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Melatonin therapy protects against renal injury before and after release of bilateral ureteral obstruction in rats

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ABSTRACT

Aim: Blockage of the urinary tract is often connected with renal function impediment, including reductions in glomerular filtration rate (GFR) and the power to control sodium as well as water elimination through urination. Melatonin, known to be the primary product of the pineal gland, prevents renal damage caused by ischemic reperfusion. However, the effects of melatonin on urinary obstruction, as well as release of obstruction induced kidney injury are still largely unknown. The aim of present study was to investigate the effect of melatonin on mediating protection against renal injury triggered from either bilateral ureteral obstruction (BUO) or BUO release (BUO-R).

Main methods: Adult male Sprague-Dawley rats (n = 60) were clustered into six treatment groups: sham treated-1; BUO-non-treated (24 h BUO only); BUO + melatonin; sham treated-2; BUO-48hR (24 h of BUO and then release for 2 days); and BUO-48hR + melatonin. Kidney tissues, blood and urine samples were obtained for further assessment.

Key findings: It was found that melatonin treatment remarkably promoted the recovery of the handling capacity of urinary excretion of water as well as sodium in BUO and BUO-48hR models. Melatonin treatment partially inhibited inflammatory cytokine expression and the downregulation of aquaporin (AQPs, AQP-1, -2 and -3) expression in these two models. Moreover, the cytoarchitecture of BUO rats exposed to melatonin was well preserved.

Significance: Melatonin treatment potently prevents BUO or BUO-R induced renal injury, which may be partially attributed to restoring the expression of AQPs and inhibition of inflammatory response, as well as preserving renal ultrastructural integrity.

1. Introduction

Obstructive nephropathy (ON) is the main cause of end-stage kidney failure in both children and adults. Bilateral ureteral obstruction (BUO) is considered to be a grave clinical condition. In children, BUO is always caused by congenital renal as well as ureter anomalies leading to pediatric BUO. Stones, benign prostatic hyperplasia and urinary tumors happen to be the primary reasons behind BUO onset in the adult population. BUO results in increased ureteral cavity pressure, renal tubules as well as pelvic pressures. Anuria is a severe aftermath of BUO. Polyuria is a common clinical manifestation after BUO release (BUO-R).

The pathophysiological features of BUO involved in renal tubular epithelial cell injury, including apoptosis, proliferation, loss of differentiation and atrophy; recruitment of interstitial inflammatory cells composed predominantly of macrophages and T cells; and interstitial fibrosis characterized by an increase and activation of interstitial fibroblasts, deposition of extracellular matrix proteins and loss of peritubular capillaries [1,2]. Complete ureteral obstruction reduces renal blood flow (RBF), glomerular filtration rate (GFR), and tubular function [3]. The principal abnormalities in tubular function that develop consequent to the tubular epithelial cell injury are impaired reabsorption of solutes and water, and reduced capacity to concentrate the urine [1]. Most of these tubular abnormalities are especially evident after relief of obstruction and may persist as permanent abnormalities, depending on the duration of ureteral obstruction [4–7]. In addition, urinary tract obstruction is also associated with a marked interstitial

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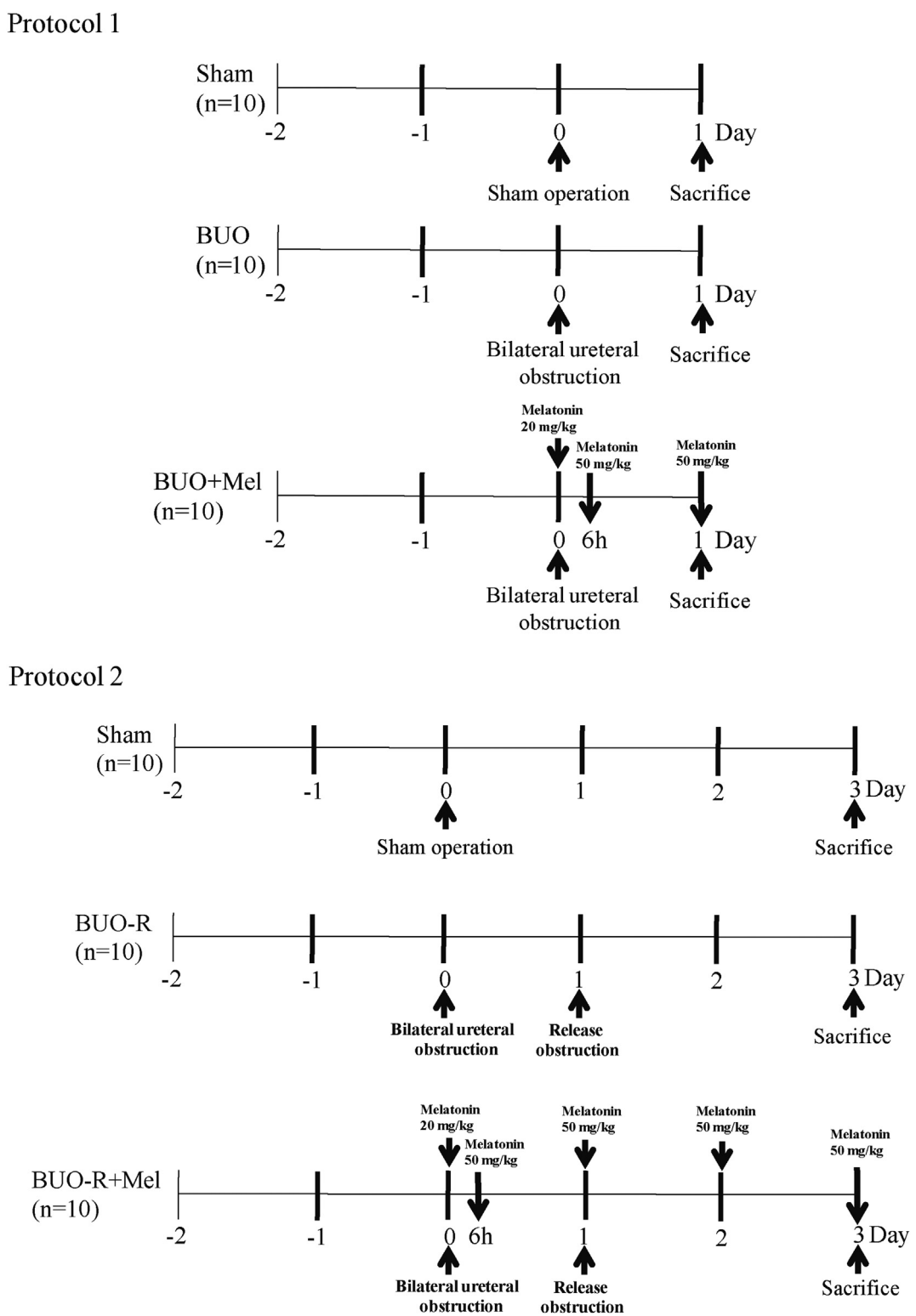

