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The effect of oral melatonin on renal ischemia–reperfusion injury in transplant patients: A double-blind, randomized controlled trial

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ABSTRACT

Background: One of the important factors in the occurrence of acute kidney injury (AKI) among renal transplant patients (RTPs) is ischemia reperfusion injury (IRI). The current study aimed at determining the anti-inflammatory and anti-oxidative effects of melatonin on the complications of IRI and the level of Klotho expression in these patients.

Methods: A total of 40 renal transplant candidates were randomly assigned into placebo or melatonin group receiving the same dose of 3 mg/day. In order to measure serum melatonin levels, inflammatory and oxidative stress factors, renal function biomarkers, and Klotho gene/protein expression, venous blood samples were taken from patients over two different time points, i e, 24 h before the transplantation and at discharge from hospital.

Results: Melatonin was associated with improvement in renal transplantation, since the serum level of neutrophil gelatinase-associated lipocalin, as a renal functional marker, significantly decreased ($P < .001$). The effect of melatonin as a suppressor of inflammation and oxidative stress was also evident in the melatonin group due to a significant reduction in the serum levels of MDA, CP, 8-OHdG, and TNF-α markers ($P < .001$).

Conclusions: Reduction in serum levels of renal function and oxidative stress/inflammatory markers in the melatonin group indicates that melatonin can inhibit IRI outcomes in RTPs through its anti-oxidant and anti-inflammatory properties. However, these properties do not appear as a result of influence on the level of Klotho gene/protein expression.

1. Introduction

Ischemia reperfusion injury (IRI), which is a main cause of acute kidney injury (AKI), represents a major clinical problem that aggravates morbidity and mortality in renal transplant patients [1]. When an organ is retrieved from the body of the donor, ischemia occurs. On the other hand, reperfusion injury is created when the vascular anastomoses are completed and organ is revascularized. There are two main factors in the incidence of ischemia; the severity of tissue destruction and the duration and extent of occluded arterial blood flow. Hypoxia, inflammatory cytokine, and free radical injury are the underlying causes of renal IRI. Increase in reactive oxygen and inflammation involved in this process result in cell death; i e, necrosis and apoptosis in AKI [2,3]. It was shown that NF-kB (p65/p50 heterodimeric form) is a critical inflammatory and apoptosis regulator. When IRI occurs, infiltrating leukocytes and tubular epithelial cells are the main sources of chemokines and inflammatory cytokines such as interleukin-1 beta (IL1-β) and tumor necrosis factor alpha (TNF-α). Therefore, decrease in production, distribution of inflammatory cytokines, free radicals, and oxidative damage may be therapeutic targets [4]. Melatonin (*N*-acetyl-5-methoxytryptamine) and its metabolites are receptor-independent intracellular free-radical scavengers with potent antioxidant capacity and

Abbreviations: AKI, acute kidney injury; RTPs, renal transplant patients; IRI, ischemia reperfusion injury; BUN/Cr ratio, blood urea nitrogen /creatinine; NGAL, neutrophil gelatinase-associated lipocalin; MDA, malondialdehyde; CP, Carbonyl protein; TNF-α, Tumor necrosis factor alpha.

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anti-inflammatory properties widely used to tackle the IRI pharmacologically. There are mechanisms, which directly neutralize free radical species and numerous anti-oxidative enzymatic stimulators, which stabilize cell membranes [5]. Klotho (Kl) is a known anti-aging gene; although Kl gene is expressed in different tissue, it is primarily expressed in healthy kidneys. Klotho gene is a single-pass transmembrane protein. It also has a soluble secretion form (S-KL) that can be derived thorough alternative splicing or cleavage from membrane Kl. S-Kl exerts multiple actions including anti-oxidation, anti-inflammation, anti-apoptosis, and anti-insulin [6]. According to accumulated data, renal Klotho protein and mRNA decreased in rats a day after ischemia reperfusion. In addition, Hu et al., demonstrated that although the rat Klotho decreased in the plasma and urine, it was detectable as early as 3 h after injury. During transplantation procedure, decreased Klotho expression, increased inflammatory cytokines as well as oxidative stress factors lead to kidney failure. In fact, acute renal failure is the main cause of kidney transplant rejection [7–9]. Relevant research demonstrate that melatonin plays a remedial role in renal IRI [2]. There were a few studies on the effects of melatonin using ex vivo renal transplant models. As a result, the current study aimed at evaluating the anti-inflammatory and antioxidant effects of melatonin on IRI complications. Furthermore, its effect on Klotho protein expression is examined in order to reduce the incidence of acute renal failure among renal transplant patients (RTPs).

2. Objective

In the present study we evaluated and compared the effects of oral melatonin on inflammatory and oxidative stress factors, renal function biomarkers, and Klotho gene/protein expression in kidney transplant patients.

3. Materials and methods

3.1. Subject recruitment

The current two-center, double-blind, randomized clinical trial (RCT) evaluated the effect of oral melatonin (3 mg, 99.5% pure synthetic melatonin, Wellness Resources Inc., US) on IRI and Klotho expression in Shahid-Modarres Hospital (Tehran, Iran) and Sheykh al-Reis Clinic (Tabriz, Iran). Patients were recruited from January 2017 to August 2018. The study protocol was approved by the Ethics Committee of Tabriz University of Medical Sciences (TBZMED) (ethical code: IR.TBZMED.REC 1395.756); the trial was also registered in the Iranian Registry of Clinical Trials (http://www.irct.ir/, IRCT: IRCT201610203812N5).

