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Melatonin Attenuates Mitochondrial Damage in Aristolochic Acid-Induced Acute Kidney Injury

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Abstract

Aristolochic acid (AA), extracted from Aristolochiaceae plants, plays an essential role in traditional herbal medicines and is used for different diseases. However, AA has been found to be nephrotoxic and is known to cause aristolochic acid nephropathy (AAN). AA-induced acute kidney injury (AKI) is a syndrome in AAN with a high morbidity that manifests mitochondrial damage as a key part of its pathological progression. Melatonin primarily serves as a mitochondria-targeted antioxidant. However, its mitochondrial protective role in AA-induced AKI is barely reported. In this study, mice were administrated 2.5 mg/kg AA to induce AKI. Melatonin reduced the increase in Upro and Scr and attenuated the necrosis and atrophy of renal proximal tubules in mice exposed to AA. Melatonin suppressed ROS generation, MDA levels and iNOS expression and increased SOD activities *in vivo* and *in vitro*. Intriguingly, the *in vivo* study revealed that melatonin decreased mitochondrial fragmentation in renal proximal tubular cells and increased ATP levels in kidney tissues in response to AA. *In vitro*, melatonin restored the mitochondrial membrane potential (MMP) in NRK-52E and HK-2 cells and led to an elevation in ATP levels. Confocal immunofluorescence data showed that puncta containing Mito-tracker and GFP-LC3A/B were reduced, thereby impeding the mitophagy of tubular epithelial cells. Furthermore, melatonin decreased LC3A/B-II expression and increased p62 expression. The apoptosis of tubular epithelial cells induced by AA was decreased. Therefore, our findings revealed that melatonin could prevent AA-induced AKI by attenuating mitochondrial damage, which may provide a potential therapeutic method for renal AA toxicity.

Key Words: Melatonin, Aristolochic acid, Acute kidney injury, Mitochondrial damage, Mitophagy, Oxidative stress

INTRODUCTION

Aristolochic acid (AA) and its derivatives, isolated from the Aristolochiaceae plant family, are a group of nitrophenanthrene carboxylic acids (Balachandran *et al*., 2005; Yang *et al*., 2013). Applications prepared from Aristolochiaceae plants, including AA, have been used for the treatment of diverse diseases, such as arthritis, gout, rheumatism, hypertension, urinary tract infection, and festering wounds (Debelle *et al*., 2008; Anger *et al*., 2020). However, these applications are reported to be nephrotoxic. Aristolochic acid nephropathy (AAN) is a common nephropathy caused by AA (Chen *et al*., 2012; Wang *et al*., 2015). In AAN, most patients rapidly deteriorate to end-stage renal disease (ESRD) (Luciano and Perazella, 2015). Patients with AAN exhibit increased serum creatinine (Scr), severe anemia, rapid tubulointerstitial injury, loss of renal proximal tubules, and tubule atrophy (Priestap *et al*., 2012). Recently, AA has been shown to cause acute kidney

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injury (AKI) and chronic kidney disease (CKD), which are often used for research on the transition from AKI to CKD (Wang *et al*., 2020). Interstitial fibrosis is the chronic pathological sign of CKD (Fu *et al*., 2018). AKI is a syndrome characterized by a sudden decline in renal function. Despite the fact that AKI is a reversible illness, patients who recover from AKI have a higher tendency for developing CKD (Jones *et al*., 2012). Thus, the investigation of AA-induced AKI has important clinical significance.

Proximal tubular epithelial cells are considered the primary target of AA (Chevalier, 2016). Researches have implicated AA causes mitochondrial damage, resulting in the apoptosis and necrosis of tubular epithelial cells (Romanov *et al*., 2015; Anger *et al*., 2020). Reactive oxygen species (ROS) play critical roles in this pathological progression. Damaged mitochondria increase ROS generation, contributing to oxidative stress, which in turn causes further damage to mitochondria (Tang *et al*., 2015). Mitochondrial damage promotes the release of proapoptotic factors, such as cytochrome c, to activate the cell apoptosis pathway (Szeto *et al*., 2017). Recently, the prevention of AAN has been limited to glucocorticoids. Moreover, most patients with ESRD still require renal replacement therapy, such as dialysis or kidney transplantation (Luciano and Perazella, 2015). Therefore, finding protective agents to prevent AA-induced AKI has become an urgent problem to be solved.

Melatonin (N-acetyl-5-methoxytryptamine), a circadian hormone, is mainly secreted by the pineal gland during the night (Singh and Jadhav, 2014). It has acquired a variety of activities at different stages of evolution in mammals, such as immunomodulatory, antiproliferative, antioxidative, anti-inflammatory, and mitochondrial protective activities (El-Sokkary *et al*., 2002; Tan *et al*., 2010; Han *et al*., 2019). Abnormalities in melatonin are associated with cancer, hypertension, diabetes, and neurodegenerative disease (Cardinali *et al*., 2008; Ganguly *et al*., 2010; Hardeland, 2012). We previously reported that melatonin attenuated pulmonary hypertension in rats and inhibited the proliferation of breast cancer cells (Jin *et al*., 2014; Wang *et al*., 2018; Wang *et al*., 2022). Mitochondria are the major organelles of melatonin synthesis (Tan and Hardeland, 2021). It is worth noting that melatonin, a potent protector of mitochondria, decreases ROS production, maintains the mitochondrial membrane potential (MMP), and elevates mitochondrial fusion, thereby protecting mitochondria (Tan *et al*., 2016). Studies have revealed that melatonin-pretreated mitochondria inhibit oxidative stress, inflammation, cellular stress and mitochondrial damage in acute liver ischemia–reperfusion injury (Ko *et al*., 2020). Even though previous work has reported that melatonin exhibites a protective effect against AAN in a CKD mouse model via inhibiting oxidative stress and inflammation, its mechanisms in AA- induced AKI were not clarified (Kim *et al*., 2019). In the present study, we aimed to explore whether melatonin can prevent AA-induced AKI via attenuating mitochondrial damage *in vivo* and *in vitro*.

MATERIALS AND METHODS

Materials

Aristolochic acid I (A5512) was purchased from YUAN YE (Shanghai, China). Melatonin (M5250) was purchased from Sigma Aldrich (Shanghai, China). The human kidney proximal tubular epithelial cell line HK-2 (CRL-2190) and rat kidney proximal tubular epithelial cell line NRK-52E (CRL-1571) were kindly provided by Dr. Feng Zheng (Dalian Medical University, Dalian, China). Antibodies against LC3A/B (12741) caspase-3 (9662), and cleaved caspase-3 (9664) were obtained from Cell Signaling Technology (MA, USA). An antibody against iNOS (18985-1-AP) was purchased from Proteintech (Wuhan, China). An antibody against SQSTM1/p62 (WL02385) was purchased from Wanleibio (Shenyang, China). The ROS assay kit (DCFH-DA, S0033), superoxide dismutase (SOD) assay kit (S0101), malondialdehyde (MDA) assay kit (S0131), adenosine triphosphate (ATP) assay kit (S0026), BCA protein assay kit (P0012) and TUNEL assay kit (C1088) were purchased from Beyotime (Shanghai, China). A tissue ROS assay kit (BB470512) was purchased from Bestbio (Shanghai, China). Mito-tracker (40741ES50) and JC-1 probes (40705ES03) were purchased from YESEN (Shanghai, China). A periodic acid Schiff (PAS) kit (G1280) and Hoechst 33342 (C0030) were purchased from Solarbio (Beijing, China). A creatinine assay kit (C011-2) and urine protein (Upr) assay kit (C035-2) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animal models

Twenty-four Kunming male mice (10 weeks old, 25-30 g) were purchased from the Animal Laboratory Center of Dalian Medical University. Animal experimental protocols and care methods were approved by the Animal Care and Use Ethics Committee of Dalian Medical University. The mice were randomly divided into four groups (six mice per group): (1) control group treated with vehicle; (2) melatonin group treated with melatonin; (3) AA group treated with AA; and (4) AA+melatonin group treated with AA and melatonin. For groups (3) and (4), mice were injected intraperitoneally with AA (2.5 mg/kg/day) for 3 days. For groups (2) and (4), mice were injected intraperitoneally with melatonin (30 mg/kg/day) from 3 days before AA exposure for 10 days, which was performed 30 min prior to AA injection. AA was diluted in saline with 5% dimethyl sulfoxide (DMSO, #D8370, Solarbio). Melatonin was dissolved in phosphate-buffered saline (PBS) with 10% propylene glycol (PEG, #3015708, AR, Sinopharm Chemical Reagent, Co., Ltd, Shanghai, China). The injection volume was 0.1 mL/10 g/mouse. All mice were euthanized on Day 11 after melatonin treatment. Blood and urine were collected for further investigations. Kidney tissue was fixed in 4% paraformaldehyde. Paraffin-embedded renal sections (4 μm) stained with hematoxylin-eosin (HE) and PAS were prepared for histological analysis.