Fig. 1. Diagram of study design. Bilateral ureteral obstruction (BUO) was established by temporary bilateral ureters obstruction for 24 h, and then released (BUO-R). The rats were monitored in the following 1 or 3 days in protocol 1 and 2, respectively. Sham rats were operated without bilateral ureteral occlusion. In protocol 1, rats were divided into three groups: (1) Sham, (2) BUO, (3) BUO treated with melatonin (Mel) (BUO + Mel). Mel 20 mg/kg was given (intraperitoneally) at the onset of BUO, and 50 mg/kg after 6-h post-obstruction on operation day (day 0). In protocol 2, rats were divided into three groups: (1) Sham, (2) BUO-R, (3) BUO-R treated with Mel (BUO-R + Mel). Mel 20 mg/kg was given (intraperitoneally) at the onset of BUO, and 50 mg/kg after 6-h post-obstruction on operation day (day 0), and then daily for 3 days. Vehicle (0.9% NaCl) was administered to sham-operated, the untreated BUO, and the untreated BUO-R rats. In protocol 1 and 2, plasma was collected at the time of sacrifice for measurement of concentrations of potassium, sodium, urea nitrogen, and creatinine. In protocol 2, rats were maintained in metabolic cages allowing monitoring of urine excretion rates. Urine osmolality, creatinine, urea nitrogen, and sodium were measured.

inflammatory response that has been suggested to play a vital role in the pathophysiological changes in renal function of the obstructed kidney [8].

More and more evidence have showed that the renal lesions induced by BUO not only persist, but also progress long after ureteral obstruction is relieved [4,9–11]. It has been reported that there was persisted tubular cell proliferation and apoptosis, and elevated transforming growth factor (TGF)-β, leading to progressive tissue injury and loss of kidney function and proteinuria 1 year after relief of obstruction [10]. Ito et al. also found that relief of complete ureteral obstruction of a 3 day duration in adult rats restored RBF and GFR; however, interstitial inflammation, interstitial fibrosis and tubular cell apoptosis persisted at 28 days after the relief [11].

Melatonin (*N*-acetyl-5-methoxytryptamine) is a potent anti-inflammatory compound and has been shown to prevent ischemia-reperfusion induced acute kidney injury [12–15]. Several mechanisms of the anti-inflammatory action induced by melatonin treatment have been identified: 1) downregulation of proinflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-8 (IL-8), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). 2) Inhibition inflammation-promoting processes such as nitric oxide (NO) release, activation of cyclooxygenase-2 (COX-2), inflammasome NLRP3 and toll-like receptor-4 (TLR4). 3) Activating processes in an anti-inflammatory network, in which SIRT1 activation, upregulation of Nrf2 and downregulation of NF-κB, and release of the anti-inflammatory cytokines interleukin-10 (IL-10) are involved. It has been demonstrated that inflammatory cell infiltration induced by ureteral blockage may exacerbate renal tubule-interstitial injury through reduction of GFR and RBF [16]. Furthermore, interstitial inflammation and tubular cytokines production may be associated with the dysregulation of renal aquaporins (AQPs) that were previously observed in rats with BUO and BUO-R [4,5,17,18], as well as human studies [19–22]. Reduced AQP-1, -2 and -3 protein levels are possibly relevant to the development of impaired urinary concentration as well as post-obstructive diuresis. Here, we hypothesized that melatonin could inhibit the downregulation of AQPs via suppression of cytokine production and reducing the inflammatory reaction.

In the present study, we tested whether melatonin can possess a cytoprotective function during renal injury in rats inflicted with BUO and BUO-R. We assessed the influence of melatonin on renal function and AQP-1, -2 and -3 expression, interstitial inflammatory cell infiltration, inflammatory cytokine production, as well as renal ultrastructural integrity in rats put through BUO and release after BUO.

2. Materials and methods

2.1. Ethics

Every animal protocol was sanctioned by the Animal Care and Use Committee of The First Affiliated Hospital of Zhengzhou University. Guidelines for the Care and Use of Laboratory Animals of The First Affiliated Hospital of Zhengzhou University during animal handling were followed.

2.2. Experimental animals

Male Sprague-Dawley (SD) rats weighing 325–350 g were used for the experiments. They were reared on standard rat chow (Experimental Animal Center of Henan Province, China). The animals were provided free access to food and water during the course of the experiment. The rats were maintained in the individual cages exposing them to 12-hour artificial light/dark cycles, humidity of (55 ± 2) % and a temperature of (21 ± 2)°C, during the course of the experiment.