3.2. Inclusion and exclusion criteria

To find eligible cases, the researchers evaluated patients over 18 years old that underwent kidney transplantation from a cadaveric donor. Exclusion criteria were patients with diabetes, inflammatory disease, chronic or acute infection, autoimmune disease, chronic liver disease, high/low blood pressure, and history of depression, seizure, and bleeding. All the subjects were assessed by a nephrologist in terms of the abovementioned diseases. In addition, clinical examinations were carried out if necessary. All patients in both groups have filled and signed the consent forms, that are now kept in Tabriz University of Medical Sciences' archive.

3.3. Study power

In the current study, the sample size was 40 using Sample Size Calculation (PS) software, version 3.1.2. Subjects were recruited during 20 months. The patients were enrolled in the study as soon as they signed the written informed consent form. To investigate any interference between melatonin and immunosuppressive agents; i e, Imuran, mycophenolic acid, prednisolone, tacrolimus, and cyclosporine, the blood concentration of patients in terms of taken agents was monitored daily.

3.4. Randomization, intervention, and blood sampling

Patients entered into the study were randomly divided into the intervention and control groups. In other words, 20 subjects were randomly assigned to the control group (IR + placebo), and the rest were considered as the intervention group (IR + Melatonin). In the current study, the subjects were matched by age, gender, and body mass index. First, 24 h before the transplantation, venous blood samples were taken from the patients (first sampling). In addition, 24 h before transplantation, the intervention group received a 3 mg/day oral melatonin capsule, while the control group received placebo in the same shape and color until discharge from hospital. At the time of discharge, a second blood sample was taken from patients. The average days that patients received melatonin were 25 days that was given them during the day. All of the studied factors were separately measured and evaluated at the two time points. Blood samples were stored in three forms of whole blood, serum, and plasma at −70 °C until analysis (Fig. 1).

3.5. Biochemical parameters

By applying an automated chemical analyzer, serum creatinine (Cr) and urea levels were measured by enzymatic colorimetric methods (Abbott analyzer, Abbott Laboratories, Abbott Park, North Chicago, IL). Blood samples kept in EDTA (ethylenediaminetetraacetic acid) containers were analyzed by an automatic cell counter (COULTER LH 750 Hematology Analyzer) in order to measure the components of complete blood count including red blood cells (RBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH).

3.6. Enzyme-linked immunosorbent assay

Plasma melatonin, TNF-α, and neutrophil gelatinase-associated lipocalin (NGAL) levels were measured before transplantation and at the time of discharge using the enzyme-linked immunosorbent assay (ELISA) kit (Bioassay Technology Laboratory, BT Lab, China). The ELISA kit was also used to measure 8-hydroxyl-deoxyguanosine (8-OHdG) as a marker of DNA damage.

3.7. Chemical analyses

According to the protocol described by Lapenna et al., malondialdehyde (MDA) level, a lipid peroxidation marker, was measured spectrophotometrically using thiobarbituric acid-reactive substances (TBARS) [10].

Protein carbonyl is another marker of oxidative stress. According to the protocol of Levine et al., it was determined by the DNPH (2,4-dinitrophenylhydrazine) method [11].

3.8. Measurement of Klotho mRNA expression

Using TRIzol reagent, total RNA was extracted from whole blood cells (Ribo Ex Ls, Gene All, Songpa-gu, Korea). According to the manufacturer's instructions, complementary DNA (cDNA) strands were synthesized using DNA Synthesis kit (Hyperscript TM Strand Synthesis kit, Gene All, Songpa-gu, Korea). In fact, supplier recommended performing quantitative PCR by a Mic thermocycler (BioMolecular Systems, Upper Coomera, Australia). Specific primers were designed for Klotho gene (Forward: 5′TCCAGCCCCAGATCGCTTTAC3′; Reverse 5′ AGGGAGAATCAGGGCCCAGTC3′) and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Forward: 5′GTCGGTGTGAACGGATTTG3′:

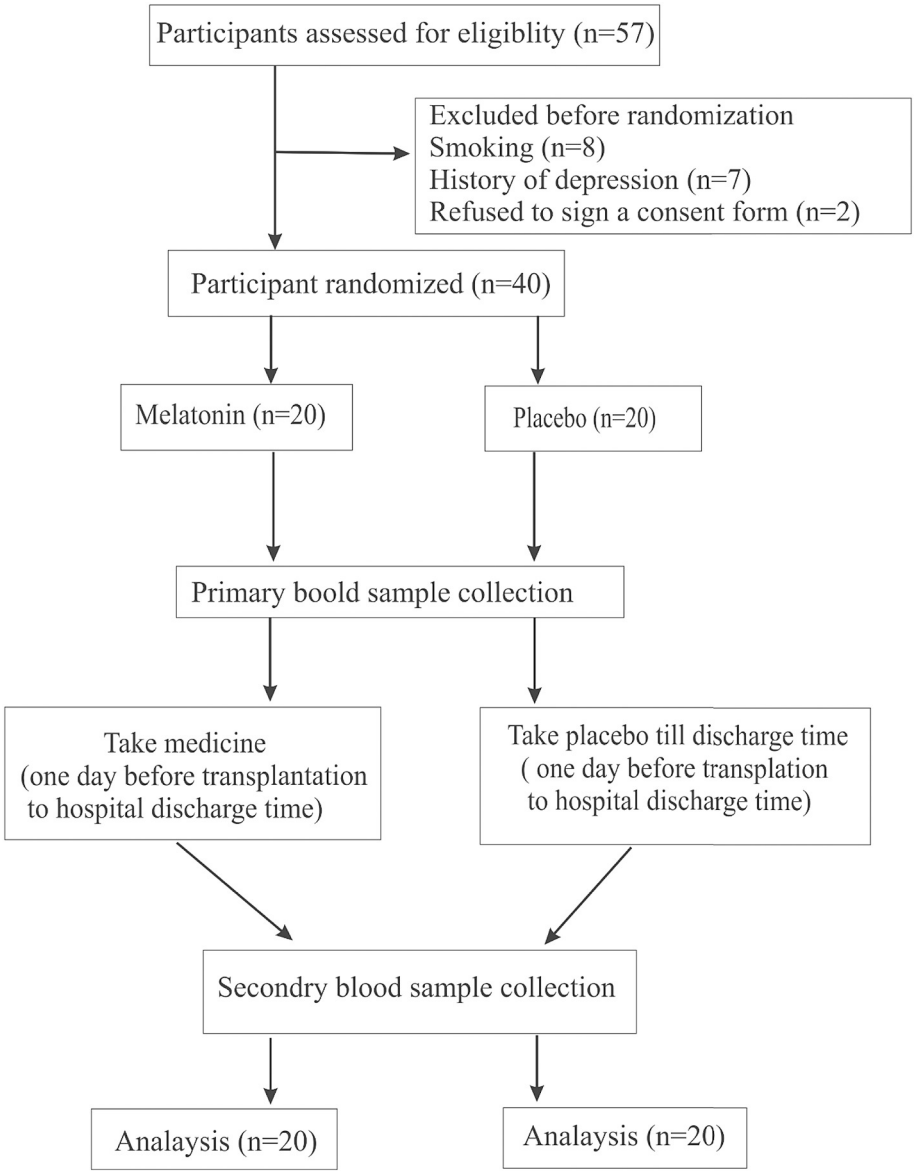

Fig. 1. Flow chart of patients in the trial.

Reverse5′TCCCATTCTCAGCCTTGAC3′) as internal gene.

3.9. Klotho Western blotting analysis

When RNA was extracted from the blood sample using TRIzol, DNA was separated. Afterwards, the protein was extracted through the following procedure: the ethanol was added to the organic phase and turned into a well-formed vortex. After the incubation and centrifugation step, the supernatant containing the phenol-ethanol phase and the protein (the deposition contains DNA) was collected. The protein was retrieved from supernatant within six consecutive steps based on the protocol provided in the catalog; TRIZOL®LS Reagent (Total RNA Isolation Reagent for Liquid Samples, GIBCO BRL, USA). The sample was ready for blotting at the end of these steps. It was possible that protein samples on PVDF (polyvinylidene difluoride) membranes (50 μg) were affected by immunoblotting and SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis; 10% gel). Klotho was examined by Western blotting using primary anti-Klotho (Santa Cruz; sc-22,220). Then, all of the membranes were produced by secondary horseradish-peroxidase-conjugated antibodies and the blots were portrayed by using the Western Blotting Luminol Reagent (Santa Cruz). ImageJ version 1.41, an image analysis software, was employed to quantify the bands on X-ray films. The density of the GAPDH bands homogenized each protein band (Santa Cruz; sc-32,233). As the fold changes the values obtained from the normal control group, the relative intensities were described.

3.10. Statistical analysis

SPSS version 21 was used for statistical analysis and results were expressed as mean ± standard deviation (SD). Initially, the distribution of continuous variables was determined using the Kolmogorov-Smirnov test. Then, Wilcoxon sign test was applied so as to examine the significance of intra-group changes (before and after). On the other hand, an independent-sample *t*-test (the Mann-Whitney U) was used to compare the results achieved from both groups. By applying the Pearson test, correlations were evaluated (based on Δ changes) and the statistical significance level was < 0.05.

Table 1
Baseline characteristics of the study population.

	Melatonin group (n = 20)	Placebo group (n = 20)	P value
Age (years)	39.25 ± 7.45	36.85 ± 8.54	0.35*
Gender, Male/female	15/5	14/6	0.31†
Weight (kg)	39.25 ± 7.46	65.55 ± 11.37	0.96*
SBP (mmHg)	136.15 ± 16.73	142.200 ± 18.66	0.21*
DBP (mmHg)	83.60 ± 6.96	87.20 ± 10.06	0.22*
BUN/Cr ratio	35.79 ± 6.12	33.81 ± 6.54	0.253*
Hemoglobin (g/dL)	9.54 ± 2.21	10.09 ± 1.96	0.56*
RBC ($\times 10^6$/mm^3)	3.95 ± 2.92	7.19 ± 2.92	0.001*
MCV (fl)	81.25 ± 16.51	84.73 ± 5.07	0.90*
MCH (Pg)	26.46 ± 3.43	26.89 ± 3.04	0.738*
MCHC (g/dl)	30.27 ± 6.24	31.80 ± 1.64	0.46*

Variables are presented as Mean ± SD, SBP: systolic blood pressure; DBP: diastolic blood pressure; BUN/Cr: blood urea nitrogen /creatinine; RBC: Red blood cell count; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration.
* Performed using independent-sample t-test.
† Performed using chi-square test.