Cell treatment

The NRK-52E or HK-2 cells were incubated overnight at 37°C with 5% CO_2. Then, the cells were subjected to different concentrations (0, 0.5, 2.5, 5, 10 μg/mL) of AA for 24, 48, and 72 h to assess cytotoxicity. For melatonin treatment, the cells were incubated with 2.5 μg/mL AA and 1 mM melatonin for 48 h.

Cell viability measurement

Cell viability was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Wang *et al*., 2018). After culturing in 96-well plates (5×10^3 cells per well) at 37°C with 5% CO_2 overnight, the cells were incubated

with 10 μL MTT (0.5 mg/mL) for 4 h at 37°C. Then, the supernatant was discarded, 150 μL DMSO was added and the cells were shaken evenly. The absorbance was detected at 490 nm with a microplate reader (Perkin Elmer, MA, USA).

Colony formation assay

NRK-52E or HK-2 cells (5×10^2 cells per well) were seeded in a 6-well plate and incubated for 10-14 days at 37°C with 5% CO_2. Then, colonies were fixed with 2% paraformaldehyde, stained with a 0.05% crystal violet solution, and counted under an inverted microscope (Leica, Solms, Germany).

ROS measurement

For ROS detection in the kidneys, the tissue homogenate was centrifuged at 1,000×g for 3 min at 4°C. Then, the precipitate was discarded. 200 μL of homogenate supernatant and 2 μL of dihydroethidium (DHE) probe were added to the 96-well plate and incubated at 37°C for 30 min in the dark. The fluorescence intensity was measured by a microplate reader (Perkin Elmer). For ROS detection *in vitro*, the cells were incubated with DCFH-DA (10 μM) for 30 min at 37°C. Fluorescence images were obtained by a fluorescence microscope (Olympus, Tokyo, Japan).

MDA, SOD and ATP content measurements

The MDA, SOD, and ATP levels were measured by commercial assay kits. A detailed manipulation process was performed according to the instructions of the manufacturer. The optical density was determined by a multifunctional enzyme marker (Perkin Elmer).

Western blot analysis

The cells were collected and lysed in RIPA buffer. Protein concentrations were measured by a BCA kit (Beyotime). The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as previously reported (Wang *et al*., 2018). The proteins were transferred to a nitrocellulose membrane for 1.5 h. Then, the membrane was blocked with 5% skim milk for 2 h at room temperature. After incubation with the primary antibodies against iNOS (1:1,000), LC3A/B (1:1,000), p26 (1:1,000), caspase 3 (1:1,000), and cleaved-caspase (1:1,000) overnight at 4°C, the membranes were exposed to the secondary antibodies for 1.5 h and analyzed by chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). The images were analyzed by Image Lab software (Bio-Rad Laboratories, CA, USA).

Transmission electron microscopy (TEM) assessment

Kidneys were first fixed with glutaraldehyde and then settled in osmium tetroxide. After dehydration in ethanol and embedding in Epon, the tissues were cut into 70 nm sections. Next, the sections were subjected to uranyl acetate and lead citrate staining. The stained sections were checked at low magnification (×3,000) to locate the renal proximal tubules. Cells

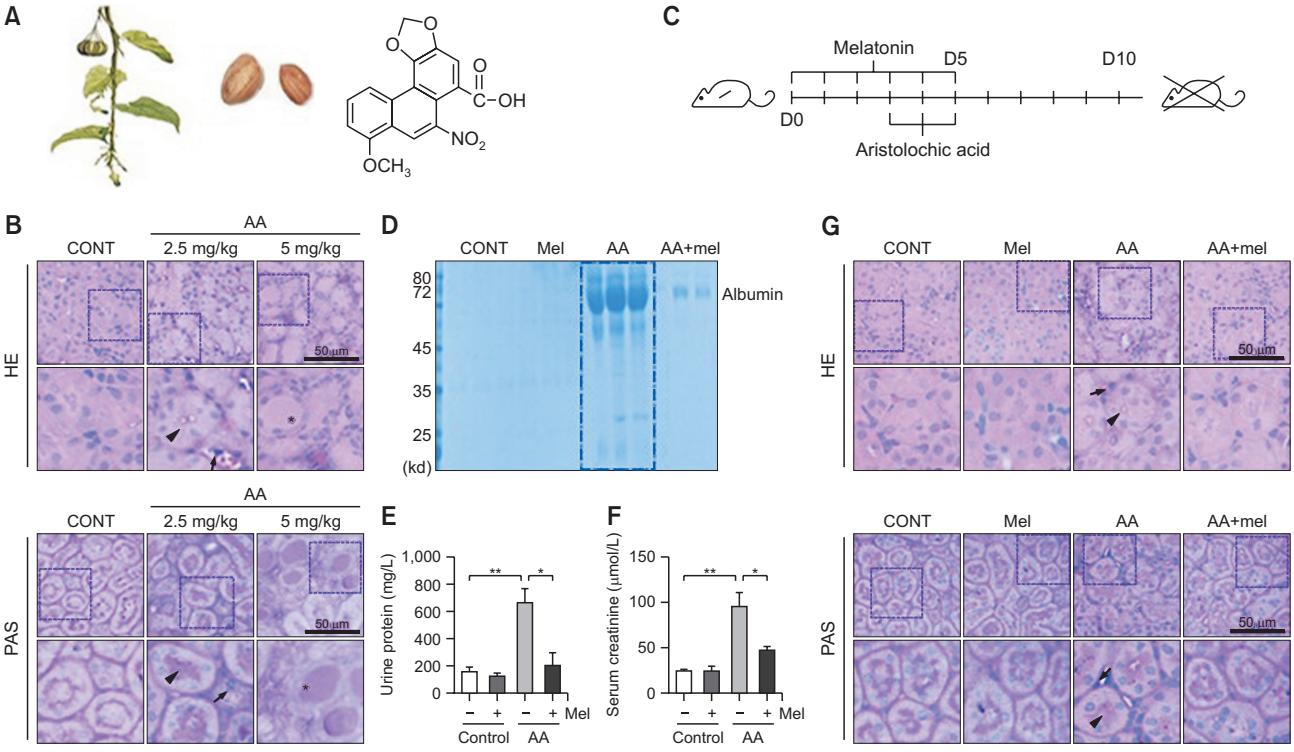

Fig. 1. Melatonin prevented AA-induced acute renal proximal tubular injury. (A) The picture of Aristolochia and the chemical structure of aristolochic acid (AA). (B) Hematoxylin-eosin (HE) and periodic acid Schiff (PAS) staining in renal sections of mice (scale bars: 50 μm). Tubular necrosis (arrow), tubular atrophy (arrowhead), and tubular casting (asterisk). (C) The treatment schedule of melatonin in AA-induced AKI mice model. (D) Coomassie blue staining detected urinary protein (Upro) at day 11 after melatonin treatment. (E) Upro and (F) serum creatinine (Scr) detection at day 11 after melatonin treatment. (G) HE and PAS staining in renal sections of mice (scale bars: 50 μm). CONT means control. Mel means melatonin. Values are means ± SEM, *p<0.05, **p<0.01, n=6.

in the proximal tubules were viewed at a high magnification (×10,000) to observe mitochondria. Finally, the images were collected by TEM (JEM-2000EX, JEOL, Tokyo, Japan). The lengths of mitochondria were measured by NIH ImageJ tracing software (NIH, MD, USA). Approximately 50 mitochondria were measured in each cell. The percentage of cells containing less than 1% long (>1 μm) mitochondria was calculated to evaluate the levels of mitochondrial fragmentation.

Mitochondrial transmembrane potential assay

The cells were cultured in a 6-well plate and treated with AA or melatonin for 24 h, followed by incubation with the JC-1 probe (2 μM) in PBS (pH 7.4) at 37°C for 30 min in the dark. Due to the sensitivity to MMP, JC-1 accumulates in the matrix of mitochondria to form aggregates at a high MMP and emits a red fluorescence. However, if JC-1 is blocked from accumulating in the matrix of mitochondria due to a low MMP, the JC-1 monomer generates a green fluorescence. Images were obtained by a fluorescence microscope (Olympus).

Immunofluorescence colocalization measurement

The cells (1-5×10^5 per well) were seeded on polyline-coated glass slides in a 6-well plate. Then, the cells were incubated with Mito-tracker Red for 30 min, fixed in 100% methanol for 20 min at –20°C, washed with PBS, and placed in 0.2% Triton-X-100 for 5 min. After incubation with an anti-LC3A/B (1:200) antibody at 4°C overnight, the slides were treated with the fluorescent secondary antibody (1:100, Abbkine, Wuhan, China) for 2 h. The cell nuclei were stained with DAPI (62248, Invitrogen, Carlsbad, CA, USA). The images were captured by a confocal laser scanning microscope LSM780 (Carl Zeiss, Oberkochen, Germany).