2.3. Experimental protocols

2.3.1. Protocol 1

Thirty SD rats were equally segregated into three groups (i.e. n = 10/each group) (Fig. 1): Sham-operated control group (which received laparotomy and free dissection of bilateral ureters but were not ligated using intraperitoneal administration of 3.0 ml of 0.9% saline solution), BUO-non-treated (underwent similar procedures as the sham control with the exception of renal ureter obstruction for 24 h), BUO + melatonin (intraperitoneal administration of melatonin 20 mg/kg at the onset of BUO and 50 mg/kg after 6 h).

2.3.2. Protocol 2

Thirty SD rats were equally segregated into three groups (i.e. n = 10/each group) (Fig. 1): Sham-operated control group (which received laparotomy and free dissection of bilateral ureters but were not ligated using intraperitoneal administration of 3.0 ml of 0.9% saline solution), BUO-R-non-treated (received similar treatment as BUO-non-treated with the exception of ureteral obstruction being released after 24 h and animals were monitored for a duration of 2 d following release), BUO-R + melatonin (20 mg/kg at the onset of BUO and 50 mg/kg after 6 h, then 50 mg/kg after release and daily for 2 days).

There are reports that have detailed the procedure and protocol of BUO and BUO-R [5]. In brief, 2.0% isoflurane was used to anesthetize animals in six groups through inhalation, and kept them on a heated table for regulating rectal temperature to a range of 37–38 °C. Both ureters were exposed through a midline abdominal incision and 5-mm of bisected polyethylene tubing (PE-50) was placed around the quarter of each ureter. The ureter was occluded through the tightening of tubing using a 5–0 silk ligature. For the BUO group, after 24 h the animals were sacrificed, blood and urine samples, as well as both kidneys were collected. For the BUO-R group, after 24 h BUO surgery, the rats were re-anesthetized and the obstructed ureters were decompressed by ligature and PE tubing removal. After 48 h the rats were sacrificed, blood and urine samples, as well as both kidneys were collected.

The dosage and time points of melatonin (Cat. No M5250, SIGMA-ALDRICH, St. Louis, MO, USA) administration to the experimental animals in different groups (Group 3 and Group 5) was based on previously published reports [13].

2.4. Renal function examination in rats with BUO or BUO release

The possible differences in plasma creatinine and urea from the blood of every experimental subject across the groups were assessed. Each animal was kept in a metabolic cage for 24 h with free access to food and water for the collection of urine samples after 24 h. The parameters, such as daily urine osmolality, urine volume, creatinine, and sodium and potassium concentrations, were determined by collecting urine samples at intervals of 24 h and 48 h, post release of BUO. The plasma and urine concentrations of creatinine and urea were investigated using colorimetric assays according to the manufacturer's instructions (BioAssay Systems, USA). Osmolality of the urine was determined with a vapor pressure osmometer according to the manufacturer's instructions (Osmomat 030, Gonotec, Berlin, Germany). The plasma and urine concentrations of sodium and potassium were measured with a chemistry analyzer according to the manufacturer's instructions (Vitros 950, Johnson & Johnson).

2.5. Immunohistochemical (IHC) staining

The 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) was used to fix the kidney samples belonging to each experimental group for 24 h. IHC staining was involved in the initial treatment of rehydrated four-micrometer-thick paraffin sections in 3% hydrogen peroxide at room temperature for 30 min. Treatment of kidney sections using 0.01 mol/L citrate buffer (pH 6.0) and heating in a microwave oven for

10 min aided antigen retrieval. The sections were incubated using primary antibodies specifically against AQP1 (dilution of 1/500, Abcam, Massachusetts, USA), AQP2 (at dilution of 1/200, Santa Cruz, USA), AQP3 (1/100, Santa Cruz, CA, USA), CD68 (1/200, Abcam, Massachusetts, USA), CD3 (1/200, Elabscience, China) and CD11b (1/300, Elabscience, China) at 4 °C overnight. Afterwards, the sections were incubated using biotinylated goat anti-rabbit IgG for 30 min, and finally the slides were stained in diaminobenzidine. The immune-labeling was examined using a Leica DM4B microscope and Image Pro Plus 6.0 analysis software was used for image analysis.

2.6. Western blot analysis

Tissue lysates were obtained through tissue lysis using RIPA lysis buffer supplemented with cocktail protease inhibitors on ice for 30 min. The protocol for Western blot analysis described in a recent report published by our research group was followed [23]. Equal amounts (50 μg) of protein extracts were resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). During post-resolution of the proteins, they were transferred into polyvinylidene difluoride (PVDF) membranes. Membranes were incubated overnight at 4 °C with the following primary antibodies: rabbit anti-AQP1 (1:1000; Abcam, USA), mouse anti-AQP2 (1:1000; Santa Cruz, USA), rabbit anti-AQP3 (the least detectable dose was 0.5 μg/ml; Abcam, USA), rabbit anti-IL-1β (Affinity Biosciences, USA; 1:2000), rabbit anti-MCP-1 (1:2000; Affinity Biosciences, USA), rabbit anti-TNF-α (1:1000; Abcam, USA), rabbit anti-iNOS (1:3000; Affinity Biosciences, USA), rabbit anti-COX-2 (1:1000; Proteintech, USA) and mouse anti-GAPDH (1:1000; Santa Cruz, USA). Post incubation with HRP-conjugated anti-rabbit IgG or anti-mouse IgG, the immune-labeled proteins were detected through enhanced chemiluminescence (ECL; Merck Millipore, USA) and the signal was captured on Biomax L film (Kodak, Rochester, NY, USA). The intensity of the indicated bands was estimated using ImageJ software.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Supernatant was obtained by homogenization and centrifugation of about 100 mg of kidney tissues. IL-6 expression was determined by following the recommended protocol of avidin-biotin complex-ELISA. ELISA kits were procured from Boster Biotechnology (Wuhan, China). All experiments were performed in triplicate.