4. Results

4.1. Patients' characteristics

According to inclusion and exclusion criteria, 57 renal transplant candidates from cadaver were screened in order to be included in the study; however, 40 individuals were finally studied (Fig. 1). These patients were randomly assigned either to the melatonin or placebo group. Before the initiation of the intervention, the general characteristics of the patients in the placebo and melatonin groups were investigated (Table 1). There was no significant difference between the groups in terms of baseline characteristics such as age, weight, gender, hypertension, and BUN/Cr ratio. Furthermore, there were no significant differences in MCV, MCH, MCHC, and HB levels except for RBC level, between the groups (Table 1). All of the 40 patients took the allocated trial medicine. Patients continued to receive trial medication until their discharge from hospital.

4.2. Melatonin levels

In order to determine the relationship between melatonin prescription and its effect on the studied parameters, melatonin serum levels were measured both in placebo and melatonin groups. There was no significant difference in the melatonin serum concentration (before and after) in the placebo group (P = .06), but a significant difference was observed in the serum melatonin concentration (before and after) in the intervention group (P = .001). Also, there was a significant difference in serum level of melatonin between both groups (P = .001) (Table 2).

4.3. Melatonin improves renal function

BUN and Cr serum concentrations were measured to evaluate the function of transplanted kidney reported as BUN/Cr ratio. This index showed a significant difference in the placebo and melatonin groups during, before, and after interventions (P = .001). Nevertheless, this difference was more significant among melatonin recipients. Furthermore, BUN/Cr ratio was significantly different between the two groups (P = .06). NGAL parameter measurement was done to evaluate the IRI and transplanted kidney function. Unlike the placebo group (P = .42), there was a significant difference in the level of NGAL serum among melatonin group subjects (P = .001) before and after treatment. Moreover, there was a significant difference between the melatonin and the placebo groups in terms of NGAL serum concentration (P = .06). The other parameters measured in both groups were four- and 24-hour urine output immediately after the transplantation and the 24-hour urine output at discharge from hospital in order to evaluate the function of transplanted kidney. The obtained results showed no significant difference in the two groups in terms of the four-hour (P = .26) and 24-hour (P = .72) urine output after the transplantation and the output of 24-hour urine at discharge time (P = .65) (Table 3).

4.4. Melatonin alleviates renal inflammation and oxidative stress

The results of the current study showed that the prescriptive intervention was associated with a decrease in the serum level of oxidative stress markers and TNF-α. In other words, the serum level of oxidative stress markers; i e, MDA, CP, and 8-OHdG (as lipid peroxidation, protein oxidation, and DNA damage markers, respectively) significantly decreased in the melatonin group in comparison with the placebo group. Such reduction was also true for TNF-α (Table 4).

4.5. Melatonin and Klotho expression

Data analysis results of RT-qPCR and Western blot showed no significant difference between the levels of Klotho expression in the melatonin and placebo groups (P = .52) and no difference in Klotho protein expression (P = .36) (Fig. 2). In other words, prescriptive melatonin can impose its effects independent of antioxidant and anti-inflammatory role of Klotho. In order to determine the effects of prescribed melatonin on variables, the correlation between degree of melatonin changes (Δ melatonin) and the degree of variable changes (Δ variables) is shown in Table 5. The results showed a significant and negative correlation between melatonin (Δ Melatonin) and Δ BUN/Cr ratio, ΔTNF-α, ΔMDA, ΔCP, ΔNGAL and Δ8-OHdG. Besides, the relationship between other variables is also reported in Table 5.

5. Discussion

The current study, conducted for the first time in the human kidney transplantation model, hypothesized that prescribing melatonin can reduce the complications of IRI due to its renoprotective effects. The

Table 2
Serum level of melatonin in Placebo and melatonin groups presented as ng/L.

Melatonin	Before	After	Differences in results before & after treatment
Placebo (3 mg/day)	5.18 ± 0.21	5.13 ± 0.02#	−0.04 ± 0.21
Melatonin(3 mg/day)	5.08 ± 0.04	5.71 ± 0.12 *	0.63 ± 12
P value	0.56†	0.001††	0.001**

Values are presented as mean ± SD.
P > .05 before treatment versus after treatment with placebo.
* p < .001 before treatment versus after treatment with melatonin.
** P < .001 Comparison of the differences (before and after treatment) between Placebo and Melatonin groups (independent samples t-tests).
† Comparison of between two before values (placebo and melatonin groups).
†† Two after values (placebo and melatonin groups).

Table 3
Comparison of acute kidney injury biomarkers in the Placebo and Melatonin Group.