TUNEL assay

For the TUNEL assay of kidney tissues, the slides were deparaffinized, rehydrated, incubated with proteinase K free of DNase (20 μg/mL) for 20 min at 37°C, and washed with PBS. Then, TdT-labelled nucleotide mix was added, and the cells were culture at 37°C for 1 h in the dark. For the TUNEL assay of cells, the slides were assessed as described above

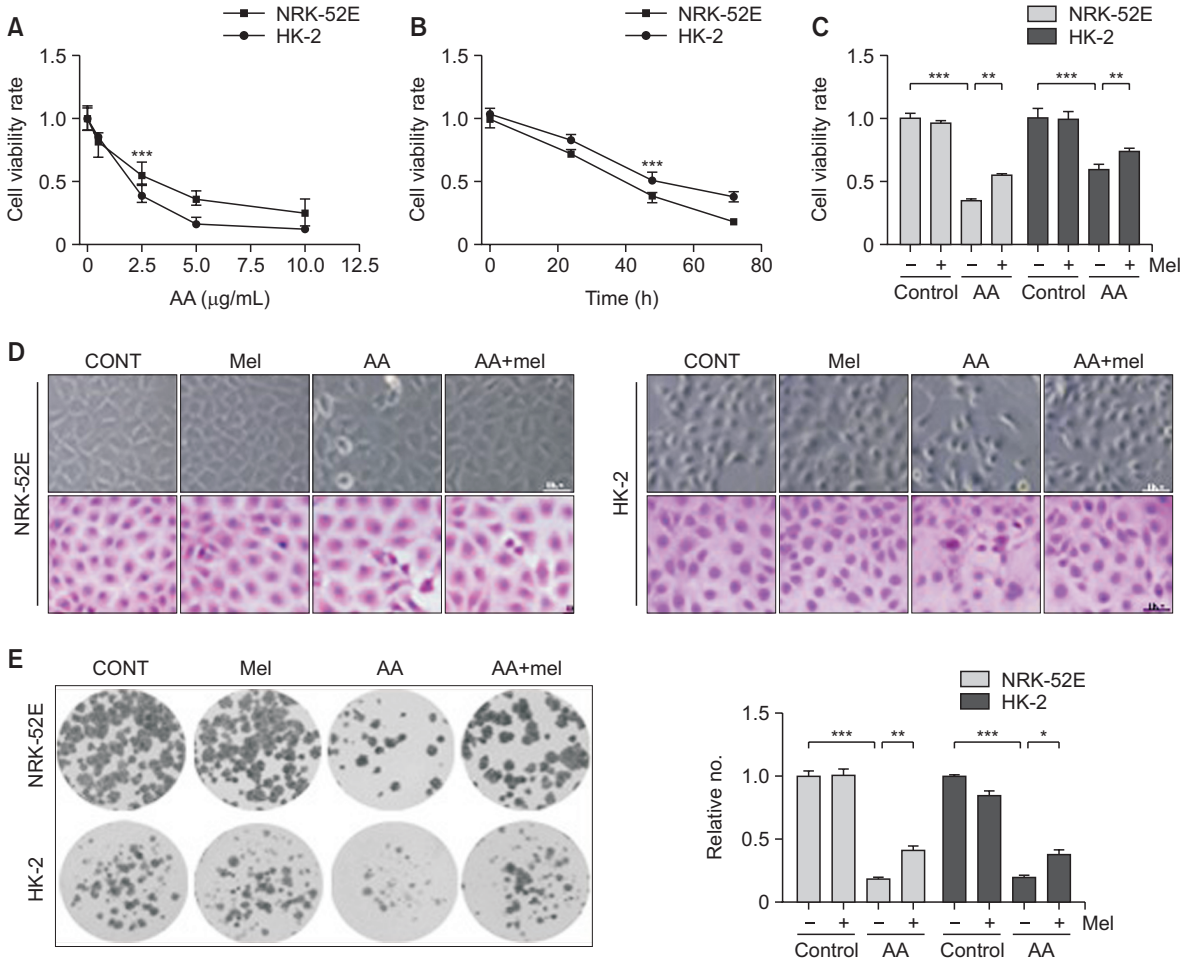

Fig. 2. Melatonin inhibited the cytotoxicity induced by AA in renal proximal tubular epithelial cells. (A, B) MTT assay of NRK-52E and HK-2 cells after AA exposure at different concentrations (0.5, 2.5, 5, 10 μg/mL) and different time points (24, 48, 72 h). (C) MTT assay of NRK-52E and HK-2 cells after melatonin (1 mM) treatment in response to AA (2.5 μg/mL). (D) HE staining of NRK-52E and HK-2 cells (scale bars: 50 μm). (E) Colony formation assay. (F) The statistic result of colony formation rate. CONT means control. Mel means melatonin. Values are means ± SEM, *p<0.05, **p<0.01, ***p<0.001, n=3.

after being fixed with 4% paraformaldehyde for 30 min. The cell nucleus was stained with DAPI. The images were captured by a fluorescence microscope, and the TUNEL positive cells were analyzed by imaging software ImageJ (NIH). The percentage of TUNEL positive cells in 500 cells of each slide was calculated.

Statistical analysis

Statistical analyses were performed by GraphPad Prism 8.0 (GraphPad Software, CA, USA). Comparisons between groups were analyzed by a two-sided Student's t test. Values are presented as the mean ± SEM. Statistically significant differences were considered significant when $p<0.05$.

RESULTS

Melatonin attenuated AA-induced AKI in mice

The appearance of Aristolochia and the chemical structure of aristolochic acid are shown in Fig. 1A. Mice were injected intraperitoneally with AA (2.5 or 5.0 mg/kg) every day for 3 days to induce AKI. HE and PAS staining were used to determine the morphological changes in kidney tissues (Fig. 1B). The results showed that renal proximal tubular necrosis and atrophy were observed in the kidney after exposure 2.5 mg/kg AA. Substantial renal proximal tubular casting was formed after exposure 5.0 mg/kg AA. The kidneys of mice in the control group showed a typical structure.

Next, we chose 2.5 mg/kg AA to explore the effect of melatonin on AKI in mice. The treatment schedule is shown in Fig. 1C. As reflected by a significant increase in urinary protein (Upro) and Scr levels, AKI was observed in mice of the AA group (Fig. 1D-1F). However, melatonin (30 mg/kg) treatment repressed the increase in Scr and Upro in response to AA. Scr and Upro showed no significant change in the melatonin alone treatment group. As demonstrated in Fig. 1G, renal proximal tubular necrosis and atrophy were observed in the AA exposure group. After melatonin treatment, these morphological

