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Melatonin attenuates cisplatin-induced acute kidney injury in rats via induction of anti-aging protein, Klotho

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ABSTRACT

This study investigated the protective effects of melatonin (MT) against cisplatin (CP)-induced acute kidney injury in rats as well as its possible mechanism of action associated with anti-aging protein Klotho. The following four experimental groups were evaluated: vehicle control, CP (7 mg/kg), CP&MT20 (20 mg/kg/day), and CP&MT40 (40 mg/kg/day). The concomitant administration of MT significantly ameliorated CP-induced acute kidney injury in rats, as evidenced by increased kidney weight, increased serum levels of blood urea nitrogen and creatinine, and increased incidence of histopathological alterations with renal tubular cell apoptosis. In addition, MT treatment protected kidney tissue against oxidative damages and significantly upregulated the expression level of Klotho decreased by CP treatment, resulting in reduced phosphorylation of protein kinase B (AKT) and forkhead box O (FOXO) as well as reduced expression levels of B-cell lymphoma 2-associated X protein (Bax) and caspase-3. MT not only partially regulated oxidative stress via AKT/FOXO signaling, but also reduced apoptosis caused by CP by inhibiting the Bax/caspase-3 pathway. Our results indicated that MT could prevent acute kidney injury induced by CP in rats, presumably through upregulating the expression of Klotho, resulting in elevated anti-oxidant and anti-apoptotic properties.

1. Introduction

Klotho, originally identified as an anti-aging protein, can exist in transmembrane form and soluble form with different roles between the two (Kuro-o et al., 1997; Kurosu et al., 2005; Matsumura et al., 1998). Soluble Klotho has various functions, including the regulation of multiple ion channels, oxidative stress, inflammation, and apoptosis (Chang et al., 2005; Kuro-o, 2008; Panesso et al., 2014). Since the soluble Klotho is produced through alternative splicing at exon or cleavage of transmembrane Klotho, the overexpression of membrane Klotho can also lead to high levels of soluble Klotho (Kurosu et al., 2005). Recent studies demonstrated that ant-oxidant and anti-apoptotic effects of Klotho via various pathways, which indicate that Klotho has a possibility of target protein in oxidative stress and apoptosis mediated-disease (Mytych et al., 2019; Cui et al., 2018; Zhu et al., 2017). In particular, as Klotho is highly expressed in renal tubular epithelial cells, Klotho can be used as a biomarker and protective agent in acute kidney injury induced by ischemia reperfusion as well as chemicals such as cisplatin (*cis*-diamminedichloroplatinum-II, CP) (Andrade et al., 2018; Hu and Moe, 2012; Ning et al., 2018).

CP is an effective chemotherapeutic agent for the treatment of various solid tumors including cancers of the ovary, cervix, colon, lung, and testis (Dasari and Tchounwou, 2014; Galluzzi et al., 2012). Nevertheless, its clinical usefulness has frequently been limited by undesirable side effects it has had on the gastrointestinal track, liver, peripheral nerves, inner ear, testes, and kidney (Florea and Busselberg, 2011; Ko et al., 2014). This is particularly and issue for kidney tissue, which is more sensitive than other tissue, because it takes up CP at

Abbreviations: AKT, protein kinase B; Bax, B-cell lymphoma 2-associated X protein; Bcl-2, B-cell lymphoma 2; BUN, blood urea nitrogen; CRE, creatinine; DAB, 3-3′-diaminobenidine; FOXO, forkhead box O; GR, glutathione reductase; GSH, glutathione; GST, glutathione *S*-transferase; H&E, hematoxylin and eosin; IHC, immunohistochemistry; MDA, malondialdehyde; MT, melatonin; ROS, reactive oxygen species; SD, standard deviation; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP-end labeling

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higher concentrations than other tissues. In the kidney, CP can inhibit antioxidant enzyme by conjugating with glutathione, leading to excess production of reactive oxygen species (ROS). ROS can lead to mitochondrial damage, lysis, and dysfunction (Kharbangar et al., 2000; Yang et al., 2014). In addition, CP can induce apoptosis through various pathways, including the extrinsic or intrinsic pathway, endoplasmic reticulum stress-mediated pathway, and DNA damage-mediated pathway (Pabla and Dong, 2008; Nho et al., 2018). Therefore, it is essential to minimize the potential side effects induced by CP via co-administration with effective antioxidants that can inhibit free radical generation and apoptosis. It has been demonstrated that certain effective antioxidants confer some protection on CP-induced oxidative stress and apoptosis in the kidney (Choi et al., 2013; Surendran et al., 2012).

Melatonin (*N-acetyl*-5-methoxytryptamine, MT) is a secretory product of the pineal gland. It has many important physiological functions (Reiter et al., 2010). A number of studies have demonstrated that MT has various pharmacological properties including anti-oxidant, anti-cancer, anti-inflammatory, and immunomodulatory effects (Calvo et al., 2013; Mauriz et al., 2013; Stehle et al., 2011). The anti-apoptotic effects of MT via mitochondrial pathways have been well documented in previous reports (Okatani et al., 2003; Petrosillo et al., 2006). It has been also reported that MT attenuates oxidative stress and the associated memory impairment induced by Klotho deficiency (Shin et al., 2014). Based on the results of previous studies, we hypothesized that MT can effectively protect against CP-induced acute kidney injury via anti-oxidant and anti-apoptotic effects stem from modulating Klotho.

The present study investigated the protective effect of MT against CP-induced acute kidney injury in rats, focusing on its effect on reducing oxidative stress and apoptosis. In addition, this study examined the change in expression levels of Klotho, as modulated by MT in acute kidney injury induced by CP, as well as its effects on changes in expression levels of pro-apoptotic, anti-apoptotic, and oxidative stress-linked proteins.

2. Materials and methods

2.1. Animals and environmental conditions

Twenty-six male Sprague–Dawley rats were obtained at six weeks of age from a specific pathogen-free colony at Samtako Co. (Osan, Republic of Korea); they were quarantined and acclimated for one week upon arrival. Following this acclimatization period, two animals per cage were housed in a room maintained at a temperature of 23 ± 3 °C and relative humidity of 50 ± 10% with artificial lighting from 08:00 to 20:00, and 13–18 air changes per h. Commercial rodent chow (Samyang Feed Co., Wonju, Republic of Korea) and tap water sterilized by radiation were available *ad libitum*. The animal study protocol was approved by the Institutional Animal Care and Use Committee of Chonnam National University. Animals were cared for in accordance with the Guidelines for Animal Experiments of Chonnam National University.

2.2. Test chemicals and treatment

CP (CAS No. 15663-27-1) and MT were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). All other chemicals were of the highest grade commercially available. CP was dissolved in sterilized normal saline. It was freshly prepared immediately prior to treatment. MT was dissolved in ethanol and diluted in saline at a final concentration of 5%. The application volumes of CP (10 ml/kg body weight) and MT (10 ml/kg body weight) were calculated based on the most recently recorded body weight of each individual animal.