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol (Invitrogen, USA) was used to extract the complete pool of RNA from rat kidneys and convert it into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Invitrogen, USA). Fast SYBR Master Mix (Applied Biosystems, B.V.) was used for qRT-PCR on Applied Biosystems 7500 Sequence Detection System. Table 1 enlists sequence-specific primers for AQP1, AQP2, AQP3, IL-1β, MCP-1, TNF-α, COX-2 and iNOS which were designed and synthesized by Sangon Biotech, China. β-actin was used as an endogenous reference gene. For the quantitative analysis, all samples were analyzed using the formula of $2^{-\Delta\Delta}$ Ct and normalized to β-actin mRNA expression.

2.9. Transmission electron microscopy (TEM)

The kidney tissues were immediately immersed in 2.5% glutaraldehyde solution post collection. Each sample was subjected to trimming, immediately fixed in 4% formaldehyde, 2.5% glutaraldehyde, and 0.03% picric acid suspended in 100 mmol/L cacodylate buffer, at pH 7.2, and placed in a thermal box cooled to 4 °C for 2 h. Following the above step, the samples were fixed in 1% osmium tetroxide in sodium cacodylate buffer, and then dehydrated using a graded ethanol series and propylene oxide. Semi-thin sections (1.0 mm) were obtained and stained in toluidine blue for observation. Uranyl acetate and lead citrate were used for staining the ultrathin sections and were tested at 80 kV under transmission electron microscopy (TEM) (JEOL 1400, Japan).

2.10. Statistical analysis

The quantitative data are expressed in the form of means ± standard derivation (SD) of a minimum of three independent observations. ANOVA followed by Bonferroni multiple comparison post hoc test, along with a two-sided Student's *t*-test were used for the statistical analyses. Statistical Package for Social Sciences (SPSS version 17.0, SPSS Inc., Chicago, USA) was used for all analytical calculations. A $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Melatonin treatment partially inhibited BUO and BUO release mediated impaired renal function

24 h after BUO, the rats had significantly increased plasma urea and creatinine levels in comparison with control rats (Table 2). Moreover, as opposed to that of the sham-operated group, the concentrations of plasma potassium in BUO rats were obviously elevated, but plasma sodium levels decreased significantly, as demonstrated in previous studies [4,6]. It is well known that BUO-R has been found to have an effect on the reabsorption of renal water and salts, declined urine concentration capacity and polyuria [24]. In accordance with the above findings, urine osmolality was significantly decreased in rats 48 h after the release of 24 h-BUO (BUO-48hR) in contrast with that of controls (508 ± 42 vs. 2076 ± 169 mosmol/kgH_2O, $P < 0.05$, Table 2), suggesting impaired renal function of concentration. Nevertheless, urine FE_{Na} levels in BUO-48hR rats were remarkably elevated than that of the control set, indicating decreased renal tubular reabsorption of sodium. Moreover, the BUO-48hR group exhibited an appreciably higher output of urine, while urinary potassium, sodium and creatinine

Table 1
Quantitative real-time polymerase chain reaction (qRT-PCR) primers for analysis.

Gene	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	Production size (bp)
AQP1	ACCTGCTGGCCATTGACTAC	CCAGGGCACTCCCAATGAAT	129
AQP2	CTTGGCCACGCTCCTTTTTG	AAGGAGACATGGCAACCCAC	198
AQP3	TGTCTGGAGCCCACTTGAAC	CTTGATCCAGGGCTCTCGTG	71
IL-1β	CAGGATGAGGACCCAAGCAC	GTCGTCATCATCCCACGAGT	81
MCP-1	TGATCCCAATGAGTCGGCTG	TGGACCCATTCCTTATTGGGG	127
TNF-α	ACTGAACTTCGGGGTGATCG	GCTTGGTGGTTTGCTACGAC	153
COX-2	GATGACGAGCGACTGTTCCA	TGGTAACCGCTCAGGTGTTG	98
iNOS	GGAGAAAACCCCAGGTGCTA	GTGAGGAACTGGGGGAAACC	90
β-actin	CACCCGCGAGTACAACCTTC	CCCATACCCACCATCACACC	207

AQP1, aquaporin 1; AQP2, aquaporin 2; AQP3, aquaporin 3; IL-1β, interleukin-1β; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α; COX-2, cyclooxygenase 2; iNOS, inducible nitric oxide synthase.

Table 2
Changes in renal functional data in BUO for 24 h or followed by release of BUO for 48 h treated with or without melatonin and sham-operated controls.

	n	Pcrea, μmol/L	Purea, mmol/L	Pk, mmol/L	PNa, mmol/L	Uosm, mosmol/kgH_2O	Uvol, μl/min/kg	Uk, mmol/L	UNa, mmol/L	Ucrea, μmol/L	UFE_{Na} %
BUO											
Sham	10	29.8 ± 2.1	6.3 ± 0.3	4.2 ± 0.6	138.6 ± 5.8						
Nontreated	10	243.4 ± 14.1*	57.8 ± 6.5*	7.5 ± 0.5*	130.8 ± 4.7*						
Melatonin	10	237.5 ± 11.2*	52.7 ± 5.8*	7.2 ± 0.4*	137.2 ± 5.1						
BUO-48hR											
Sham	10	30.2 ± 2.6	7.5 ± 0.4	4.3 ± 0.7	139.2 ± 5.6	2076 ± 169	30 ± 3	168.3 ± 7.9	152.7 ± 10.8	4219 ± 578	0.62 ± 0.03
Nontreated	9	128.4 ± 24.4*	24.6 ± 3.7*	6.3 ± 0.7*	136.8 ± 4.9	508 ± 42*	77 ± 6*	130.8 ± 8.5*	108.6 ± 5.9*	2408 ± 459*	1.74 ± 0.05*
Melatonin	8	56.4 ± 19.6*†	12.7 ± 2.8*†	5.6 ± 0.8*	141.3 ± 4.2	623 ± 79*	72 ± 8*	146.6 ± 9.2*†	137.2 ± 6.3*†	3409 ± 438*†	0.98 ± 0.04*†