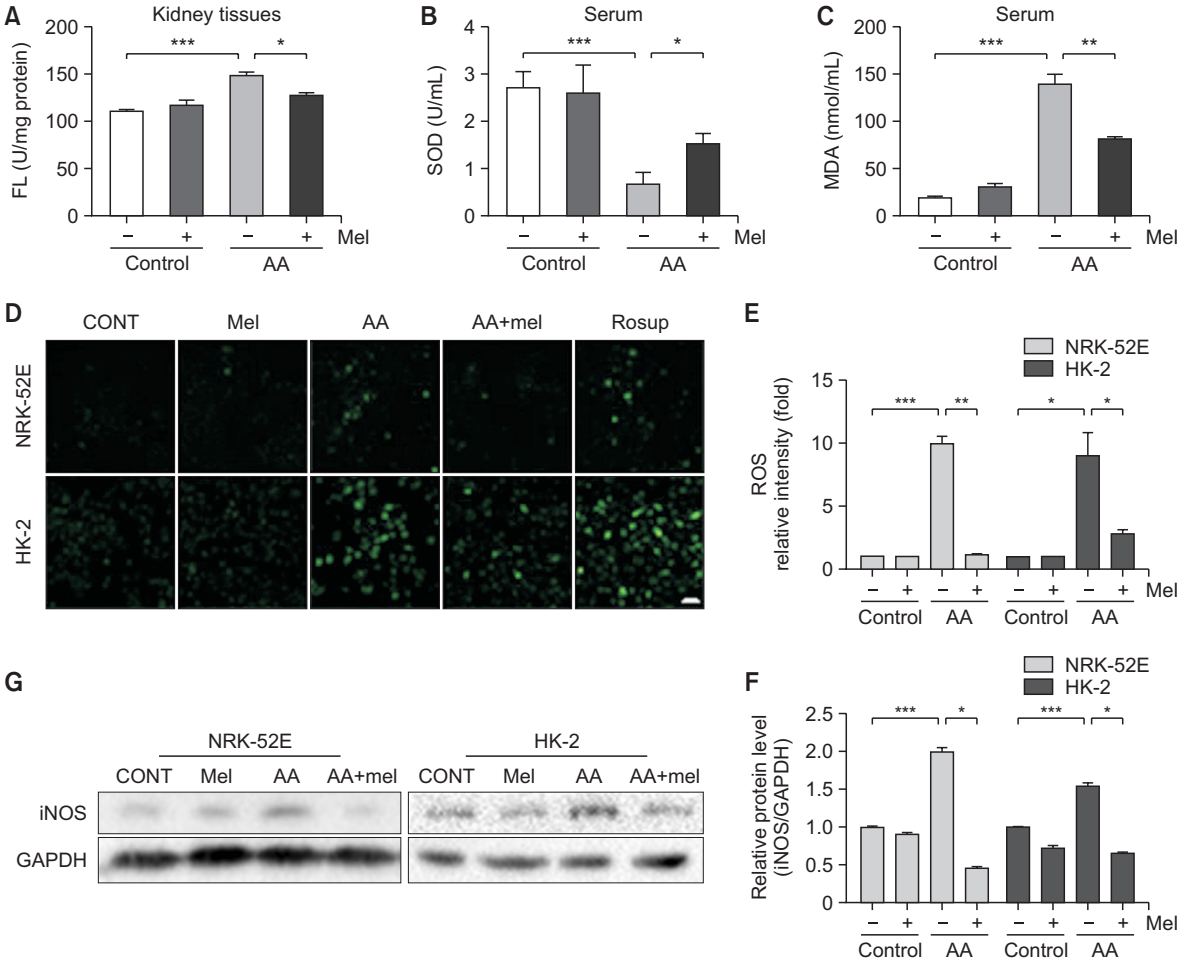

Fig. 3. Melatonin repressed the oxidative stress induced by AA *in vivo* and *in vitro*. (A) ROS detection using DHE probe in kidney tissues of mice (n=6). (B) SOD detection in serum of mice (n=6). (C) MDA detection in serum of mice (n=6). (D) DCFD-DA immunofluorescence detection of intracellular ROS levels in NRK-52E and HK-2 cells after melatonin (1mM) treatment in response to AA (2.5 μg/mL) (scale bars: 50 μm). (E) The statistic result of relative DCFH-DA fluorescence intensity in NRK-52E and HK-2 cells (n=3). (F) Western blot analysis of iNOS expression in NRK-52E and HK-2 cells. (G) The statistic result of relative expression of iNOS to GAPDH. CONT means control. Mel means melatonin. Values are means ± SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

changes were limited to a few proximal tubules. The renal proximal tubules remained a typical structure in the melatonin alone treatment group. Interstitial fibrosis was not observed, and no abnormality was detected within the glomeruli of mice in any group. These data suggested that melatonin attenuated AA-induced AKI in mice.

Melatonin inhibited the cytotoxicity induced by AA in renal proximal tubular epithelial cells

To explore the role of melatonin in AA-induced cytotoxicity *in vitro*, HK-2 and NRK-52E cells were used. AA decreased cell viability in a time- and dose-dependent manner. Nevertheless, melatonin (1 mM) increased cell viability after exposure AA (2.5 μg/mL) (Fig. 2A-2C). The cell morphological results showed that melatonin reversed the cell necrosis induced by AA (Fig. 2D). Consistent with the MTT results, the colony formation results showed that melatonin enhanced the cell colony formation rate (Fig. 2E). Collectively, these data demonstrated that AA led to cytotoxicity in NRK-52E and HK-2 cells. Interestingly, melatonin preserved cell viability.

Melatonin repressed the oxidative stress induced by AA *in vivo* and *in vitro*

Melatonin is a strong mitochondria targeted antioxidant

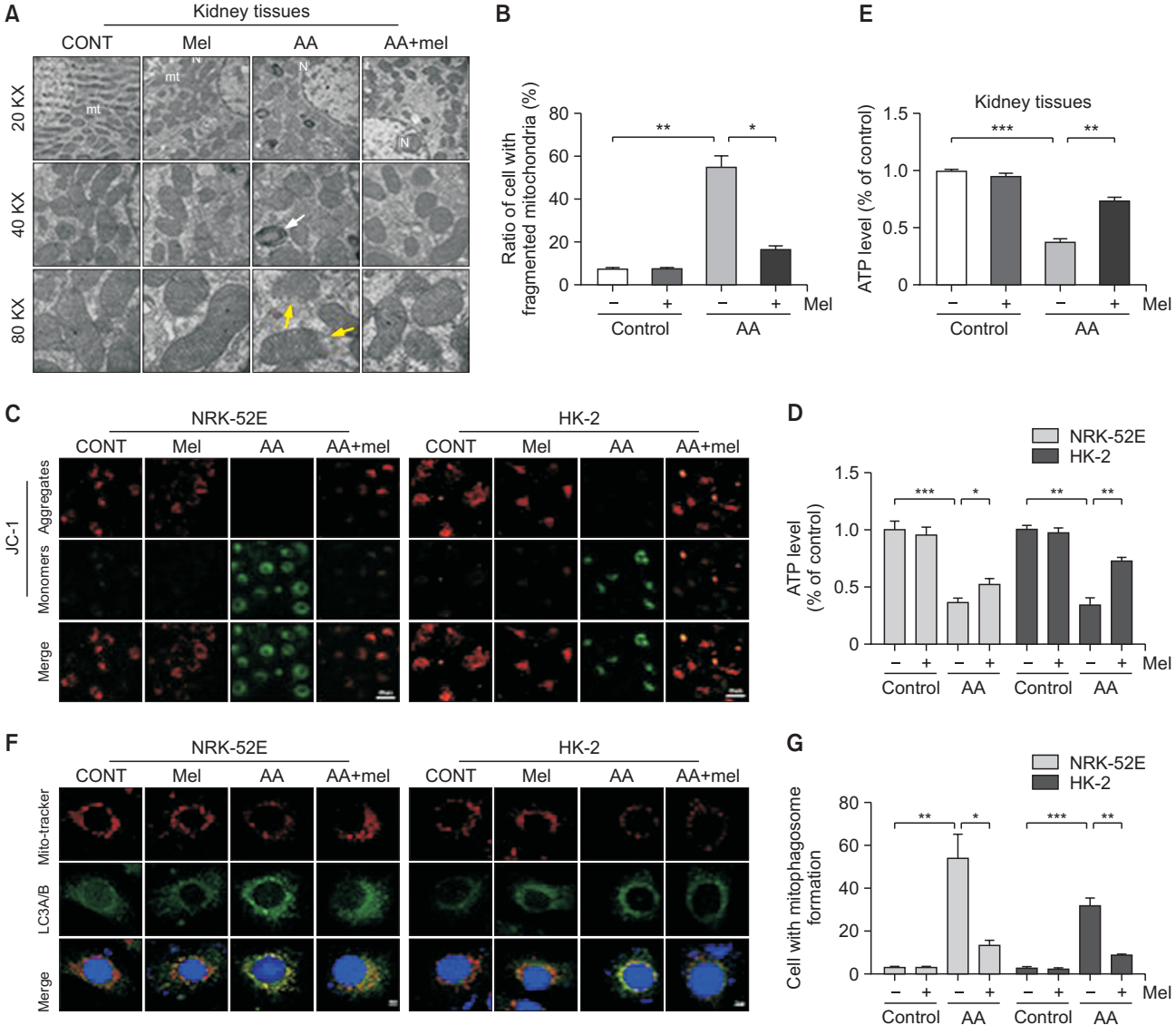

Fig. 4. Melatonin attenuated mitochondrial damage induced by AA. (A) Transmission electron microscopy (TEM) images of mitochondria in renal proximal tubular epithelial cells of mice. Mt: mitochondria, N: nucleus, yellow arrow: damaged mitochondria, white arrow: mitophagosome. (B) The statistic result of mitochondrial fragmentation (n=3). (C) Mitochondrial transmembrane potential detection by JC-1 probes in NRK-52E and HK-2 cells after melatonin (1 mM) treatment in response to AA (2.5 μg/mL) (scale bars: 20 μm) (n=3). (D) ATP levels in NRK-52E and HK-2 cells (n=4). (E) ATP levels in kidney tissues of mice (n=6). (F) Immunofluorescence colocalization detection in NRK-52E and HK-2 cells using GFP-LC3A/B antibody (green) and Mito-tracker (red) (scale bar: 5 μm). The cell nuclei were stained with DAPI (blue). (G) The statistical result of mitophagosome formation (n=3). CONT means control. Mel means melatonin. Values are means ± SEM, **p*<0.05, ***p*<0.01, ****p*<0.001.