2.3. Experimental groups and dose selection

A total of 24 healthy male rats were randomly assigned into four experimental groups with six rats per group: 1) vehicle control group (received a 10-day repeated intraperitoneal (i.p.) injection of 5% ethanol and a single i.p. injection of saline on test day 6 at 1 h after 5% ethanol injection), 2) CP group (received a 10-day repeated i.p. injection of 5% ethanol and a single i.p. injection of CP at 7 mg/kg on test day 6 at 1 h after 5% ethanol injection), 3) CP&MT20 group (received a 10-day repeated i.p. injection of MT at 20 mg/kg and a single i.p. injection of CP at 7 mg/kg on test day 6 at 1 h after MT administration), and 4) CP&MT40 group (received a 10-day repeated i.p. injection of MT at 40 mg/kg and a single i.p. injection of CP at 7 mg/kg on test day 6 at 1 h after MT administration). The nephrotoxic dose of CP used in this study was based on the results of a previous study (Atessahin et al., 2005), and effective doses of MT were based on previous reports as well (Wang et al., 2009; Xu et al., 2007).

2.4. Clinical observation and body weights

All animals were observed daily throughout the study period for any clinical signs of toxicity or mortality. Abnormal signs were recorded individually by noting the type, observation day, and time. The body weight of each rat was measured every other day.

2.5. Necropsy, serum biochemistry, and organ weights

All treated animals were euthanized by carbon dioxide inhalation for blood collection on the scheduled termination day (test day 10). Blood samples were drawn from the inferior vena cava. Serum samples were obtained by centrifuging blood samples at 800 × *g* for 10 min within 1 h after blood collection. Samples were stored at −80 °C until analysis. Blood urea nitrogen (BUN) and creatinine (CRE) were measured with an autoanalyzer (Dri-chem 4000i, Fujifilm Co., Tokyo, Japan). The absolute weights of the kidneys and their kidney weights relative to their body weights were then calculated.

2.6. Histopathological examination

The right kidney was fixed in 10% neutral buffered formalin solution for two weeks. The left kidney was quickly frozen on dry ice and stored at −80 °C for biochemical analysis. Fixed tissues were routinely processed, embedded in paraffin, then sectioned at 4 μm thickness, deparaffinized, and rehydrated using standard technique. These kidney sections were stained with hematoxylin and eosin (H&E) in order to identify histological changes in the kidneys. All observations were made manually in a totally blinded manner using a light microscope with × 5, × 10, × 20, and × 40 objective lenses and a × 100 oil immersion lens. The following variables were used to assess the histological changes in the kidney: (1) proximal renal tubular dilation, (2) hyaline cast, and (3) epithelial cell detachment.

2.7. Determination of lipid peroxidation, glutathione, and antioxidant enzymes activities

The removed left kidney was cut in half and homogenized in a glass-Teflon homogenizer with 50 mM phosphate buffer (pH 7.4) in order to obtain 1:9 (w/v) whole homogenate. The homogenates were then centrifuged at 11,000 × *g* for 15 min at 4 °C so as to remove any cell debris. The supernatant was used to measure malondialdehyde (MDA) and glutathione (GSH) contents. The concentration of MDA was assayed by monitoring the formation of thiobarbituric acid-reactive substance using the method described by Berton et al. (1998). GSH content was measured using the method described Moron et al. (1979). The activities of antioxidant enzymes such as catalase, glutathione reductase (GR), and glutathione *S*-transferase (GST) were determined using colorimetric commercial assay kits (Cayman Chemical, Ann Arbor, MI, USA). Total protein content was determined according to the method described Lowry et al. (1951), using bovine serum albumin as a

Table 1
Body weight changes and kidney weights in male rats treated with cisplatin and/or melatonin.

Items	Groups			
	VC	CP	CP&MT20	CP&MT40
No. of rats	6	6	6	6
Body weight (g)				
Day 6	251.4 ± 15.58[a]	252.0 ± 2.54	245.2 ± 8.59	246.4 ± 3.41
Day 8	259.6 ± 17.65	245.45 ± 4.67	235.5 ± 8.35	240.4 ± 1.15
Day 10	277.8 ± 17.02	231.15 ± 6.73	230.3 ± 11.13	230.3 ± 12.84
Day 10-Day 6	26.4 ± 2.96	−20.8 ± 3.05**	−14.9 ± 5.18	−10.9 ± 7.96†
Kidneys				
Per body weight (%)	0.38 ± 0.02	0.66 ± 0.09**	0.57 ± 0.05	0.44 ± 0.14††

** Significant difference at $P < 0.01$ level as compared to the vehicle control group.
† Significant difference at $P < 0.05$ level as compared to the cisplatin group.
†† Significant difference at $P < 0.01$ level as compared to the cisplatin group.
VC, vehicle control; CP, cisplatin treatment (7 mg/kg) with PBS treatment; CP&MT20 and CP&MT40, cisplatin treatment (7 mg/kg) with melatonin treatment (20 and 40 mg/kg/day, respectively).
[a] Values are presented as means ± SD.

standard.

2.8. Immunohistochemistry (IHC) analysis for Klotho and caspase-3

The fixed tissues were processed routinely, embedded in paraffin, then sectioned to 4 μm thickness, deparaffinized, and rehydrated using standard techniques for IHC analysis. Following incubation with a protein block (anti rabbit-specific horseradish peroxidase HRP/3-3′-diaminobenidine (DAB) IHC Kit; Abcam, Cambridge, MA, USA), the sections were incubated overnight with Klotho (1:200; Novus Biological, Littleton, CO, USA) and caspase-3 antibody (1:200; Cell Signaling Technology, Beverly, MA, USA) at 4 °C. The expression levels of Klotho and caspase-3 were visualized using an IHC kit (Abcam) according to the manufacturer's protocol. Secondary antibody biotinylated goat anti-rabbit IgG (Abcam), was applied, followed by streptavidin HRP and then DAB chromogen with substrate buffer. The sections were counterstained with Harris's hematoxylin prior to being mounted. Each slide was examined manually in a totally blinded manner using a light microscope (Leica, Wetzlar, Germany) with × 10 or × 20 objective lenses and a × 100 oil immersion lens.

2.9. Terminal deoxynucleotidyl transferase-mediated dUTP-end labeling (TUNEL) assay

Apoptotic changes in the kidneys were detected via TUNEL assay kit (ApopTag® Peroxydase In Situ Apoptosis Detection Kit; Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instruction. The slides were visualized with DAB chromogen and counterstained with Harris's hematoxylin before being mounted. Each slide was examined manually with a light microscope (Leica) with × 10 or × 20 objective lenses and a × 100 oil immersion lens in a totally blinded manner.