Values are means ± SD. BUO, bilateral ureteral obstruction; *n,* number of rats; Pcrea, plasma creatinine; Purea, plasma urea; Pk, plasma potassium; PNa, plasma sodium; Uosm, urine osmolality; Uvol, urine volume; Uk, urine potassium; UNa, urine sodium; Ucrea, urine creatinine; UFE_{Na}, urine fractional excretion of sodium. BUO-48hR, rats subjected to BUO and then release 48 h later. *$P < 0.05$ compared with sham-operated controls. †$P < 0.05$ compared with nontreated rats with release of BUO.

concentrations showed a significant decrease. Meanwhile, rats in the BUO-48hR group showed a remarkable elevation in the levels of urea, plasma creatinine and potassium compared with that of the sham-treated controls (Table 2).

The influence of melatonin on renal function was investigated. Both BUO and BUO-48hR rats were introduced to melatonin. Melatonin administration did not alter the decreased osmolality of urine in BUO-48hR rats. In contrast, BUO-48hR rats receiving melatonin showed attenuation of elevated plasma creatinine (128.4 ± 24.4 μmol/L in non-treated rats vs.56.4 ± 19.6 μmol/L in melatonin-administered animals, $P < 0.05$, Table 2). In a similar fashion, melatonin administration was related to decreased urea levels in the plasma of BUO-48hR rats. Moreover, melatonin treatment inhibited the decreased urine creatinine levels and concentrations of potassium and sodium in BUO-48hR animals (Table 2). But in the meantime, melatonin treatment facilitated the decline of urine FE_{Na} in BUO-48hR rats (1.74 ± 0.05% in non-treated rats vs. 0.98 ± 0.04% in melatonin-treated rats, $P < 0.05$, Table 2).

3.2. Melatonin prevented downregulation of AQP-1, -2, and -3 in BUO as well as BUO-48hR animals

Similar to the results of previous research [4,17], both Western blotting and qRT-PCR results revealed that BUO and BUO-48hR are involved in the significant decrease of AQP-1, -2, and -3 mRNA and protein expression compared with that of the sham-operated group (Fig. 2). On the contrary, melatonin treatment significantly suppressed reduction in AQP-1, -2, and -3 in BUO and BUO-48hR groups compared with that of the sham group (Fig. 2).

IHC staining confirmed the reduction of AQP-1 prevalence in untreated renal proximal tubules in reaction to 24-h BUO as well as BUO-48hR treatment (Fig. 3A and B). The labeling was enhanced in BUO and BUO-48hR rats in reaction to melatonin exposure (Fig. 3A and B), but still lower than that of sham controls (Fig. 3A and B). Likewise, IHC showed apical plasma membranes to be AQP2 positive especially in collecting duct principal cells and AQP3 in the cortical collecting duct basolateral membranes. After 24 h of BUO, AQP2 and AQP3 levels in the collecting duct of BUO rats was significantly down-regulated than that of the sham-operated controls. After releasing of BUO for 2 days, the expression of AQP2 and AQP3 had partially recovered. When treated with melatonin, the AQP2 and AQP3 staining density appeared more prominent, but still lower than that of the sham controls (Fig. 3A and B).

3.3. Ultrastructural study

Tubular features of the proximal as well as collecting ducts were assessed in different experimental groups though ultrastructural analysis. Compared with the sham-operated group, ultrastructural features of interstitial fibrosis and tubular damage were evident in kidneys of BUO rats (Fig. 4A, B, D, and E). Brush border detachment as well as abnormal mitochondria with disrupted cristae was evident in the proximal tubules (Fig. 4B and E). In contrast, kidneys of sham-operated rats presented well-defined renal ultrastructure involving the elongated mitochondrial shape and well-preserved cristae in the proximal tubules (Fig. 4A and D). When analysis of the 24 h BUO rats treated with melatonin was carried out, remarkable restoration of “normal” ultrastructure features in the tubules along with regular and continuous brush borders was found (Fig. 4C and F). In addition, the proximal tubules displayed numerous, well preserved and elongated mitochondria possessing clear cristae.

3.4. Melatonin treatment alleviated inflammatory response in BUO or BUO-48hR rats

It has been demonstrated that prolonged ureteric blockage induces inflammatory and fibrotic damage in the affected kidney. It is marked with the infiltration of immune cells such as lymphocytes and macrophages, and activation of fibroblasts as well as tubule-interstitial deposition of attendant extracellular matrix [25]. In order to explore whether melatonin plays a role in regulating inflammation response and inflammatory cell infiltration induced by BUO or BUO-48hR, levels of IL-1β, TNF-α and MCP-1, which are considered to be pro-inflammatory mediators, were also investigated. Meanwhile, the expression of CD68 (a marker for macrophages), CD11b (a marker for neutrophils) and CD3 (a marker for lymphocytes) were also observed. As described in Fig. 5A and Fig. 6A, CD68, CD11b and CD3-positive cells were rarely detectable in sham-operated controls, while an excess of positive cells were found in the interstitium in 24 h BUO or BUO-48hR, suggesting that the infiltration of macrophages, neutrophils and lymphocytes into injured kidneys had increased (Fig. 5B and Fig. 6B). However, melatonin introduction remarkably reduced the number of CD68, CD11b and CD3-positive inflammatory cells in 24 h BUO or BUO-48hR rats. Indeed, after 24 h BUO, inflammatory cytokine levels of TNF-α, IL-1β and MCP-1, as well as IL-6 were significantly upregulated. As illustrated in Fig. 7, melatonin treatment strongly prevented the expression of these inflammatory cytokines induced by 24 h BUO or BUO-48hR. In a nutshell, melatonin treatment decreased inflammatory cell infiltration and suppressed the release of inflammatory cytokines.