(Tan *et al*., 2016; Reiter *et al*., 2017). Therefore, we next investigated the role of melatonin in AA-induced oxidative stress. *In vivo*, ROS production was enhanced in the kidneys of the AA group compared with those of the control group (Fig. 3A). Nevertheless, melatonin significantly inhibited the generation of ROS induced by AA. Then, SOD and MDA, as oxidative stress biomarkers were measured in the serum of mice. Melatonin increased the SOD activities and decreased the MDA levels in the AA+melatonin group (Fig. 3B, 3C). *In vitro*, we evaluated the ROS level in NRK-52E and HK-2 cells by DCFH-DA staining. NRK-52E and HK-2 cells incubated with AA showed a dramatic increase in the fluorescence intensity of DCFH-DA. However, melatonin reversed the increased fluorescence intensity (Fig. 3D, 3E). Additionally, iNOS expression was increased in NRK-52E and HK-2 cells after AA incubation, and melatonin inhibited the expression of iNOS (Fig. 3F, 3G). Thus, we proved that melatonin repressed oxidative stress in AA-induced AKI.

Melatonin attenuated mitochondrial damage in AA-induced AKI

Given that melatonin inhibited AA-induced oxidative stress, we further explored whether this effect was related to its mitochondrial protective activity. Mitochondrial morphology in the renal proximal tubules of mice was examined by TEM (Fig. 4A, 4B). AA caused mitochondrial fragmentation and loss of cristae and mitophagosomes in the renal proximal tubular epithelial cells of mice. Melatonin treatment reversed these changes induced by AA. The mitochondria of tubular epithelial cells were normal in size and shape in the melatonin alone treatment group. *In vitro*, a JC-1 probe was used to evaluate the mitochondrial transmembrane potential of tubular epithelial cells (Fig. 4C). JC-1 formed red fluorescent aggregates in the mitochondrial matrix of NRK-52E and HK-2 cells in the control group. AA blocked the formation of JC-1 aggregates, leading to the generation of JC-1 monomers (green fluorescence) in the mitochondria, suggesting that AA damaged the MMP. However, melatonin reversed the changes induced by AA. The ATP levels were also assessed (Fig. 4D, 4E). AA repressed ATP levels compared with the control group *in vitro* and *in vivo*. However, melatonin increased the levels of ATP.

Since the TEM study showed that melatonin reduced mitophagosomes in tubular epithelial cells *in vivo*, we assessed mitophagy in NRK-52E and HK-2 cells by Immunofluorescence colocalization detection using Mito-tracker and GFP-LC3A/B. The results showed that puncta containing Mito-tracker and GFP-LC3A/B were more abundant in the AA group, indicating increased autophagosome formation in mitochondria. Notably, melatonin reduced the puncta induced by AA (Fig. 4F, 4G). These results demonstrated that melatonin reversed the mitochondrial damage in AA-induced AKI.

Melatonin prevented the autophagy of renal proximal tubular epithelial cells induced by AA

Next, we further explored whether melatonin inhibited autophagy of tubular epithelial cells induced by AA. Immunofluorescence staining assays were performed in NRK-52E and HK-2 cells using an anti-LC3A/B antibody (Fig. 5A, 5B). LC3A/B were increased in the AA group compared with the control group, indicating that AA caused autophagosome formation in NRK-52E and HK-2 cells. Melatonin repressed the autophagy induced by AA. In accordance with the immuno-

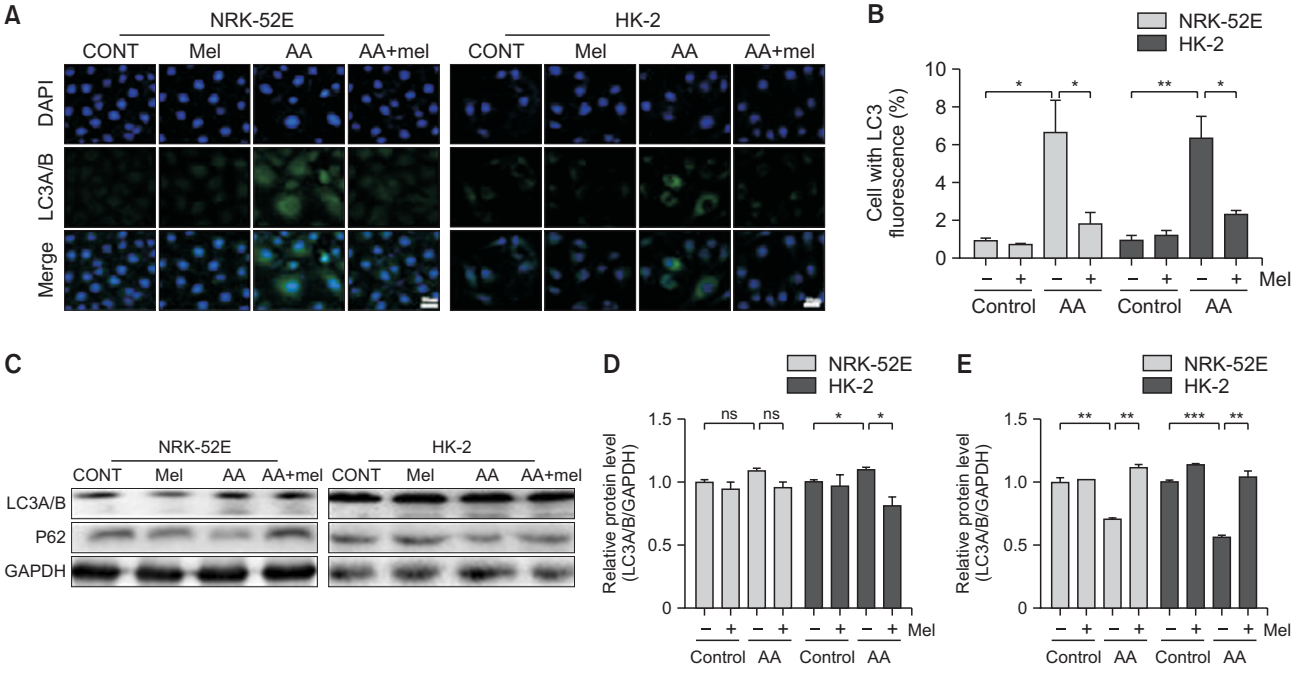

Fig. 5. Melatonin prevented the autophagy of renal proximal tubular epithelial cells induced AA. (A) Immunofluorescent detection of LC3A/B expression in NRK-52E and HK-2 cells after melatonin (1 mM) treatment after exposure AA (2.5 μg/mL) (scale bars: 20 μm). Cell nuclei were stained with DAPI (blue). (B) The statistic result of LC3A/B expression. (C) Western blot analysis of LC3A/B-I/II and P62 expression. (D, E) The statistic result of LC3A/B-I/II and P62 expression to GAPDH. CONT means control. Mel means melatonin. Values are means ± SEM, *p<0.05, **p<0.01, ***p<0.001, n=3.

fluorescence results, western blot analysis showed that the autophagy-related protein LC3A/B-II was increased and that the autophagy substrate p62 was reduced after AA exposure. Melatonin reduced the protein levels of LC3A/B-II in HK-2 cells and increased the protein levels of p62 in both NRK-52E and HK-2 cells (Fig. 5C-5E). Our data confirmed that melatonin suppressed AA-activated autophagy in renal proximal tubular epithelial cells.

Melatonin inhibited the apoptosis of renal proximal tubular epithelial cells caused by AA

To determine the role of melatonin in the apoptosis of renal proximal tubular epithelial cells after exposure AA, a TUNEL assay was performed on the mouse kidneys. As shown in Fig. 6A and 6B, there were fewer TUNEL-positive cells in the kidneys in the AA+melatonin group than in the AA group. *In vitro*, AA increased the number of TUNEL-positive in NRK-52E and HK-2 cells; however, melatonin reversed the change induced by AA (Fig. 6C, 6D). The above results were confirmed by cleaved caspase-3 expression (Fig. 6E, 6F). AA enhanced the expression of cleaved caspase-3. After melatonin treatment, cleaved caspase-3 protein levels were reduced. These findings suggested that melatonin inhibited AA-mediated apoptosis of tubular epithelial cells *in vivo* and *in vitro*.

