, but did not influence other markers of inflammation and oxidative stress, neither gene expression for IL-1, TNF-α and TGF-β. We have previously indicated that melatonin can decrease MDA and protein carbonyl in patients under methadone maintenance treatment.[43] Melatonin also increased SOD-1 activity and reduced MDA levels in patients with diabetes.[44] Melatonin intake improved antioxidative defense by increasing catalase activity and decreasing MDA levels in patients with metabolic syndrome.[7] Melatonin also reduced lipid peroxidation during exercise, and increased antioxidative enzyme activities.[45] In another study, patients with chronic obstructive pulmonary disease receiving melatonin showed a decrease in 8-isoprostane levels.[46] Melatonin intake increased glutathione peroxidase and decreased MDA levels in obese patients.[47] Melatonin may affect diabetes and associated metabolic disturbances not only by controlling insulin secretion, but also by providing protection against free radicals and reactive oxygen species.[40] Melatonin has multiple advantages over other antioxidants. It is an amphiphilic substance, which can pass through all biological membranes and stimulates directly antioxidant enzymes, while also scavenging free radicals. It also increases glutathione levels, which is an antioxidant by inducing gamma-glutamyl synthase activity. Melatonin is the ultimate antioxidant, unlike vitamin E or vitamin C that uses glutathione to revive its oxidative form.[48,49] Melatonin can modulate renal ischemia/reperfusion injury in diabetes via activating of sirtuin 1/ nuclear factor erythroid 2-related factor 2/heme oxygenase-1 signaling pathway.[50] Melatonin also improves mitochondrial function and impairs glomerular apoptosis which results in reversing diabetic renal fibrosis and maintenance of renal function by activating monophosphate-activated protein kinase/ proliferator-activated receptor γ coactivator 1-α pathway.[51]

LIMITATIONS

This study has some limitations. We did not assess plasma or salivary melatonin levels. Also, we were unable to determine the impact of melatonin administration on inflammatory factors such as IL-6 and IL-8. In the current study, sample size was small. Futher studies are needed with larger sample size to confirm our findings. In addition, we did not match participants according to the level of renal failure in the beginning of the study. This should be considered in the interpretation of our findings.

CONCLUSION

This study indicated that melatonin administration for 12 weeks in DN patients had beneficial effects on glycemic control, HDL-cholesterol, TAC and GSH levels, and gene expression of PPAR-γ, but did not have any effect on other metabolic parameters.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

MS, FB and ZA contributed in conception, data collection and manuscript drafting. ZR, AS, EA and NK contributed in conception, data collection and manuscript drafting. All authors read and approved the final version of the paper.

CLINICAL REGISTRATION

http://www.irct.ir: IRCT20150606022562N5.

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