4. Discussion

This study successfully established complete BUO, as well as BUO-R models of rats. It was observed that complete 24 h ureteral blockage induced a remarkable increase in plasma urea, creatinine and potassium, which was sustained for the next 48 h after release of the

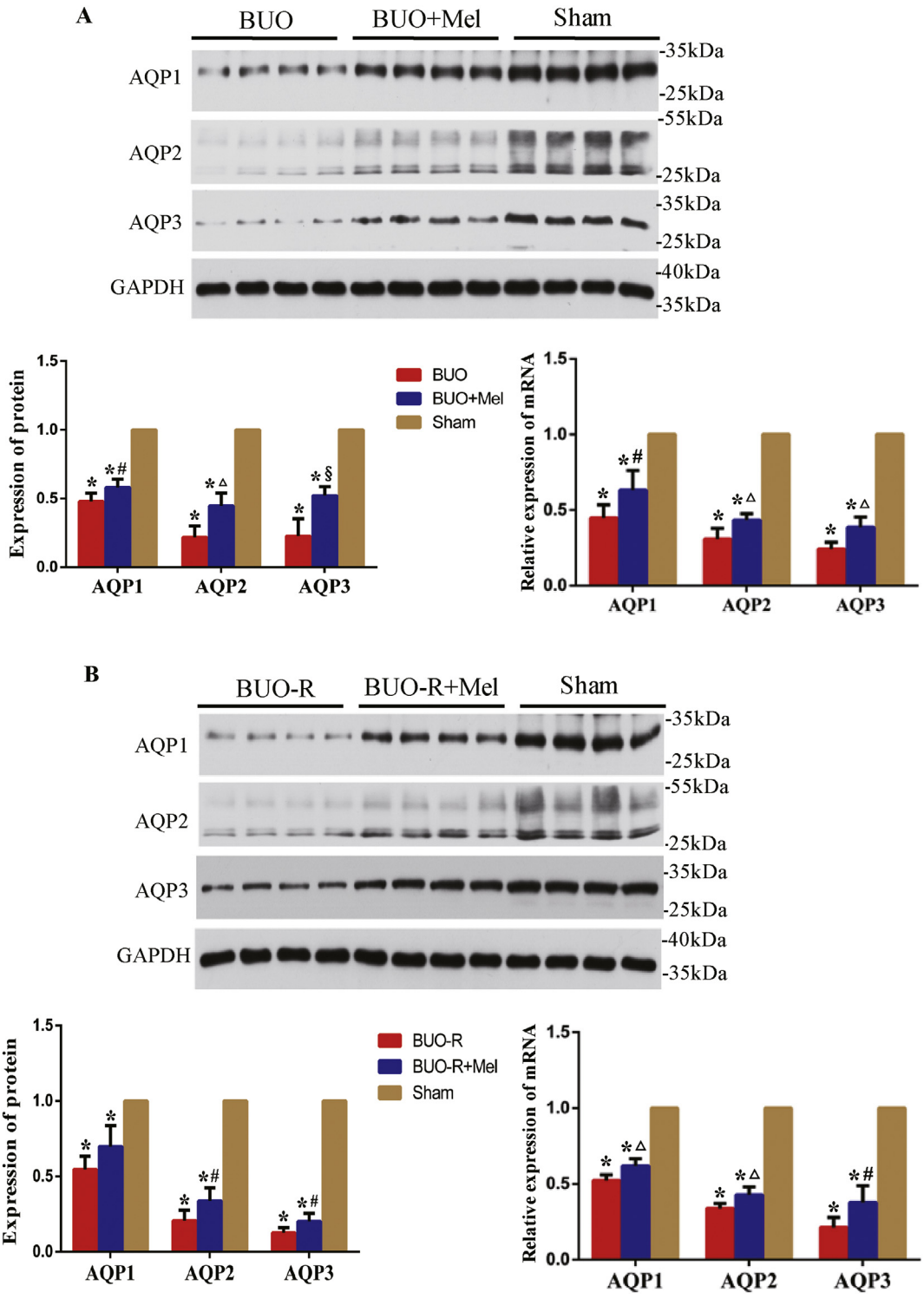

Fig. 2. Effect of melatonin treatment on renal aquaporin-1 (AQP1), AQP2 and AQP3 protein and mRNA expressions in bilateral ureteral obstruction (BUO) rats (*protocol 1*) and BUO followed by release for 48 h (BUO-R) rats (*protocol 2*).

(A) Kidney tissues were analyzed by Western blot and quantitative RT-PCR for AQP1, AQP2 and AQP3 expression in Sham, BUO and BUO treated with melatonin groups. $^{*}P < 0.001$ compared with Sham group, $^{\#}P < 0.05$, $^{\triangle}P < 0.01$, $^{\S}P < 0.001$ compared with BUO group.

Bars in A represent means ± standard deviation (SD), n = 10/group.

(B) Kidney tissues were analyzed by Western blot and quantitative RT-PCR for AQP1, AQP2 and AQP3 expression in Sham, BUO-R and BUO-R treated with melatonin groups. $^{*}P < 0.001$ compared with Sham group, $^{\#}P < 0.05$, $^{\triangle}P < 0.01$ compared with BUO-R group.

Bars in B represent means ± standard deviation (SD), n = 10 for Sham; n = 9 for BUO-R group; n = 8 for BUO-R treated with melatonin group.