Variables	Before	After	Differences in results before & after treatment
BUN/Cr ratio Placebo	30.17 ± 4.81	16.09 ± 3.14	−17.46 ± 5.81
BUN/Cr ratio Melatonin	32.04 ± 5.18	14.57 ± 3.23	14.07 ± 5.64
P value	0.24*	0.13**	0.71#
NGAL (ng/l) Placebo	6.68 ± 0.83	6.72 ± 0.72	0.03 ± 0.39
NGAL (ng/l) Melatonin	6.56 ± 0.75	5.52 ± 0.43	−1.04 ± 0.80
p-value	0.51*	0.001**	0.001#
4-h UV (ml) Placebo			2276 ± 876.70
4-h UV (ml) Melatonin			1784.50 ± 484.40
P value			0.102†
24-h UV (ml) Placebo			5170 ± 2340.11
24-h UV (ml) Melatonin			4700 ± 1219.25
P value			0.73†
24-h UV (ml) Placebo			3050 ± 1459.00
24-h UV (ml) Melatonin			4135 ± 646.67
P value			0.02†

Values are presented as mean ± SD.
NGAl: neutrophil gelatinase-associated lipocalin;4-h UV: urine volume;24-h UV: urine volume.
A Comparison of the differences (before and after treatment) between Placebo and Melatonin groups.
† Comparison of urine output (4 h, 24 h after transplantation and hospital discharge time respectively) between placebo and melatonin groups.
* A comparison of between two before values (placebo and melatonin groups).
** Two after values (placebo and melatonin groups).

Table 4
Comparisons of serum MDA, 8-OHdG, CP and TNF-α Levels in the study groups.

Variables	Before	After	Differences in results before & after treatment
MDA (nmol/ml) Placebo	2.24 ± 0.48	2.31 ± 0.50	0.06 ± 0.15
MDA (nmol/ml) Melatonin	2.03 ± 0.57	1.27 ± 0.46	−0.75 ± 0.73
P value	0.19*	0.001**	0.001#
CP (nmol/ml) Placebo	194.87 ± 11.18	193.92 ± 11.62	−0.95 ± 17.99
CP (nmol/ml) Melatonin	190.82 ± 25.60	139.01 ± 19.37	−51.81 ± 32.42
P value	0.77*	0.001**	0.001#
8-OHdG (ng/mL) Placebo	1.88 ± 0.56	1.84 ± 0.55	−0.03 ± 0.28
8-OHdG(ng/mL) Melatonin	1.78 ± 0.47	1.00 ± 0.41	−0.77 ± 0.61
P value	0.96*	0.001**	0.001#
TNF-α (ng/L) Placebo	3.79 ± 0.13	3.77 ± 0.25	−0.02 ± 0.29
TNF-α (ng/L) Melatonin	3.80 ± 0.11	3.08 ± 0.18	−0.71 ± 0.22
P value	0.96*	0.001**	0.001#

Values are presented as mean ± SD. 8-OHdG: 8-hydroxydeoxyquanosine; MDA: malondialdehyde, CP: Carbonyl protein, TNF-α: Tumor necrosis factor alpha.
A Comparison of the differences (before and after treatment) between Placebo and Melatonin groups.
* Comparison of between two before values (placebo and melatonin groups).
** Two after values (placebo and melatonin groups).

results of the current study findings showed that prescribing 3 mg/day melatonin to RTP from 24 h before the transplant until the time of discharge from hospital led to a decrease in the serum level of TNF-α, and also reduced the lipids peroxidation, proteins, and nucleic acids oxidation. Therefore, the concentration of related markers such as MDA, CP, and 8-OHdG also showed a decrease in serum level. In addition, it was observed that renal function markers- i e, BUN, Cr, especially NGAL- significantly reduced in the melatonin group compared to the placebo group. However, there was no significant difference between the two groups regarding the volume of urine four and 24 h after transplantation and 24-hour urine volume at the time of discharge. The importance of the NGAL diagnostic biomarker was used to replace it as a serum creatinine level to show IRI-induced kidney damage. The reason was that serum and urinary levels of NGAL immediately increased 2 h after tissue ischemia. However, the serum creatinine levels increased 48–72 h after IRI. Although evidence suggests a direct relationship between serum and urinary levels of NGAL, the increase in urine volume has less specificity than serum levels [12–14]. The results of the study by Tesipanlis also showed an inverse relationship between serum OX-LDL (oxidized low-density lipoprotein) levels and telomerase activity in patients underground hemodialysis in comparison with the control subjects. Reducing telomerase activity acts as a signal for apoptosis induction; therefore, shortening the telomere length is a serious problem for organ rehabilitation and recovery after IRI [15]. Overall, results of the present study showed that melatonin had a protective effect on IR-induced renal injury. This means that renal dysfunction indicating AKI is reversible through melatonin prescription. IRI is known to be a factor that worsens the outcome of the transplant. AKI is due to clinical and experimental IR syndrome, which can be characterized by severe drop in glomerular filtration rate, glomerular injury, extensive tubular damage, and tubular cell necrosis. Based on evidence, it is assumed that most of these glomerular and tubular defects occur during the reperfusion period, after the ischemic stage, and the production of free oxygen radicals. Free oxygen radicals are a potential contributor to reperfusion injury. Oxygen, the only absent substrate during ischemia is introduced into system with a high concentration in the reperfusion stage results in tissue damage through the production of free oxygen radicals. Clinical and experimental studies show that preventing or reducing the complications of IRI can lead to improved renal transplant function. Although there is no definitive treatment to prevent complications from IRI yet, some interventions are proposed to reduce the problems caused by this injury. In line with these goals, the attention of researchers is also focused on melatonin. Melatonin, the major secretion product of the pineal gland, is a direct