DISCUSSION

In the present study, we successfully established an AA-induced AKI mouse model characterized by increased Upr and Scr, necrosis, and atrophy in the renal proximal tubules. Melatonin remarkably prevented the biochemical and morpho-

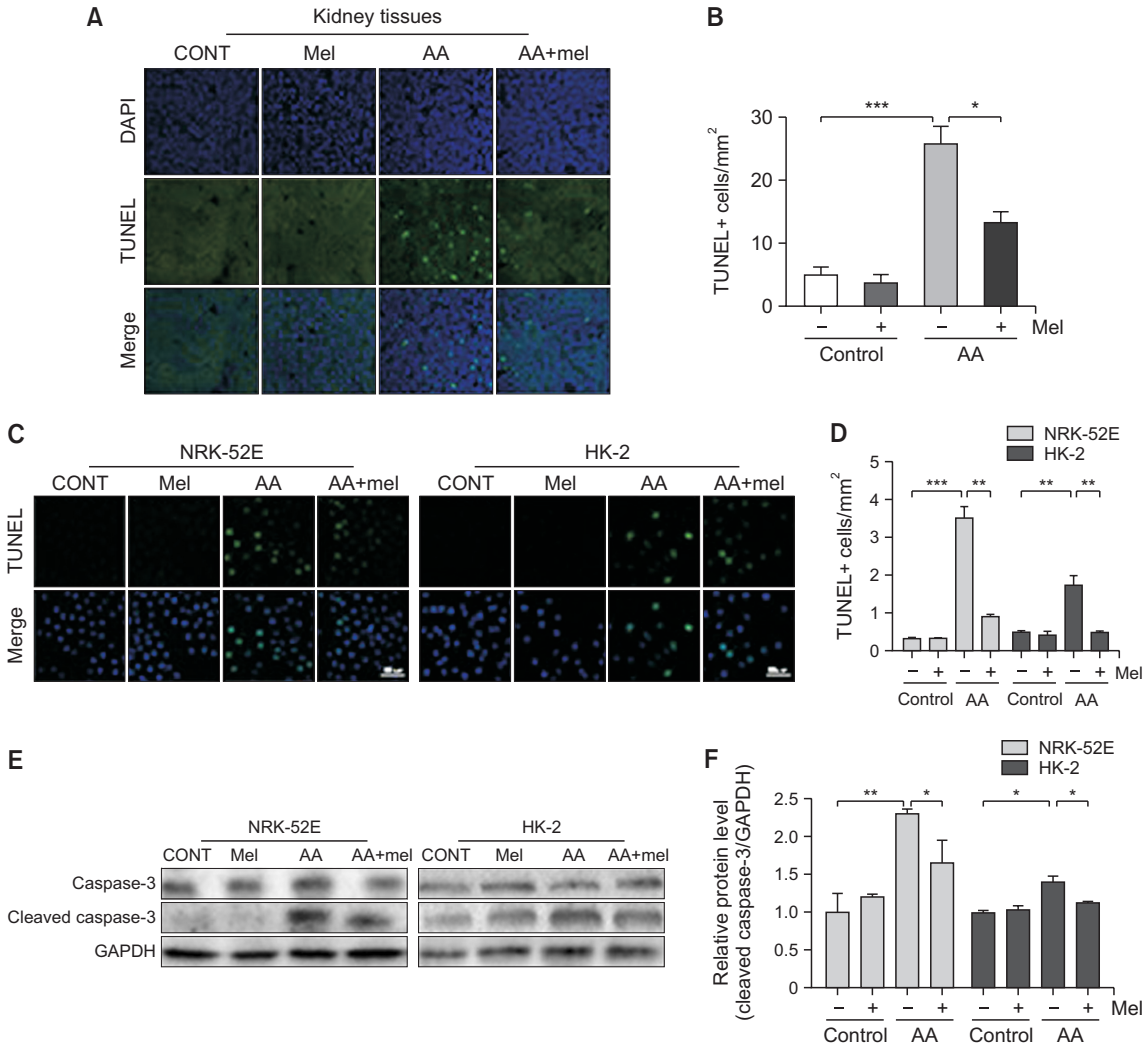

Fig. 6. Melatonin inhibited the apoptosis of renal proximal tubular epithelial cells induced by AA *in vivo* and *in vitro*. (A) TUNEL staining in kidney sections of mice (scale bars: 20 μm). (B) The statistic result of the percentage of TUNEL positive cells in kidney sections of mice (n=6). (C) TUNEL staining in NRK-52E and HK-2 cells after melatonin (1 mM) treatment under AA (2.5 μg/mL) exposure (scale bars: 20 μm). (D) The statistical result of the percentage of TUNEL positive cells in NRK-52E and HK-2 cells (n=3). (E) Western blot analysis of caspase-3 and cleaved caspase-3 expression. (F) The statistic result of relative expression of cleaved caspase-3 to GAPDH (n=3). CONT means control. Mel means melatonin. Values are means ± SEM, *p<0.05, **p<0.01, ***p<0.001.

logical changes, along with the oxidative stress caused by AA. In particular, melatonin reduced mitochondrial fragmentation, restored MMP, increased ATP levels and repressed the mitophagy of renal proximal tubular epithelial cells responding to AA. Eventually, autophagy and apoptosis of tubular epithelial cells exposed to AA were reversed, and cytotoxicity was inhibited. Therefore, our study revealed that melatonin prevents AA-induced AKI by attenuating mitochondrial damage (Fig. 7).

An increasing number of reports have suggested that damaged mitochondria are implicated in the pathogenesis of acute and chronic kidney diseases, including AAN, which lead to significant changes in mitochondrial morphology and function (Hall and Schuh, 2016; Tang *et al*., 2018; Anger *et al*., 2020). Reversing mitochondrial dysfunction has been shown to prevent tubular epithelial cells death and maintain renal function (Brooks *et al*., 2009; Ishimoto and Inagi, 2016; Suzuki *et al*., 2016). Mitochondria are intracellular organelles and function as the powerhouse because of their ability to generate ATP, which provides energy to support basic cell activities (Li *et al*., 2006). AA induces a decrease in ATP levels and mitochondrial membrane depolarization in renal proximal tubular epithelial cells (Qi *et al*., 2007). Melatonin, a potent protector of mitochondria, was shown to be more effective than mitochondrial-targeted antioxidative products, such as vitamin E (MitoE) and coenzyme Q10 (MitoQ) (Tan *et al*., 2016). Mitochondria are biological targets of melatonin, playing an antitumor role through the mitochondrial apoptosis pathway (Huo *et al*., 2017; Yu *et al*., 2018). Besides, melatonin has been shown to rescue mitochondrial function from renal fibrosis in diabetic mice and regulate mitochondrial bioenergetics to protect mesenchymal stem cells in chronic kidney disease (Han *et al*., 2019; Tamarindo *et al*., 2019). In this study, melatonin reduced fragmented mitochondria, restored MMP, and increased levels of ATP in tubular epithelial cells. In addition, autophagy has been found to promote AA-induced kidney injury (Yang *et al*., 2013). Mitophagy is a form of autophagy that selectively eliminates dysfunctional and damaged mitochondria to autophagosomes. However, excessive activation of mitophagy may deteriorate the pathological progression of diabetic nephropathy (Higgins and Coughlan, 2014). In the present study, we demonstrated that AA caused autophagy and mitophagy in renal proximal tubular epithelial cells in AA-induced AKI. However, melatonin reduced the expression of the autophagy marker LC3A/B-II expression and enhanced the expression of the autophagy substrate p62 expression, thereby inhibiting mitophagy and autophagy in tubular epithelial cells. All these data suggested that melatonin protected mitochondria from AA-induced damage in AKI.

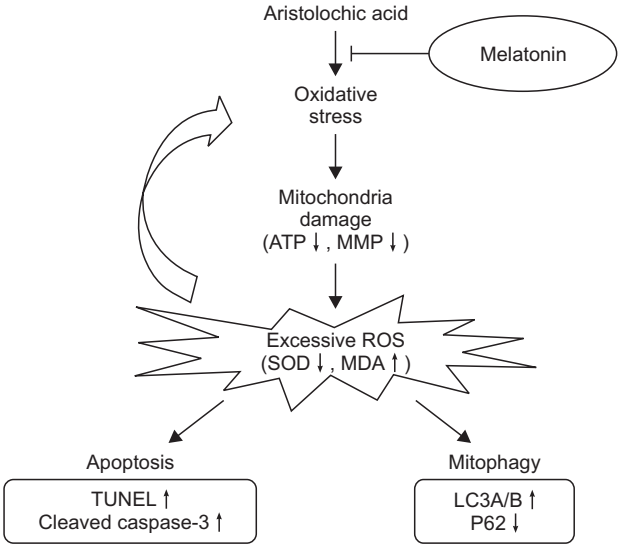

Fig. 7. Schematic diagram of this study. Melatonin prevents AA-induced AKI via attenuating mitochondrial damage.