2.10. Western blotting analysis

Equal amounts of protein (40 μg/well) from each sample were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Whatman, Maidstone, UK), and blocked in blocking buffer (150 mM NaCl in 10 mM Tris, pH 7.5, containing 5% nonfat dry milk) for 1 h at room temperature. The membranes were then incubated with the following primary antibodies at 4 °C for 18 h: rabbit antibodies against rat Klotho, forkhead box O (FOXO), phosphor (p)-FOXO (1:1000; Novus Biological), B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), Bcl-2, caspase-3, protein kinase B (AKT), p-AKT, and β-actin (1:1000; Cell Signaling Technology). The membranes were then washed three times with washing buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.1% Tween 20) and incubated with HRP-conjugated secondary antibodies (1:2000; Jackson Immuno Research, West Grove, PA, USA) for 1 h at room temperature. After washing three times with washing buffer, protein bands were detected with enhanced chemiluminescence reagents. Protein concentrations were determined using a Bicinchoninic acid Protein Assay kit (Pierce, Rockford, IL, USA). Protein expression was quantified based on band density using TINA 20 Image software (Raytest Isotopenmessgeraete GmbH, Straubenhardt, Germany). The relative intensities of protein bands were normalized against the intensity of loading control.

2.11. Statistical analyses

The results were expressed as means ± standard deviation (SD). All statistical comparisons were made by means of one-way analysis of variance followed by Dunnett's multiple comparison test. Differences with a p value ≤ 0.05 were considered to be statistically significant.

3. Results

3.1. Effects of MT on clinical signs, body weight, and kidney weight

No treatment-related mortality was observed in animals treated with CP and/or MT during the study period. However, the incidence and severity of clinical signs in the CP group were increased as compared to those in the vehicle control group, including nasal discharge, soiled perineal region, piloerection, and depression (data not shown). CP treatment also resulted in a statistically significant decrease in body weight gain during the study period as well as significant increase in relative kidney weight when compared to those in the vehicle control group (Table 1). By contrast, the incidence and severity of clinical signs in the CP&MT treated groups tended to be decreased as compared to those in the CP group (data no shown). The CP&MT treated groups also showed an increment in body weight gain and a decrement in relative kidney weight as compared to those of the CP group (Table 1).

3.2. Effect of MT on serum biochemistry

As shown in Fig. 1A, the BUN level was significantly higher in the CP group (188.8 ± 73.9 mg/dL) than the vehicle control group (18.0 ± 1.70 mg/dL). As compared to the CP group, the CP&MT groups had significantly lower BUN levels (122.3 ± 18.12 mg/dL in the CP&MT20 group and 64.6 ± 23.45 mg/dL in the CP&MT40 group). Serum CRE levels showed a similar pattern to BUN levels (Fig. 1B); CRE level was significantly higher in the CP group (4.0 ± 1.69 mg/dL) than

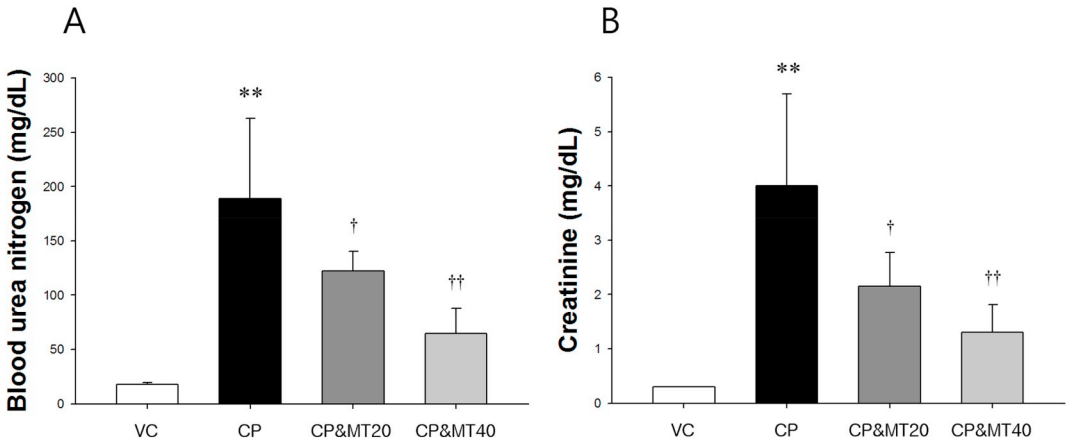

Fig. 1. Effect of melatonin on blood urea nitrogen and creatinine levels in male rats treated with cisplatin and/or melatonin. (A) Blood urea nitrogen level; (B) Creatinine level. Each bar represents the means ± SD ($n = 6$). $^{**}P < 0.01$ as compared to the VC group; $^{\dagger,\dagger\dagger}P < 0.05$ and $p < 0.01$ as compared to the CP group, respectively. VC, vehicle control; CP, cisplatin treatment (7 mg/kg) with PBS treatment; CP&MT20 and CP&MT40, cisplatin treatment (7 mg/kg) with melatonin treatment (20 and 40 mg/kg/day, respectively).

the vehicle control group (0.3 ± 0.00 mg/dL). As compared to the CP group, the CP&MT groups had a significantly lower CRE level (2.15 ± 0.62 mg/dL in the CP&MT20 and 1.30 ± 0.51 mg/dL in the CP&MT40 group).

3.3. Effect of MT on renal histopathology

As shown in Fig. 2, the kidneys of the vehicle control group exhibited normal architecture. By contrast, the kidneys of the CP group showed significant histopathological alterations represented by proximal tubular dilation. In addition, the CP group showed hyaline cast and epithelial cell detachment. These histopathological alterations were observed in all animals in the CP group (Table 2). Although these findings were also observed in the CP&MT groups, the incidence and severity of histopathological lesions in those groups were decreased as compared to those of the CP group.