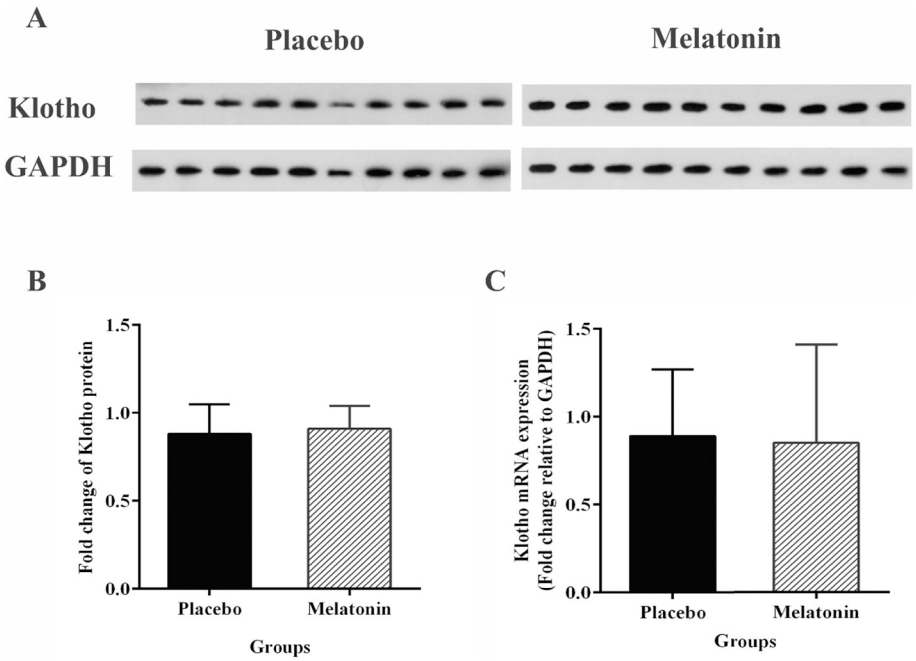

Fig. 2. Effect of melatonin and placebo on klotho protein level (A, B), and Klotho gene expression(C). Protein levels were semi-quantitatively assayed by western blotting. Gene expressions were assessed by RT-qPCR.

Table 5
Correlations between the changes in BUN/Cr ratio, NGAL, MDA,8 -OHdG, TNF-α and CP after melatonin or placebo treatment.

Variables	ΔBUN/Cr ratio	Δ NGAL	Δ MDA	Δ CP	Δ 8-OHdG	Δ TNF-α
ΔMelatonin						
r	−0.401	−0.657	−0.531	−0.653	−0.481	−0.648
P value	0.010	0.001	0.001	0.001	0.002	0.001
Δ BUN/Cr						
r	–	0.319	-	0.283	0.300	0.327
p-value	–	0.045	-	0.077	0.060	0.039
Δ NGAL						
r	–	–	–	0.598	0.527	0.497
P value	–	–	–	0.001	0.001	0.001
Δ MDA						
r	–	–	–	0.508	0.347	0.444
P-value	–	–	–	0.001	0.028	0.004
Δ CP						
r	–	0.598	0.508	–	0.693	0.465
P value	–	0.001	0.001	–	0.001	0.003
Δ 8-OHdG						
r	–	–	0.347	0.693	–	0.379
P value	–	–	0.028	0.001	–	0.016

BUN/Cr ratio: blood urea nitrogen /creatinine, NGAL: neutrophil gelatinase-associated lipocalin. MDA: malondialdehyde, CP: Carbonyl protein, TNF-α: Tumor necrosis factor alpha. Δvariable = variable (after melatonin or placebo treatment) minus Variable (baseline).

scavenger of free radicals and an indirect antioxidant. When describing the role of melatonin as a scavenger, it should be noted that it can neutralize OH, superoxide anion radical, singlet oxygen, peroxyl radical, and $OONO^{\pm}$ [16]. The finding of Dun-Xian Tan's study showed that melatonin has potent hydroxyl radicals scavenging ability compared to other well-known free radical scavenger such as glutathione and mannitol, however, the doses of melatonin used was much larger than its physiologic range. This capability depends on the unique chemical structure of melatonin, i.e. Highly lipophilic, resonance-stabilized and methoxylated indoleamine properties. The methyl group located on 5-OH position of the indole group is necessary for its scavenging function because 5-Hydroxytryptamin(5-HT), lacking the methyl group in its indole ring, didn't show this function [17]. The study by Sergio Rodríguez-Reynoso showed that melatonin improved IRI. They reported that melatonin reduced the damage to the organ through controlling the oxidative response in the primary and the end reperfusion during the process; in other words, melatonin antioxidant function is probably due to its stimulating effect on superoxide dismutase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase and its inhibitory effect on nitric oxide synthase [18]. The results of the study by Aktoz indicated that melatonin had a protective effect on reperfusion-induced ischemic injury. They reported that MDA serum level significantly reduced in rats affected by IRI after being treated by melatonin. Furthermore, catalase and glutathione peroxidase serum levels also increased with this treatment. Also, melatonin helped to strengthen cell membranes against oxidation attacks through stabilizing them [2]. The results of the study by Sener G showed that rat treatment with melatonin resulted in a decrease in serum levels of MDA and TNF-α, while there was an increase in serum level of anti-apoptotic bcl-2 factor after IRI. It is also reported that the biochemical parameters of kidney- i e, BUN and Cr- significantly reduced in treated rats compared with control rats [19]. It should be mentioned that melatonin has regulatory effects on organ reperfusion injury, such as antithrombotic effect, anti-inflammatory action and microvascular regulatory property, it induces these effects via different mechanisms. Hao Zhou and colleges showed melatonin has protective role in cardiac I/R via suppression of palette hyperactivity through restoration platelets' PPARγ content [20]. Zhewei Zhao study demonstrated melatonin has neuroprotective effect on brain IRI in rats and humans which is induced via reducing NF-κB, S100β and stimulating Nrf2 and antioxidant enzymes [21] . Also, Amanda Lochner has been documented cardioprotective effects of melatonin which is mediated via its various receptors [22]. The current study focused on Klotho protein for the first time to clarify mechanism of the melatonin action as an anti-inflammatory and antioxidant. The current study hypothesized that melatonin can induce its effects by increasing the expression of the Klotho protein (as a key messenger). Klotho is a transmembrane protein expressed extensively in the surface of the kidney tubular epithelial cells. This protein is cleaved by a disintegrin and metalloproteinase (ADAM) 10/17 and released into the bloodstream [23].The study by Zhong Zuo showed Klotho's role as an