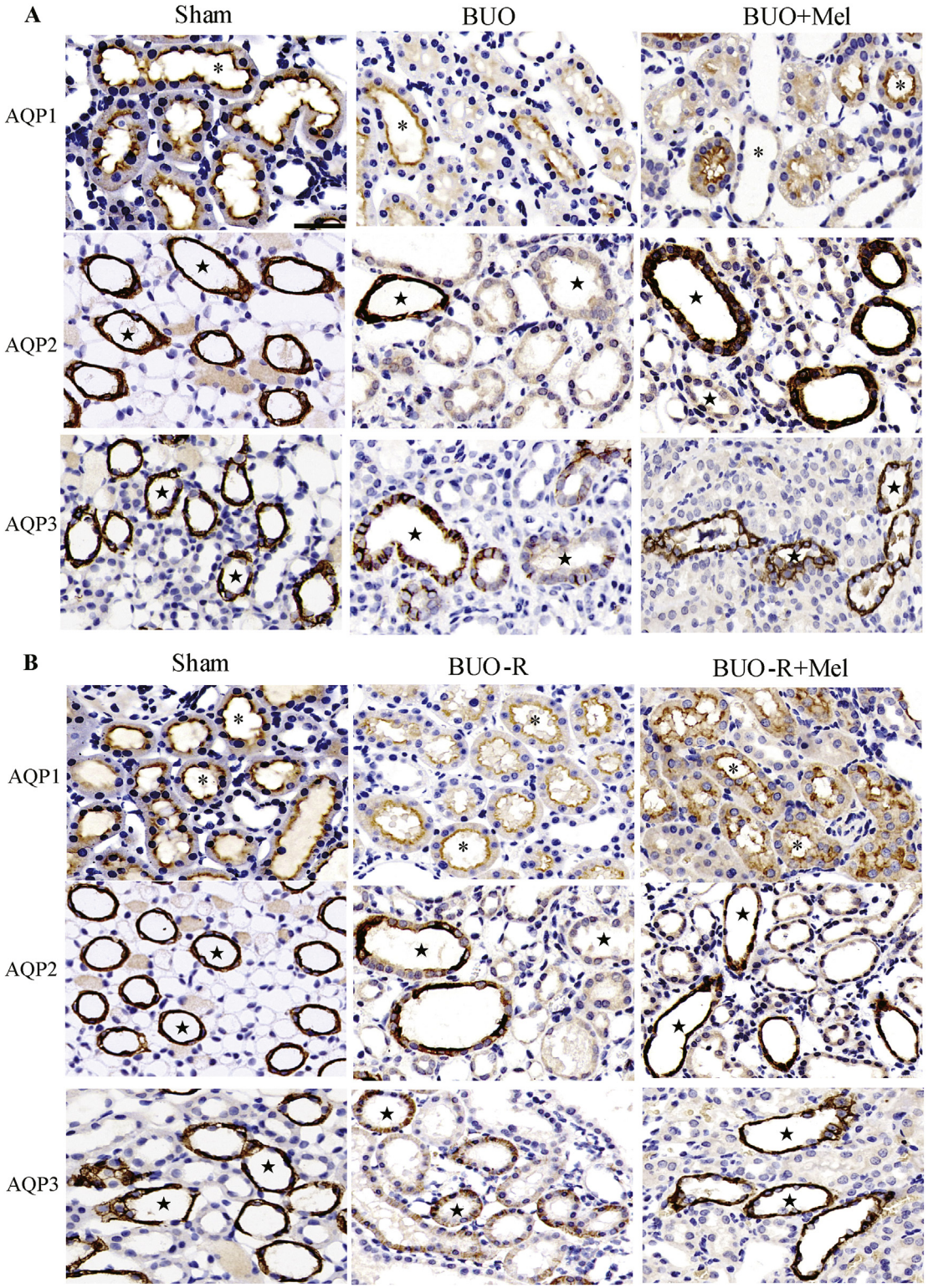

Fig. 3. Immunoperoxidase localization of aquaporin-1 (AQP1), AQP2 and AQP3 in kidney from bilateral ureteral obstruction (BUO), melatonin-treated BUO, BUO followed by release for 48 h (BUO-R), melatonin-treated BUO-R, and sham-operated rats.

(A) Abundant labeling of AQP-1 is associated with apical and basolateral plasma membranes of proximal tubules (*) in sham-operated rat kidneys. In kidney cortex from rats with BUO, there was a marked reduction in the AQP-1 labeling of proximal tubule. In melatonin-treated BUO rats, AQP-1 labeling is slightly reduced compared with sham controls. In sham-operated rats, AQP2 labeling is associated with the apical plasma membrane domains of collecting duct (⋆). A marked decrease in AQP2 labeling is seen in the collecting duct from BUO rats without melatonin treatment. Comparing to sham, AQP2 labeling in collecting duct was slightly decreased in melatonin-treated BUO rats. In sham-operated rats, AQP3 labeling was intensive in the basolateral membranes of collecting tubule. AQP3 labeling was significantly reduced in the collecting tubules from BUO kidney. Compared with sham-operated rats, AQP3 labeling in collecting tubules was slightly decreased in melatonin-treated BUO rats (magnification ×400). Scale bar = 50 μm.

(B) Immunoperoxidase microscopy demonstrated decreased AQP1 immunolabeling in the proximal tubules (*), AQP2 in the collecting ducts (⋆), as well as AQP3 in the collecting ducts (⋆) in nontreated kidneys in response to BUO-R. When treated with melatonin, the AQP1, AQP2 and AQP3 staining density appeared more prominent, but still lower than that in sham-operated rats (magnification ×400). Scale bar = 50 μm.