3.4. Effects of MT on MDA and GSH contents

The MDA content in rats treated with CP (1.3 ± 0.25 mol/mg protein) was significantly higher than in the vehicle control group (0.9 ± 0.10 mol/mg protein) (Fig. 3A). The MDA contents in the CP&

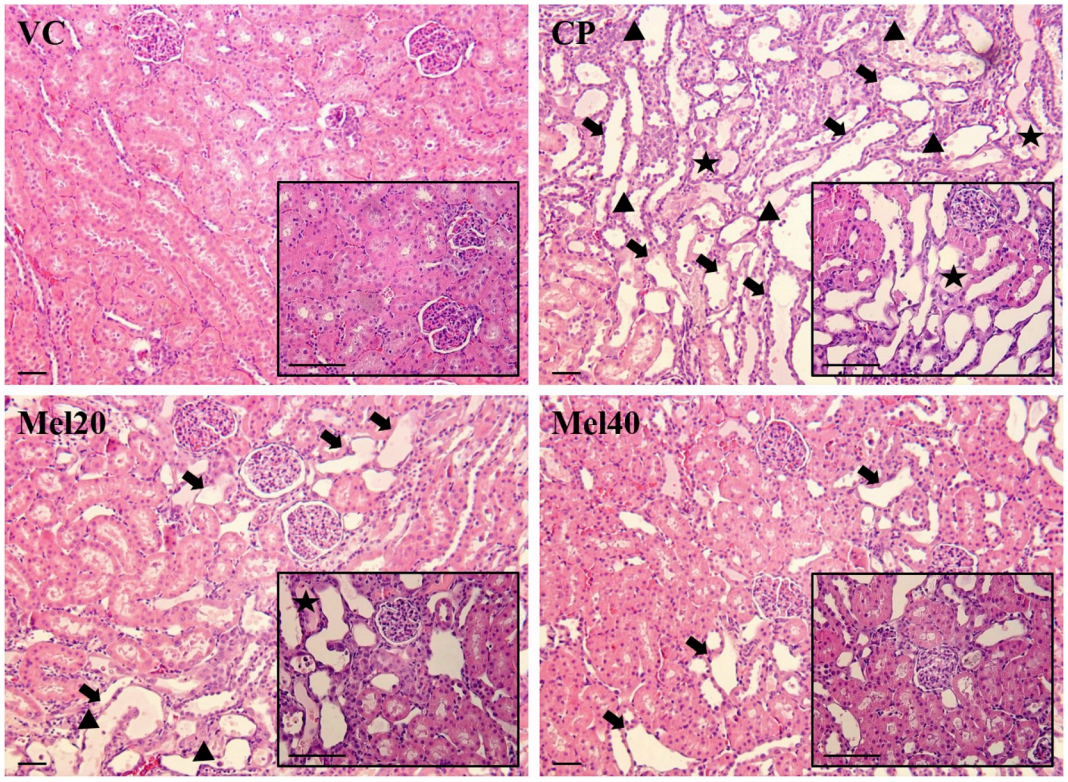

Fig. 2. Effect of melatonin on renal histopathology. Renal cortex in the VC group showed normal appearance. Renal cortex in the CP group showed proximal tubular dilation (closed arrow), hyaline cast (asterisk), and epithelial cell detachment (closed arrowhead). Renal cortex in the CP&MT20 and CP&MT40 groups showed decreased histological alterations and incidence. H&E stain. Bar = 50 μm (× 200). VC, vehicle control; CP, cisplatin treatment (7 mg/kg) with PBS treatment; CP&MT20 and CP&MT40, cisplatin treatment (7 mg/kg) with melatonin treatment (20 and 40 mg/kg/day, respectively).

Table 2
Histopathological findings in kidney of rats treated with cisplatin and/or melatonin.

Findings	Groups			
	VC	CP	CP&MT20	CP&MT40
Tubular dilation				
Appears normal	6[a]	0	0	0
Mild	0	0	0	2
Moderate	0	0	4	2
Severe	0	6	2	2
Hyaline cast				
Appears normal	6	0	0	1
Mild	0	0	2	4
Moderate	0	2	4	1
Severe	0	4	0	0
Epithelial cell detachment				
Appears normal	6	0	0	0
Mild	0	0	4	5
Moderate	0	4	2	1
Severe	0	2	0	0

VC, vehicle control; CP, cisplatin treatment (7 mg/kg) with PBS treatment; CP&MT20 and CP&MT40, cisplatin treatment (7 mg/kg) with melatonin treatment (20 and 40 mg/kg/day, respectively).
[a] No. of rats with the histopathological findings.

MT groups (1.2 ± 0.18 mol/mg protein in the CP&MT20 group and 1.1 ± 0.12 mol/mg protein in the CP&MT40 group) were lower than that of the CP group. However, the differences between the MDA contents of the CP group and CP&MT groups were not statistically significant.

The GSH content in the CP group (11.9 ± 0.80 mol/mg protein) was significantly lower than that of the vehicle control group (19.4 ± 1.40 mol/mg protein) (Fig. 3B). By contrast, the CP&MT groups had significantly higher GSH contents (13.6 ± 0.40 mol/mg protein for the CP&MT20 group and 14.7 ± 0.90 mol/mg protein for the CP&MT40 group) than the CP group.

3.5. Effects of MT on antioxidant enzymes activities

The catalase activity of the CP group (9.0 ± 0.87 U/mg protein) was significantly lower than that of the vehicle control group (16.5 ± 2.01 U/mg protein) (Fig. 4A). By contrast, the CP&MT groups had significantly higher catalase activities (11.5 ± 0.80 U/mg protein for the CP&MT20 group and 12.6 ± 2.42 IU/mg protein for the CP&MT40 group) than the CP group. The results of GST (Fig. 4B) and GR (Fig. 4C) showed a similar pattern to catalase activity. The activities of GST and GR in the CP group (24.0 ± 1.80 U/mg protein and 9.9 ± 0.75 U/mg protein, respectively) were significantly lower than those of the vehicle control group (42.0 ± 2.70 U/mg protein and 13.8 ± 0.66 U/mg protein, respectively). As compared to the CP group, the CP&MT groups had significantly higher activities of GST (30.0 ± 1.20 U/mg protein for the CP&MT20 group and 33.0 ± 0.90 U/mg protein for the CP&MT40 group) and GR (11.1 ± 0.93 U/mg protein for the CP&MT20 group and 12.0 ± 0.99 U/mg protein for the CP&MT40 group).

3.6. Effects of MT on Klotho- and caspase-3-immunopositivity

In order to further confirm the protective effect of MT, we conducted IHC analysis for Klotho and caspase-3. The number of Klotho-positive cells in the CP group was considerably lower than that in the vehicle control group (Fig. 5). As compared to the CP group, the CP&MT groups had more Klotho-positive cells. Unlike Klotho-immunopositivity, the number of caspase-3-positive cells in the CP group was considerably higher than that in the vehicle control group (Fig. 6). As compared to the CP group, the CP&MT groups treated with 20 or 40 mg/kg of MT had fewer caspase-3-positive cells.

3.7. Effects of MT on the renal apoptosis

TUNEL assay was used to identify renal tubular cell apoptosis in rats. As shown Fig. 7, few TUNEL-positive cells were observed in the vehicle control group. A marked increased in the number of TUNEL-positive cells was observed in the CP group compared to the vehicle control group. However, this increase was attenuated in rats treated with 20 or 40 mg/kg of MT.