Mitochondria are also a leading source of ROS in cells. Excessive ROS generated by mitochondria lead to oxidative stress, which in turn enhances ROS production and further damages mitochondria (Tang *et al*., 2015). Numerous reports have demonstrated that ROS are increased in renal tubular epithelial cells exposed to AA (Romanov *et al*., 2015; Wang *et al*., 2019; Zhang *et al*., 2019). AA-induced oxidative stress has been associated with decreased SOD2 and glutathione synthetase mRNA levels and increased MDA contents (Kim *et al*., 2019). In this work, melatonin reduced ROS generation, increased SOD levels, and decreased MDA levels and iNOS expression *in vivo* and *in vitro*. ROS and mitochondrial membrane depolarization also contribute to DNA damage and cell apoptosis (Thangam *et al*., 2014; Dai *et al*., 2015). Increased apoptosis has been found in AA-induced cancers (Cosyns *et al*., 1999; Chang *et al*., 2007; Simoes *et al*., 2008) and cultured tubular epithelial cells exposed to AA (Hsin *et al*., 2006; Zhou *et al*., 2010). Apoptosis of tubular epithelial cells is one of the central mechanisms leading to AAN (Pozdzik *et al*., 2008; Romanov *et al*., 2015). In this study, we provided evidence to support the role of damaged mitochondria in AA-induced AKI. Damaged mitochondria produce excessive ROS and release proapoptotic factors such as cytochrome c, which may ultimately result in the death of renal tubular epithelial cells (Sweetwyne *et al*., 2017; Szeto *et al*., 2017). We found that AA caused a significant increase in TUNEL-positive cells *in vivo* and *in vitro*. Melatonin inhibited AA-induced tubular epithelial cell apoptosis and decreased the expression of cleaved-caspase3.

In summary, melatonin exhibited a protective role in AA-induced AKI *in vivo* and *in vitro*. Melatonin attenuated mitochondrial damage by reducing mitochondrial fragmentation, restoring MMP, increasing ATP levels and repressing mitophagy in renal proximal tubular epithelial cells exposed to AA. Eventually, melatonin suppressed ROS generation and oxidative stress, inhibiting autophagy and apoptosis of renal tubular epithelial cells. Therefore, our findings suggest that melatonin may offer a novel therapeutic strategy and be a potential protective drug for AA-induced AKI via protecting mitochondria.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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REFERENCES

Anger, E. E., Yu, F. and Li, J. (2020) Aristolochic acid-induced nephrotoxicity: molecular mechanisms and potential protective approaches. *Int. J. Mol. Sci.* **21**, 1157.

Balachandran, P., Wei, F., Lin, R. C., Khan, I. A. and Pasco, D. S. (2005) Structure activity relationships of aristolochic acid analogues: toxicity in cultured renal epithelial cells. *Kidney Int.* **67**, 1797-1805.

Brooks, C., Wei, Q., Cho, S. G. and Dong, Z. (2009) Regulation of mitochondrial dynamics in acute kidney injury in cell culture and rodent models. *J. Clin. Invest.* **119**, 1275-1285.

Cardinali, D. P., Esquifino, A. I., Srinivasan, V. and Pandi-Perumal, S. R. (2008) Melatonin and the immune system in aging. *Neuroimmunomodulation* **15**, 272-278.

Chang, H. R., Lian, J. D., Lo, C. W., Huang, H. P. and Wang, C. J. (2007) Aristolochic acid-induced cell cycle G1 arrest in human urothelium SV-HUC-1 cells. *Food Chem. Toxicol.* **45**, 396-402.

Chen, C. H., Dickman, K. G., Moriya, M., Zavadil, J., Sidorenko, V. S., Edwards, K. L., Gnatenko, D. V., Wu, L., Turesky, R. J., Wu, X. R., Pu, Y. S. and Grollman, A. P. (2012) Aristolochic acid-associated urothelial cancer in Taiwan. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 8241-8246.

Chevalier, R. L. (2016) The proximal tubule is the primary target of injury and progression of kidney disease: role of the glomerulotubular junction. *Am. J. Physiol. Renal Physiol.* **311**, F145-F161.

Cosyns, J. P., Jadoul, M., Squifflet, J. P., Wese, F. X. and van Ypersele de Strihou, C. (1999) Urothelial lesions in Chinese-herb nephropathy. *Am. J. Kidney Dis.* **33**, 1011-1017.

Dai, H., Deng, Y., Zhang, J., Han, H., Zhao, M., Li, Y., Zhang, C., Tian, J., Bing, G. and Zhao, L. (2015) PINK1/Parkin-mediated mitophagy alleviates chlorpyrifos-induced apoptosis in SH-SY5Y cells. *Toxicology* **334**, 72-80.

Debelle, F. D., Vanherweghem, J. L. and Nortier, J. L. (2008) Aristolochic acid nephropathy: a worldwide problem. *Kidney Int.* **74**, 158-169.

El-Sokkary, G. H., Omar, H. M., Hassanein, A. F., Cuzzocrea, S. and Reiter, R. J. (2002) Melatonin reduces oxidative damage and increases survival of mice infected with Schistosoma mansoni. *Free Radic. Biol. Med.* **32**, 319-332.

Fu, Y., Tang, C., Cai, J., Chen, G., Zhang, D. and Dong, Z. (2018) Rodent models of AKI-CKD transition. *Am. J. Physiol. Renal Physiol.* **315**, F1098-F1106.

Ganguly, K., Sharma, A. V., Reiter, R. J. and Swarnakar, S. (2010) Melatonin promotes angiogenesis during protection and healing of indomethacin-induced gastric ulcer: role of matrix metaloproteinase-2. *J. Pineal Res.* **49**, 130-140.

Hall, A. M. and Schuh, C. D. (2016) Mitochondria as therapeutic targets in acute kidney injury. *Curr. Opin. Nephrol. Hypertens.* **25**, 355-362.

Han, Y. S., Kim, S. M., Lee, J. H., Jung, S. K., Noh, H. and Lee, S. H. (2019) Melatonin protects chronic kidney disease mesenchymal stem cells against senescence via PrP(C)-dependent enhancement of the mitochondrial function. *J. Pineal Res.* **66**, e12535.

Hardeland, R. (2012) Neurobiology, pathophysiology, and treatment of melatonin deficiency and dysfunction. *ScientificWorldJournal* **2012**, 640389.

Higgins, G. C. and Coughlan, M. T. (2014) Mitochondrial dysfunction and mitophagy: the beginning and end to diabetic nephropathy? *Br. J. Pharmacol.* **171**, 1917-1942.

Hsin, Y. H., Cheng, C. H., Tzen, J. T., Wu, M. J., Shu, K. H. and Chen, H. C. (2006) Effect of aristolochic acid on intracellular calcium concentration and its links with apoptosis in renal tubular cells. *Apoptosis* **11**, 2167-2177.

Huo, X., Wang, C., Yu, Z., Peng, Y., Wang, S., Feng, S., Zhang, S., Tian, X., Sun, C., Liu, K., Deng, S. and Ma, X. (2017) Human transporters, PEPT1/2, facilitate melatonin transportation into mitochondria of cancer cells: an implication of the therapeutic potential. *J. Pineal Res.* **62**, e12390.

Ishimoto, Y. and Inagi, R. (2016) Mitochondria: a therapeutic target in acute kidney injury. *Nephrol. Dial. Transplant.* **31**, 1062-1069.

Jin, H., Wang, Y., Zhou, L., Liu, L., Zhang, P., Deng, W. and Yuan, Y. (2014) Melatonin attenuates hypoxic pulmonary hypertension by inhibiting the inflammation and the proliferation of pulmonary arterial smooth muscle cells. *J. Pineal Res.* **57**, 442-450.

Jones, J., Holmen, J., De Graauw, J., Jovanovich, A., Thornton, S. and Chonchol, M. (2012) Association of complete recovery from acute kidney injury with incident CKD stage 3 and all-cause mortality. *Am. J. Kidney Dis.* **60**, 402-408.

Kim, J. Y., Leem, J. and Jeon, E. J. (2019) Protective effects of melatonin against aristolochic acid-induced nephropathy in mice. *Biomolecules* **10**, 11.

Ko, S. F., Chen, Y. L., Sung, P. H., Chiang, J. Y., Chu, Y. C., Huang, C. C., Huang, C. R. and Yip, H. K. (2020) Hepatic (31) P-magnetic resonance spectroscopy identified the impact of melatonin-pretreated mitochondria in acute liver ischaemia-reperfusion injury. *J. Cell. Mol. Med.* **24**, 10088-10099.

Li, Y., Liu, Z., Guo, X., Shu, J., Chen, Z. and Li, L. (2006) Aristolochic acid I-induced DNA damage and cell cycle arrest in renal tubular epithelial cells *in vitro*. *Arch. Toxicol.* **80**, 524-532.

Luciano, R. L. and Perazella, M. A. (2015) Aristolochic acid nephropathy: epidemiology, clinical presentation, and treatment. *Drug Saf.* **38**, 55-64.

Pozdzik, A. A., Salmon, I. J., Debelle, F. D., Decaestecker, C., Van den Branden, C., Verbeelen, D., Deschodt-Lanckman, M. M., Vanherweghem, J. L. and Nortier, J. L. (2008) Aristolochic acid induces proximal tubule apoptosis and epithelial to mesenchymal transformation. *Kidney Int.* **73**, 595-607.