anti-aging factor by reporting increased production of superoxide and the reduction of Klotho serum levels in aged rats compared with young ones [24]. Rakugi H., also confirmed that Klotho is an aging process regulator, which increases the level of superoxide dismutase enzyme [25]. The study of Yanhua Zhao showed that Klotho's serum level reduction was associated with the increase in serum levels of inflammatory and oxidative factors. It also showed that the serum levels of these factors significantly reduced when Klotho was added [26]. According to the studies by Gastellano serum levels of Klotho significantly reduced 24 h after IRI. An inverse relationship was also observed between Klotho serum levels and delay in the function of renal transplantation in the case of transplant patients. In fact, the soluble variant of this protein decreased during IRI. Therefore, it can be said that Klotho leads to the reduction of oxidative stress caused by the IR when the expression of the antioxidant enzyme is increased [26].The results of the current study revealed no significant difference in the amount of Klotho protein expression between the control and intervention groups. It is believed that lack of changes in the amount of the Klotho protein expression can be due to the low dose of prescribed melatonin or the number of days it is prescribed. According to the studies, there is a direct correlation between the Klotho concentration reduction and the amount of antioxidant enzymes expression after IRI, and a direct relationship between melatonin prescription and the reduction of oxidative stress and anti-inflammatory agents. Therefore, it is suggested that further studies should examine the two factors of dose and the number of prescribed days to clarify the role of Klotho in the melatonin action mechanism.

5.1. Conclusion

It seems that melatonin prescription improves kidney function by reducing renal function markers such as NGAL and BUN/Cr ratio. Furthermore, melatonin prescription leads to a decrease in oxidative stress/inflammatory markers. Therefore, a reduction in the complications of ischemic reperfusion injury can be observed in patients receiving melatonin. In fact, the anti-inflammatory and anti-stressing properties of oxidative melatonin were apparent in the current study, and their effects were different from of the ones that affected Klotho protein expression.

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Declaration of Competing Interest

The authors have no conflicts of interest to disclose.