3.8. Effect of MT on the regulation of oxidative stress via Klotho

The protein expression levels of Klotho as well as phosphorylation levels of FOXO and AKT are shown in Fig. 8. The expression level of Klotho in the CP group was significantly decreased as compared to that in the vehicle control group. By contrast, the expression level of Klotho was increased significantly in the CP&MT groups as compared to in the CP group. The patterns for the phosphorylation of FOXO and AKT differed from that of Klotho. The CP group had markedly increased phosphorylation of FOXO and AKT as compared to the vehicle control group. However, the CP&MT groups had significantly lower phosphorylation of FOXO and AKT than the CP group in a dose-dependent

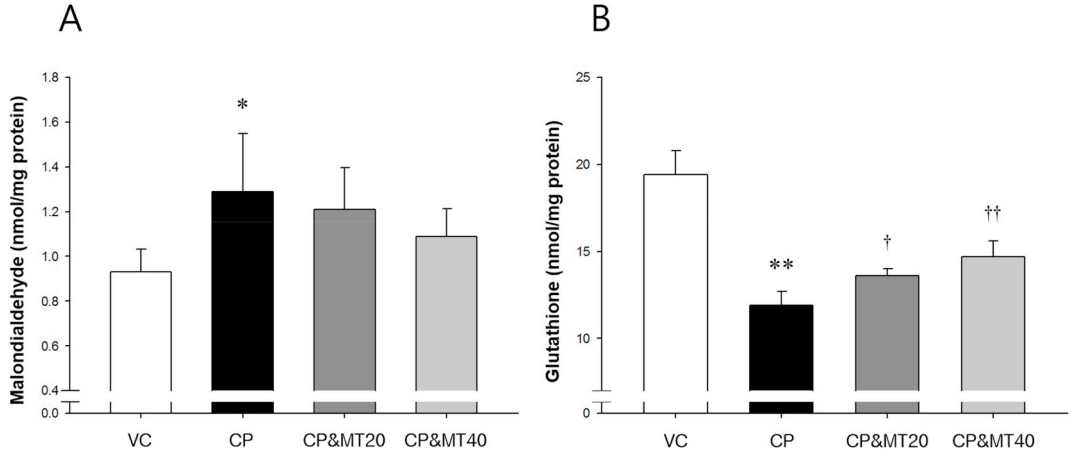

Fig. 3. Effect of melatonin on lipid peroxidation and glutathione contents in kidneys. (A) Malondialdehyde content due to lipid peroxidation; (B) Reduced glutathione contents in the kidneys of male rats treated with cisplatin and/or melatonin. Each bar represents the means ± SD ($n = 6$). $^{*,**}P < 0.05$ and $p < 0.01$ as compared to the VC group, respectively; $^{\dagger,\dagger\dagger}P < 0.05$ and $p < 0.01$ as compared to the CP group, respectively. VC, vehicle control; CP, cisplatin treatment (7 mg/kg) with PBS treatment; CP&MT20 and CP&MT40, cisplatin treatment (7 mg/kg) with melatonin treatment (20 and 40 mg/kg/day, respectively).

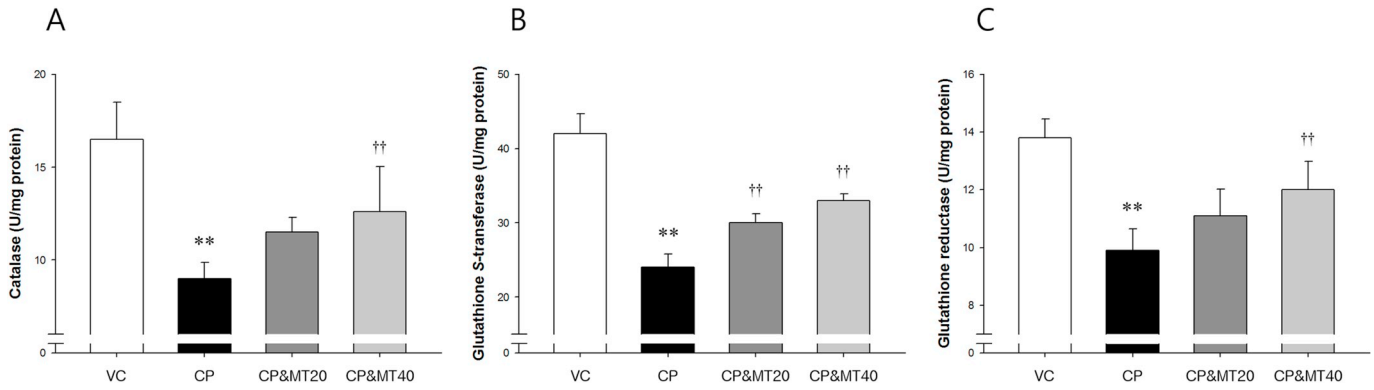

Fig. 4. Effect of melatonin on antioxidant enzyme activities in the kidneys of male rats treated with cisplatin and/or melatonin. (A) Catalase activity; (B) Glutathione reductase activity; (C) Glutathione *S*-transferase activities in kidneys. Each bar represents the means ± SD ($n = 6$). $^{**}P < 0.01$ as compared to the VC group; $^{††}P < 0.01$ as compared to the CP group. VC, vehicle control; CP, cisplatin treatment (7 mg/kg) with PBS treatment; CP&MT20 and CP&MT40, cisplatin treatment (7 mg/kg) with melatonin treatment (20 and 40 mg/kg/day, respectively).