Priestap, H. A., Torres, M. C., Rieger, R. A., Dickman, K. G., Freshwater, T., Taft, D. R., Barbieri, M. A., and Iden, C. R. (2012) Aristolochic acid I metabolism in the isolated perfused rat kidney. *Chem. Res. Toxicol.* **25**, 130-139.

Qi, X., Cai, Y., Gong, L., Liu, L., Chen, F., Xiao, Y., Wu, X., Li, Y., Xue, X. and Ren, J. (2007) Role of mitochondrial permeability transition in human renal tubular epithelial cell death induced by aristolochic acid. *Toxicol. Appl. Pharmacol.* **222**, 105-110.

Reiter, R. J., Rosales-Corral, S., Tan, D. X., Jou, M. J., Galano, A. and Xu, B. (2017) Melatonin as a mitochondria-targeted antioxidant: one of evolution's best ideas. *Cell. Mol. Life Sci.* **74**, 3863-3881.

Romanov, V., Whyard, T. C., Waltzer, W. C., Grollman, A. P. and Rosenquist, T. (2015) Aristolochic acid-induced apoptosis and G2 cell cycle arrest depends on ROS generation and MAP kinases activation. *Arch. Toxicol.* **89**, 47-56.

Simoes, M. L., Hockley, S. L., Schwerdtle, T., Gamboa da Costa, G., Schmeiser, H. H., Phillips, D. H. and Arlt, V. M. (2008) Gene expression profiles modulated by the human carcinogen aristolochic acid I in human cancer cells and their dependence on TP53. *Toxicol. Appl. Pharmacol.* **232**, 86-98.

Singh, M. and Jadhav, H. R. (2014) Melatonin: functions and ligands. *Drug Discov. Today* **19**, 1410-1418.

Suzuki, T., Yamaguchi, H., Kikusato, M., Hashizume, O., Nagatoishi, S., Matsuo, A., Sato, T., Kudo, T., Matsuhashi, T., Murayama, K., Ohba, Y., Watanabe, S., Kanno, S., Minaki, D., Saigusa, D., Shinbo, H., Mori, N., Yuri, A., Yokoro, M., Mishima, E., Shima, H., Akiyama, Y., Takeuchi, Y., Kikuchi, K., Toyohara, T., Suzuki, C., Ichimura, T., Anzai, J., Kohzuki, M., Mano, N., Kure, S., Yanagisawa, T., Tomioka, Y., Toyomizu, M., Tsumoto, K., Nakada, K., Bonventre, J. V., Ito, S., Osaka, H., Hayashi, K. and Abe, T. (2016) Mitochonic acid 5 binds mitochondria and ameliorates renal tubular and cardiac myocyte damage. *J. Am. Soc. Nephrol.* **27**, 1925-1932.

Sweetwyne, M. T., Pippin, J. W., Eng, D. G., Hudkins, K. L., Chiao, Y. A., Campbell, M. D., Marcinek, D. J., Alpers, C. E., Szeto, H. H., Rabinovitch, P. S. and Shankland, S. J. (2017) The mitochondrial-targeted peptide, SS-31, improves glomerular architecture in mice of advanced age. *Kidney Int.* **91**, 1126-1145.

Szeto, H. H., Liu, S., Soong, Y., Seshan, S. V., Cohen-Gould, L., Manichev, V., Feldman, L. C. and Gustafsson, T. (2017) Mitochondria protection after acute ischemia prevents prolonged upregulation of IL-1beta and IL-18 and arrests CKD. *J. Am. Soc. Nephrol.* **28**, 1437-1449.

Tamarindo, G. H., Ribeiro, D. L., Gobbo, M. G., Guerra, L. H. A., Rahal, P., Taboga, S. R., Gadelha, F. R. and Goes, R. M. (2019) Melatonin and docosahexaenoic acid decrease proliferation of PNT1A prostate benign cells via modulation of mitochondrial bioenergetics and ROS production. *Oxid. Med. Cell. Longev.* **2019**, 5080798.

Tan, D. X. and Hardeland, R. (2021) The reserve/maximum capacity of melatonin's synthetic function for the potential dimorphism of melatonin production and its biological significance in mammals. *Molecules* **26**, 7302.

Tan, D. X., Hardeland, R., Manchester, L. C., Paredes, S. D., Korkmaz, A., Sainz, R. M., Mayo, J. C., Fuentes-Broto, L. and Reiter, R. J. (2010) The changing biological roles of melatonin during evolution: from an antioxidant to signals of darkness, sexual selection and fitness. *Biol. Rev. Camb. Philos. Soc.* **85**, 607-623.

Tan, D. X., Manchester, L. C., Qin, L. and Reiter, R. J. (2016) Melatonin: a mitochondrial targeting molecule involving mitochondrial protection and dynamics. *Int. J. Mol. Sci.* **17**, 2124.

Tang, C., Han, H., Yan, M., Zhu, S., Liu, J., Liu, Z., He, L., Tan, J., Liu, Y., Liu, H., Sun, L., Duan, S., Peng, Y., Liu, F., Yin, X. M., Zhang, Z. and Dong, Z. (2018) PINK1-PRKN/PARK2 pathway of mitophagy is activated to protect against renal ischemia-reperfusion injury. *Autophagy* **14**, 880-897.

Tang, C., He, L., Liu, J. and Dong, Z. (2015) Mitophagy: basic mechanism and potential role in kidney diseases. *Kidney Dis. (Basel)* **1**, 71-79.

Thangam, R., Senthilkumar, D., Suresh, V., Sathuvan, M., Sivasubramanian, S., Pazhanichamy, K., Gorlagunta, P. K., Kannan, S., Gunasekaran, P., Rengasamy, R. and Sivaraman, J. (2014) Induction of ROS-dependent mitochondria-mediated intrinsic apoptosis in MDA-MB-231 cells by glycoprotein from Codium decorticatum. *J. Agric. Food Chem.* **62**, 3410-3421.

Wang, K., Feng, C., Li, C., Yao, J., Xie, X., Gong, L., Luan, Y., Xing, G., Zhu, X., Qi, X. and Ren, J. (2015) Baicalin protects mice from aristolochic acid I-induced kidney injury by induction of CYP1A through the aromatic hydrocarbon receptor. *Int. J. Mol. Sci.* **16**, 16454-16468.

Wang, L., Liu, N., Xue, X. and Zhou, S. (2019) The effect of overexpression of the enhancer of zeste homolog 1 (EZH1) gene on aristolochic acid-induced injury in HK-2 human kidney proximal tubule cells *in vitro*. *Med. Sci. Monit.* **25**, 801-810.

Wang, R., Pan, J., Han, J., Gong, M., Liu, L., Zhang, Y., Liu, Y., Wang, D., Tang, Q., Wu, N., Wang, L., Yan, J., Li, H. and Yuan, Y. (2022) Melatonin attenuates dasatinib-aggravated hypoxic pulmonary hypertension via inhibiting pulmonary vascular remodeling. *Front. Cardiovasc. Med.* **9**, 790921.

Wang, T., Liu, B., Guan, Y., Gong, M., Zhang, W., Pan, J., Liu, Y., Liang, R., Yuan, Y. and Ye, L. (2018) Melatonin inhibits the proliferation of breast cancer cells induced by bisphenol A via targeting estrogen receptor-related pathways. *Thorac. Cancer* **9**, 368-375.

Wang, X., Xue, N., Zhao, S., Shi, Y., Ding, X. and Fang, Y. (2020) Upregulation of miR-382 contributes to renal fibrosis secondary to aristolochic acid-induced kidney injury via PTEN signaling pathway. *Cell Death Dis.* **11**, 620.

Yang, C. C., Wu, C. T., Chen, L. P., Hung, K. Y., Liu, S. H. and Chiang, C. K. (2013) Autophagy induction promotes aristolochic acid-I-induced renal injury *in vivo* and *in vitro*. *Toxicology* **312**, 63-73.

Yu, Z., Tian, X., Peng, Y., Sun, Z., Wang, C., Tang, N., Li, B., Jian, Y., Wang, W., Huo, X. and Ma, X. (2018) Mitochondrial cytochrome P450 (CYP) 1B1 is responsible for melatonin-induced apoptosis in neural cancer cells. *J. Pineal Res.* **65**, e12478.

Zhang, Y., ShiYang, X., Zhang, Y., Li, Y., Shi, X. and Xiong, B. (2019) Exposure to aristolochic acid I compromises the maturational competency of porcine oocytes via oxidative stress-induced DNA damage. *Aging* **11**, 2241-2252.

Zhou, L., Fu, P., Huang, X. R., Liu, F., Lai, K. N. and Lan, H. Y. (2010) Activation of p53 promotes renal injury in acute aristolochic acid nephropathy. *J. Am. Soc. Nephrol.* **21**, 31-41.