References

[1] M. Nakamura, G. Seki, K. Iwadoh, I. Nakajima, S. Fuchinoue, T. Fujita, S. Teraoka, Acute kidney injury as defined by the RIFLE criteria is a risk factor for kidney transplant graft failure, Clin. Transpl. 26 (4) (2012) 520–528.
[2] T. Aktoz, N. Aydogdu, B. Alagol, O. Yalcin, G. Huseyinova, I.H. Atakan, The protective effects of melatonin and vitamin E against renal ischemia-reperfusion injury in rats, Ren. Fail. 29 (5) (2007) 535–542.
[3] A. Bonavia, K. Singbartl, A review of the role of immune cells in acute kidney injury, Pediatr. Nephrol. 33 (10) (2018) 1629–1639.
[4] F. Panah, A. Ghorbanihaghjo, H. Argani, M.A. Zarmehri, S.N.S. Ahmad, Ischemic acute kidney injury and klotho in renal transplantation, Clin. Biochem. 55 (2018) 3–8.
[5] M.-L. Wang, C.-H. Wei, W.-D. Wang, J.-S. Wang, J. Zhang, J.-J. Wang, Melatonin attenuates lung ischaemia–reperfusion injury via inhibition of oxidative stress and inflammation, Interact. Cardiovasc. Thorac. Surg. 26 (5) (2018) 761–767.
[6] M.C. Hu, M. Shi, N. Gillings, B. Flores, M. Takahashi, M. Kuro-o, O.W. Moe, Recombinant α-Klotho may be prophylactic and therapeutic for acute to chronic kidney disease progression and uremic cardiomyopathy, Kidney Int. 91 (5) (2017) 1104–1114.
[7] G. Castellano, A. Intini, A. Stasi, C. Divella, M. Gigante, P. Pontrelli, R. Franzin, M. Accetturo, A. Zito, M. Fiorentino, Complement modulation of anti-aging factor Klotho in ischemia/reperfusion injury and delayed graft function, Am. J. Transplant. 16 (1) (2016) 325–333.
[8] M.-C. Hu, O.W. Moe, Klotho as a potential biomarker and therapy for acute kidney injury, Nat. Rev. Nephrol. 8 (7) (2012) 423.
[9] M.-C. Hu, M. Kuro-o, O.W. Moe, Klotho and kidney disease, J. Nephrol. 23 (Suppl. 16) (2010) S136.
[10] D. Lapenna, G. Ciofani, S.D. Pierdomenico, M.A. Giamberardino, F. Cuccurullo, Reaction conditions affecting the relationship between thiobarbituric acid reactivity and lipid peroxidesin human plasma, Free Radic. Biol. Med. 31 (3) (2001) 331–335.
[11] R.L. Levine, D. Garland, C.N. Oliver, A. Amici, I. Climent, A.-G. Lenz, B.-W. Ahn, S. Shaltiel, E.R. Stadtman, Determination of carbonyl content in oxidatively modified proteins, Methods Enzymol. (1990) 464–478 (Elsevier).
[12] I.E. Hall, S.G. Yarlagadda, S.G. Coca, Z. Wang, M. Doshi, P. Devarajan, W.K. Han, R.J. Marcus, C.R. Parikh, IL-18 and urinary NGAL predict dialysis and graft recovery after kidney transplantation, J. Am. Soc. Nephrol. 21 (1) (2010) 189–197.
[13] M. Mahdavi-Mazdeh, A. Abdollahi, B.N. Heshmati, Z. Sobhani, Comparison of serum and urine neutrophil gelatinase-associated lipocalin (NGAL) with serum creatinine in prediction of kidney suitability for transplantation, Nephrourol. Mon. 5 (1) (2013) 679.
[14] Z. Rostami, M. Nikpoor, B. Einollahi, Urinary neutrophil gelatinase associated lipocalin (NGAL) for early diagnosis of acute kidney injury in renal transplant recipients, Nephrourol. Mon. 5 (2) (2013) 745.
[15] G. Tsirpanlis, S. Chatzipanagiotou, F. Boufidou, V. Kordinas, M. Zoga, F. Alevyzaki, K. Stamatelou, E. Frangou, L. Savva, C. Nicolaou, Serum oxidized low-density lipoprotein is inversely correlated to telomerase activity in peripheral blood mononuclear cells of haemodialysis patients, Nephrology 11 (6) (2006) 506–509.
[16] G. Sener, A.Ö. Sehirli, M. Keyer-Uysal, S. Arbak, Y. Ersoy, B.Ç. Yeğen, The protective effect of melatonin on renal ischemia–reperfusion injury in the rat, J. Pineal Res. 32 (2) (2002) 120–126.
[17] D.-X. Tan, Melatonin: a potent, endogenous hydroxyl radical scavenger, Endocr. J. 1 (1993) 57–60.
[18] S. Rodríguez-Reynoso, C. Leal, E. Portilla-de Buen, J.C. Castillo, F. Ramos-Solano, Melatonin ameliorates renal ischemia/reperfusion injury, J. Surg. Res. 116 (2) (2004) 242–247.
[19] G. Şener, K. Paskaloğlu, H. Toklu, C. Kapucu, G. Ayanoglu-Dulger, A. Kacmaz, A. Sakarcan, Melatonin ameliorates chronic renal failure-induced oxidative organ damage in rats, J. Pineal Res. 36 (4) (2004) 232–241.
[20] H. Zhou, D. Li, P. Zhu, S. Hu, N. Hu, S. Ma, Y. Zhang, T. Han, J. Ren, F. Cao, Melatonin suppresses platelet activation and function against cardiac ischemia/reperfusion injury via PPAR γ/FUNDC 1/mitophagy pathways, J. Pineal Res. 63 (4) (2017) e12438.
[21] Z. Zhao, C. Lu, T. Li, W. Wang, W. Ye, R. Zeng, L. Ni, Z. Lai, X. Wang, C. Liu, The protective effect of melatonin on brain ischemia and reperfusion in rats and humans: in vivo assessment and a randomized controlled trial, J. Pineal Res. 65 (4) (2018) e12521.
[22] A. Lochner, E. Marais, B. Huisamen, Melatonin and cardioprotection against ischaemia/reperfusion injury: What's new? A review, J. Pineal Res. 65 (1) (2018) e12490.
[23] S. Raeisi, A. Ghorbanihaghjo, H. Argani, S. Dastmalchi, M. Seifi, B. Ghasemi, T. Ghazizadeh, M.M. Abbasi, P. Karimi, Oxidative stress-induced renal telomere shortening as a mechanism of cyclosporine-induced nephrotoxicity, J. Biochem. Mol. Toxico. 32 (8) (2018) e22166.
[24] Z. Zuo, H. Lei, X. Wang, Y. Wang, W. Sonntag, Z. Sun, Aging-related kidney damage is associated with a decrease in klotho expression and an increase in superoxide production, Age 33 (3) (2011) 261–274.
[25] M. Ikushima, H. Rakugi, K. Ishikawa, Y. Maekawa, K. Yamamoto, J. Ohta, Y. Chihara, I. Kida, T. Ogihara, Anti-apoptotic and anti-senescence effects of Klotho on vascular endothelial cells, Biochem. Biophys. Res. Commun. 339 (3) (2006) 827–832.
[26] Y. Zhao, S. Banerjee, N. Dey, W.S. LeJeune, P.S. Sarkar, R. Brobey, K.P. Rosenblatt, R.G. Tilton, S. Choudhary, Klotho depletion contributes to increased inflammation in kidney of the db/db mouse model of diabetes via RelA (serine) 536 phosphorylation, Diabetes 60 (7) (2011) 1907–1916 DB_101262.