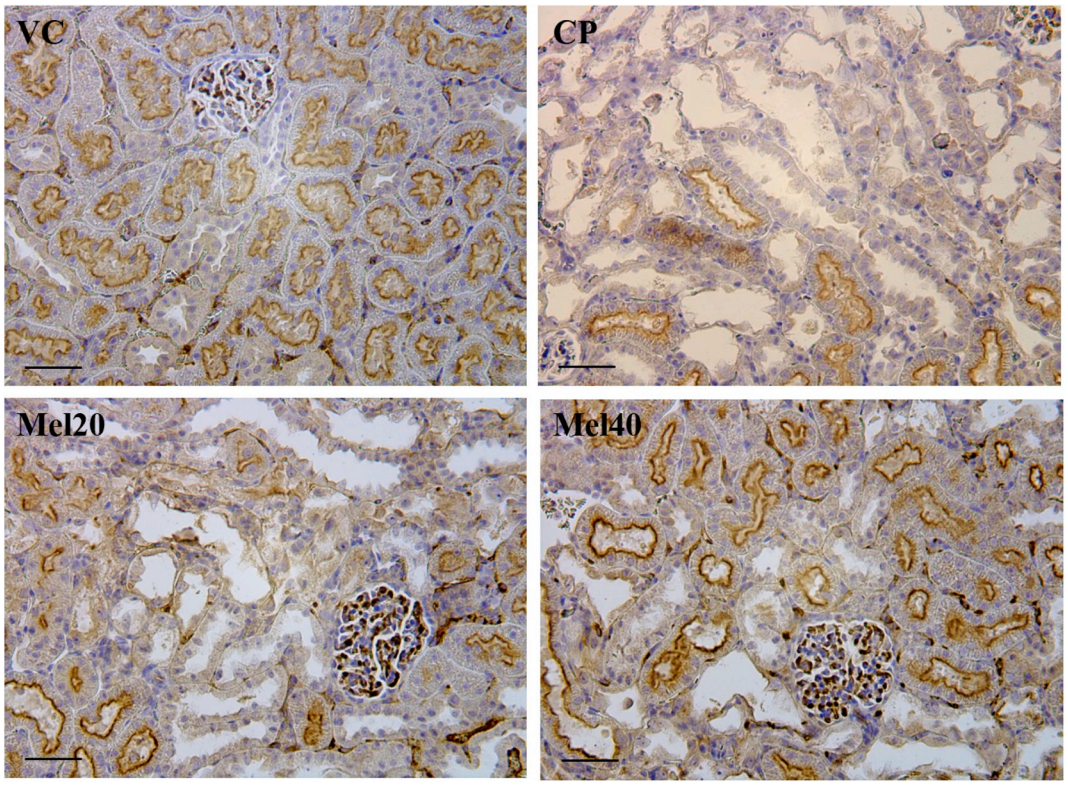

Fig. 5. Effect of melatonin on renal Klotho activation. Representative photographs of immunohistochemical analysis of Klotho performed for kidneys sections of the VC, CP, CP&MT20, and CP&MT40 groups. Bar = 50 μm (× 200). VC, vehicle control; CP, cisplatin treatment (7 mg/kg) with PBS treatment; CP&MT20 and CP&MT40, cisplatin treatment (7 mg/kg) with melatonin treatment (20 and 40 mg/kg/day, respectively).

manner.

3.9. Effect of MT on Klotho-dependent apoptotic pathway

The expression levels of Bax and caspase-3 were significantly higher in the CP group than the vehicle control group (Fig. 9). By contrast, the expression levels of Bax and caspase-3 in the CP&MT groups were significantly lower than those in the CP group. Regarding the expression levels of Klotho and Bcl-2, the CP group showed markedly decreased levels as compared to the vehicle control group, whereas the CP &MT groups had significantly increased levels compared to the CP group.

4. Discussion

Although CP is a highly active anti-neoplastic agent, its clinical utility is often limited by its undesirable side effects on various organs, particularly the kidney (Florea and Busselberg, 2011). Therefore, it is essential to minimize its potential side effects while also preserving its chemotherapeutic efficacy. MT is a potent anti-oxidant, anti-inflammatory, and anti-apoptotic agent. Recently, it has been reported that the renoprotective effect of melatonin against chemotherapy-induced nephrotoxicity is attributed to different mechanisms such as reduction of oxidative stress, apoptosis, and inflammation (Haghi-Aminjan et al., 2018). In the present study, we investigated the antioxidant and anti-apoptotic effects of MT against CP-induced acute kidney injury with a focus on Klotho. MT significantly ameliorated the

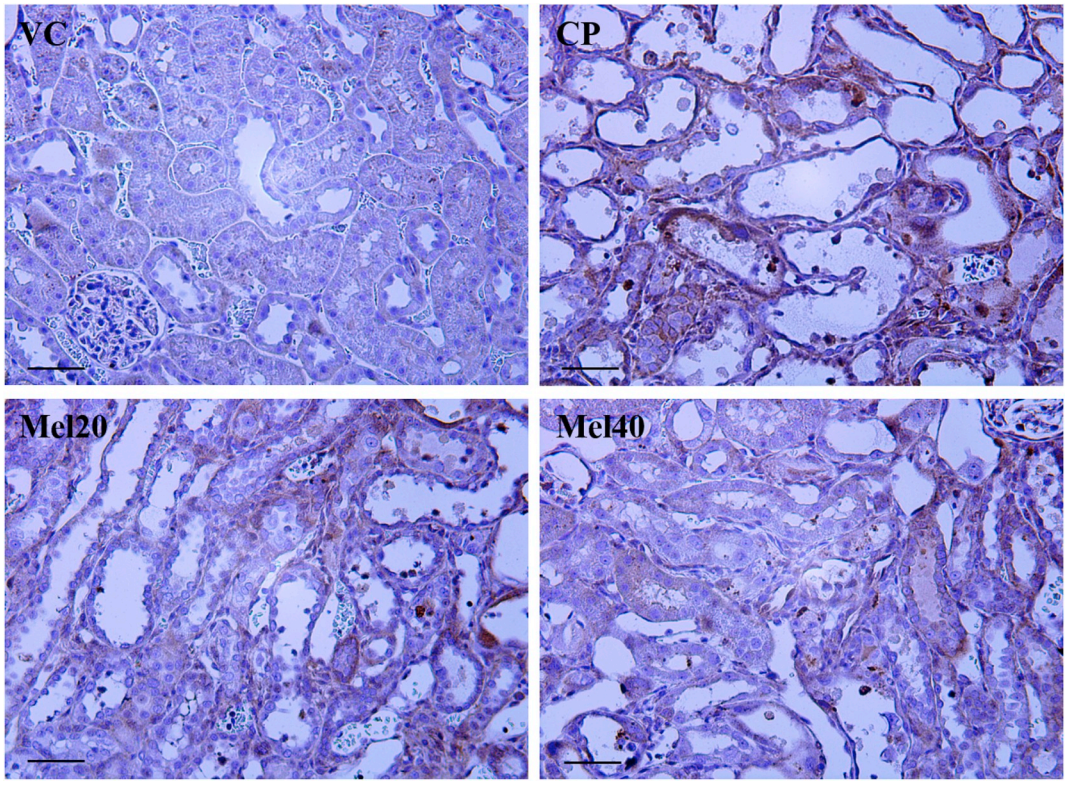

Fig. 6. Effect of melatonin on renal caspase-3 activation. Representative photographs of immunohistochemical analysis of caspase-3 performed for kidney sections of the VC, CP, CP&MT20, and CP&MT40 groups. Bar = 50 μm (× 200). VC, vehicle control; CP, cisplatin treatment (7 mg/kg) with PBS treatment; CP&MT20 and CP&MT40, cisplatin treatment (7 mg/kg) with melatonin treatment (20 and 40 mg/kg/day, respectively).

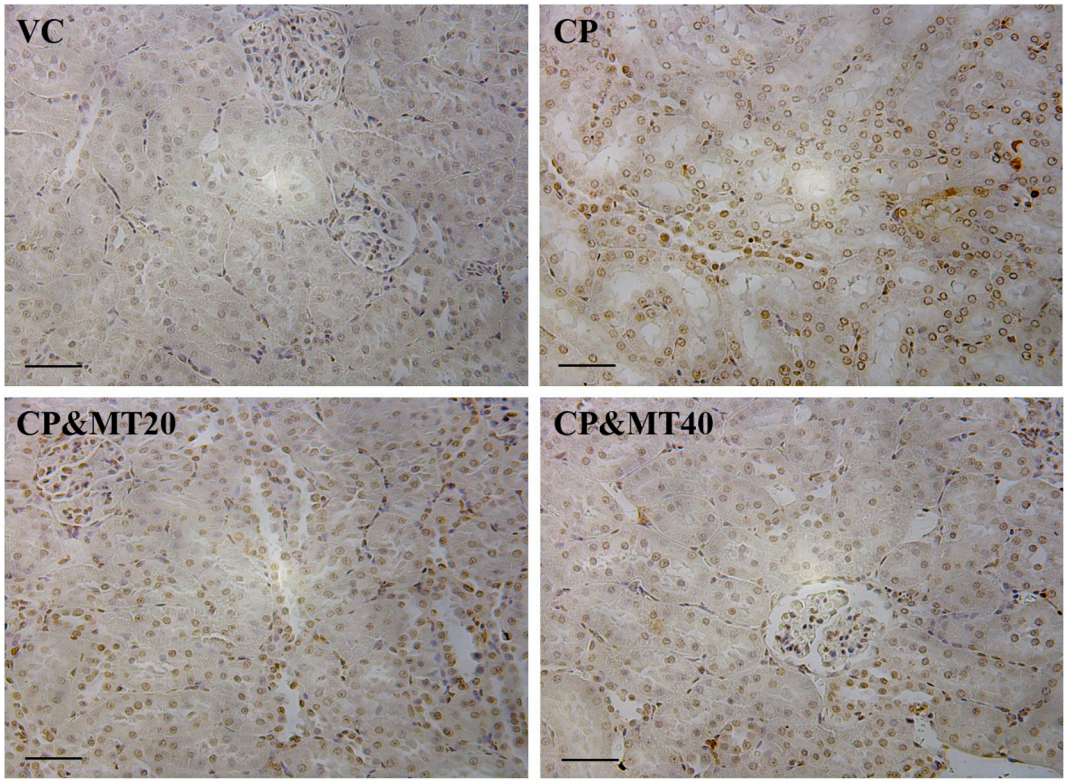

Fig. 7. Effect of melatonin on renal tubular cell apoptosis. Representative photographs of TUNEL assay performed for kidney sections of the VC, CP, CP&MT20, and CP&MT40 groups. Bar = 50 μm (× 200). VC, vehicle control; CP, cisplatin treatment (7 mg/kg) with PBS treatment; CP&MT20 and CP&MT40, cisplatin treatment (7 mg/kg) with melatonin treatment (20 and 40 mg/kg/day, respectively).

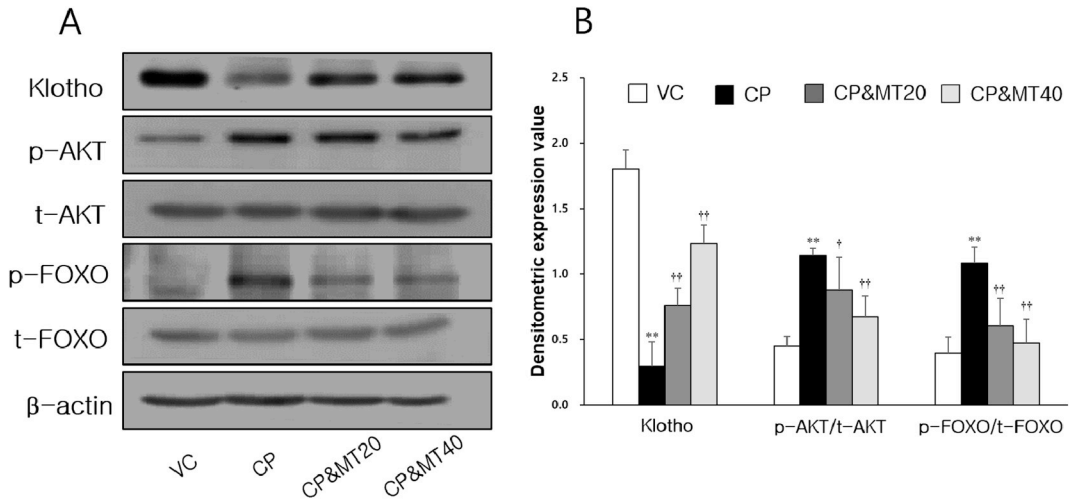

Fig. 8. Effect of melatonin on oxidative stress pathways. (A) Western blot analysis of protein levels of Klotho, AKT, FOXO, and phosphorylation of AKT and FOXO in the kidney of VC, CP, CP&MT20, and CP&MT40 groups. (B) Bar graphs showing Klotho, relative p-AKT/t-AKT ratio, and p-FOXO/t-FOXO ratio in the kidneys of the VC, CP, CP&MT20, and CP&MT40 groups. Values are presented as means ± SD ($n = 6$). $^{**}P < 0.01$ as compared to the VC group; $^{\dagger}, ^{\dagger\dagger}P < 0.05$ and $p < 0.01$ as compared to the CP group, respectively. VC, vehicle control; CP, cisplatin treatment (7 mg/kg) with PBS treatment; CP&MT20 and CP&MT40, cisplatin treatment (7 mg/kg) with melatonin treatment (20 and 40 mg/kg/day, respectively).

CP treatment-induced increases in BUN, CRE, oxidative stress, histopathological alteration, and apoptosis. In addition, MT reduced the levels of AKT, FOXO phosphorylation, Bax, and caspase-3 accompanying the induction of Klotho and Bcl-2, resulting in reduced oxidative stress and apoptosis caused by CP.

In this study, MT treatment effectively reduced the elevation of BUN and CRE induced by CP administration. Further, MT attenuated CP-induced histopathological alterations, including tubular dilation, hyaline cast, and epithelial cell detachment. BUN and CRE are removed from the blood by the kidney through glomerular filtration and proximal tubular secretion. Therefore, BUN and CRE have been used as sensitive indicators of kidney injury in many experiments investigating nephrotoxicity (Shahbazi et al., 2012). Various toxic materials can stimulate marked increases in BUN and CRE levels due to kidney tubular damage. CP is a well-known nephrotoxic material, as it can increase the levels of BUN and CRE in the blood, causing nephrotoxicity (Haghighi et al., 2012; Panesso et al., 2014). Therefore, decreases in BUN and CRE levels may be important markers of a potential therapeutic effect for test materials for the treatment of toxin-induced acute nephrotoxicity. Therefore, results of previous studies and our findings suggest that MT could attenuate CP-induced acute kidney injury.

In this study, MT decreased the MDA content and effectively restored CP-induced GSH contents depletion in a dose-dependent manner. In addition, the decreased activities of antioxidant enzymes induced by CP were significantly increased by MT treatment. These antioxidant effects of MT are well established in many studies. According to previous studies, MT reduces metal-induced oxidative stress in cerebral cortex and cerebellum (Esparza et al., 2003, 2005). Especially, the results of several studies investigating role of MT against CP indicate that the protective effect of MT against CP-induced acute kidney injury could be closely associated with its function in reducing oxidative stress (Hara et al., 2001; López et al., 2008; Sener et al., 2000). In mechanistic studies, MT suppresses CP-induced oxidative stress via different mechanism including nuclear factor erythroid-derived 2-related factor 2/heme oxygenase 1 pathway (Haghi-Aminjan et al., 2018; Kilic et al., 2013). In this study, we investigated the antioxidant effects of MT focusing on the protein expression levels of Klotho along with the phosphorylation of FOXO and AKT. FOXO up-regulates the expression of manganese superoxide dismutase gene encoding a mitochondrial enzyme that detoxifies superoxide, resulting in increased resistance to

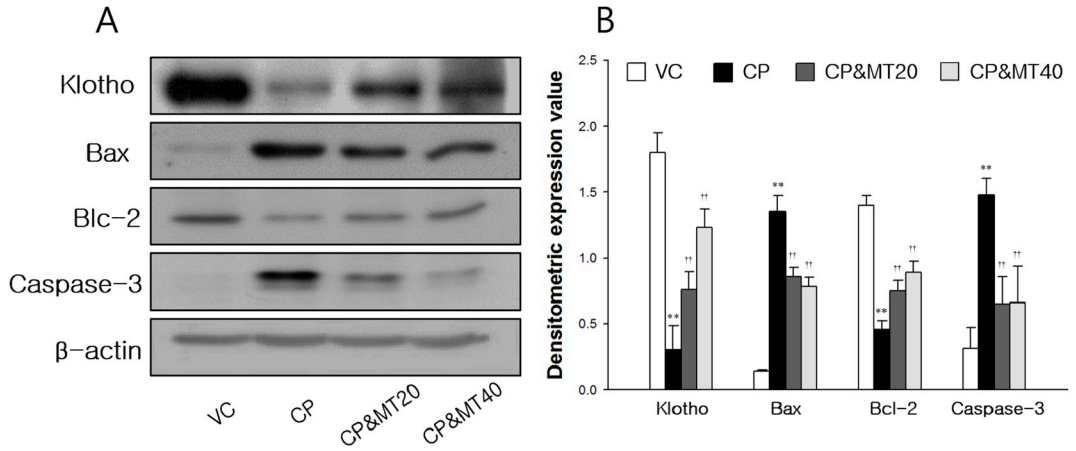

Fig. 9. Effect of melatonin on apoptotic pathways. (A) Western blot analysis of protein levels of Klotho, Bax, Bcl-2, and caspase-3 in the kidneys of the VC, CP, CP&MT20, and CP&MT40 groups. (B) Bar graphs showing Klotho, Bax, Bcl-2, and caspase-3 levels in the kidneys of the VC, CP, CP&MT20, and CP&MT40 groups. Values are presented as means ± SD ($n = 6$). $^{**}P < 0.01$ as compared to the VC group; $^{\dagger\dagger}P < 0.01$ as compared to the cisplatin group. VC, vehicle control; CP, cisplatin treatment (7 mg/kg) with PBS treatment; CP&MT20 and CP&MT40, cisplatin treatment (7 mg/kg) with melatonin treatment (20 and 40 mg/kg/day, respectively).

oxidative stress (Kops et al., 2002). A previous study shown that FOXO is negatively regulated by the phosphorylation of AKT (Yamamoto and Tatar, 2011). In addition, Yamamoto et al. (2005) have demonstrated that Klotho protein can reduce the phosphorylation of AKT and FOXO both *in vitro* and *in vivo*. In the present study, CP administration significantly decreased the expression levels of Klotho accompanied by the induced phosphorylation of AKT and FOXO. By contrast, MT increased the expression levels of Klotho but decreased the phosphorylation of AKT and FOXO. Based on these results, MT could regulate oxidative stress induced by CP via the upregulation of Klotho followed by inhibition of the phosphorylation of AKT and FOXO.

Multiple pathways of apoptosis have been reported in CP-induced acute kidney injury. For example, Wei et al. (2007) reported that CP can induce the activation of Bax pro-apoptotic protein and subsequently lead to the release of apoptogenic factor such as cytochrome *c*. Thus, modulating these factors is considered to be an important step to providing protection against CP-induced apoptosis (Jiang et al., 2007; Wang et al., 2004). It has recently been reported that Klotho is closely associated with apoptotic changes (Sugiura et al., 2005). Specifically, mice overexpressed with Klotho have lower apoptosis in CP-induced kidney injury model than wild type mice (Panesso et al., 2014). CP can induce decreases in renal Klotho protein expression (Panesso et al., 2014). Mice overexpressed with Klotho have lower TUNEL-positivity, Bax/Bcl-2 ratio, and active form of caspase-3 than wild type mice (Panesso et al., 2014). However, Klotho deficient mice show the opposite tendency. MT has been reported to possess anti-apoptotic effects against various diseases such as brain injury caused by subarachnoid hemorrhage, ischemia reperfusion-induced lung injury, and micro-radiation-induced DNA fragmentation in testicular tissue (Chen et al., 2015; Sokolovic et al., 2015; Yang et al., 2015). Considering these points of evidence, we hypothesize that, if MT could modulate the expression levels of Klotho, it would have a protective effect against CP-induced apoptosis in acute kidney injury. In the current study, CP significantly decreased the expression levels of renal Klotho and Bcl-2 but increased the levels of Bax and caspase-3. By contrast, MT reversed CP-induced pro-apoptotic and anti-apoptotic protein levels. These results were confirmed by IHC and TUNEL assay in which MT significantly reduced caspase-3- and TUNEL-positive cells induced by CP. Taken together, these results suggest that MT has an anti-apoptotic property via the upregulation of the expression of Klotho.

In conclusion, in rats, MT has a protective effect against acute kidney injury induced by the administration of CP. This protective effect of MT may be due to the increased antioxidant activity and decreased apoptotic changes associated with the upregulation of renal Klotho. Although future studies of perfect design in methodology, pharmacology, and therapeutics are needed to achieve a correct clinical uses of MT, our results suggest that MT may serve as a protective agent against CP-induced side effects, including acute kidney injury caused by oxidative stress and apoptotic changes.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.04.049.

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