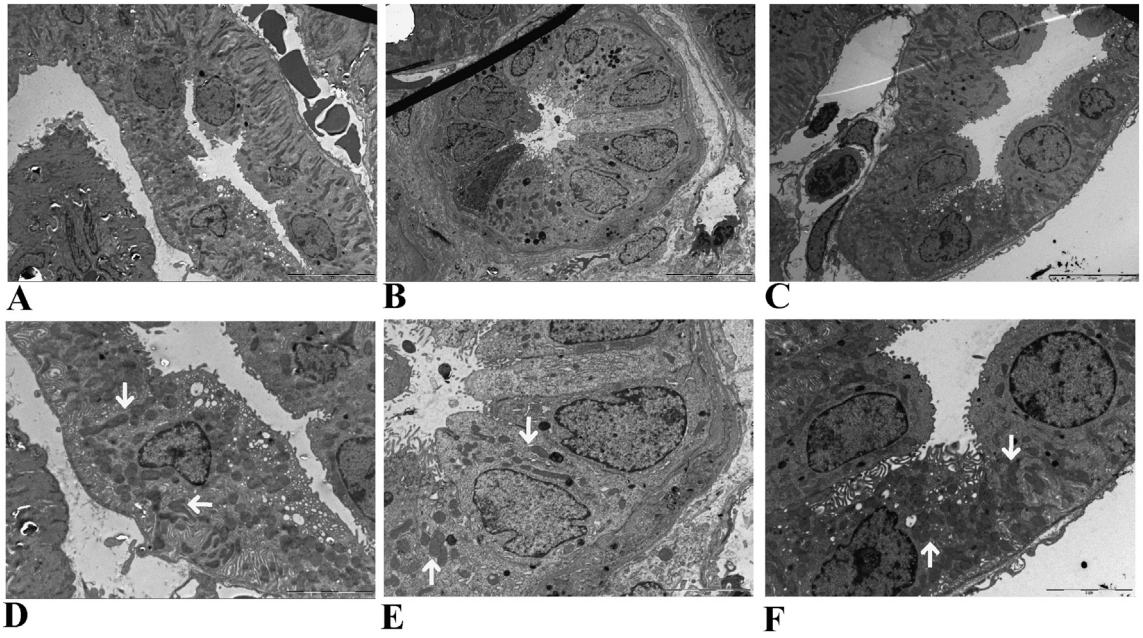

Fig. 4. Melatonin preserves kidney ultrastructural in bilateral ureteral obstruction (BUO) rats.
Photomicrographs showed that proximal tubules and proximal tubular mitochondria of Sham group (A and D), BUO rats group (B and E) and melatonin-treated BUO rats group (C and F). Arrowhead indicated mitochondria change in proximal tubules. Scale bar = 10 μm in A-C; Scale bar = 5 μm in D-F.

blockage. Moreover, impaired capacity for urinary concentration in BUO and short duration BUO-R animals was observed, which is consistent with the results of previous studies [4,5,17,18]. The results of this study showed for the first time, that melatonin influence significantly ameliorated renal function deterioration in rats undergoing BUO and BUO-R (Table 2). Furthermore, melatonin administration, to a great extent, rescued the expression of AQP-1, -2, as well as-3, especially in a backdrop of release of BUO. Meanwhile, melatonin was found to have a significant function in the inhibition of the inflammatory response, as well as in preserving renal ultrastructural integrity before and after release of BUO. Thus, these findings strongly suggest that melatonin therapy is relevant to the prevention of BUO and BUO-R, which is associated with worsening of renal function along with AQP-1, -2, and -3 downregulation, and renal ultrastructural integrity failure, as well as a series of inflammatory responses.

As far as is known, urinary tract obstruction often results in a sustained increase of intraluminal tubular pressure and progressive decrease in GFR and RBF [26]. Surprisingly, in the present study it was found that melatonin administration to animals of the test groups (with BUO or BUO-R) markedly promoted recovery of renal insufficiency. Moreover, melatonin treatment significantly normalized altered renal water and sodium handling that was observed in BUO as well as BUO-R, including increased plasma urea, creatinine, high fractional excretion of sodium (FE_{Na}) and urine output, as well as decreased urine osmotic pressure. These observations suggested that melatonin treatment might have a prominent effect on accelerating renal function recovery.

AQPs, a family of ubiquitous membrane channel protein involved in osmotic water flux regulation, have been proven to be involved in renal physiology and transepithelial water transport [27,28]. Previous studies have demonstrated that down-regulated AQPs levels are related with impaired ability of urine concentration in various animal models of study, such as UUO, BUO and BUO-R [4,6,29]. The present study confirms previous findings that AQP-1, -2, and -3 expression is appreciably decreased in BUO and BUO-48hR rats, suggesting that the decrease is a persistent post obstruction release. Importantly, this is the first report to identify that melatonin administration partially restrains the downregulation of AQP-1, -2, and -3 during 24 h BUO and 48 h after release of BUO, which are possibly involved in partial water reabsorption rescue. However, the underlying mechanisms of melatonin treatment to promote the recovery of AQPs response to BUO and BUO-R are unknown.

Numerous studies have demonstrated that angiotensin II plays a pivotal role in the pathogenesis of ON. Even if the obstruction is removed, intrarenal renin-angiotensin system (RAS) activation still exist persistently, as shown in BUO-R animal model [5] and human disease [22]. As a consequence of upregulation of the RAS, interstitial inflammation develops early in the course of ON. Accumulation of macrophages is a prominent feature [8]. Prado et al. showed that melatonin lowered angiotensin II receptor (AT1) binding, and reduced the signal transduction stimulated by angiotensin II [30]. Ishigaki et al. also found that melatonin alleviated intrarenal RAS activation and renal injury in a 5/6 nephrectomy rat model through decreasing the expression of intrarenal angiotensinogen, AT1 and angiotensin II [31]. These findings suggest that melatonin ameliorates renal injury mediated by angiotensin II. Our current data showed that BUO and BUO-R induced inflammatory responses such as neutrophil, lymphocyte and macrophage infiltration and the synthesis of inflammatory cytokines can be ameliorated by melatonin, which is consistent with the previous studies [8]. Melatonin has been reported to intercept pro-inflammatory cytokine and chemokine synthesis and function in some cases, and has been shown to have a protective effect in experimental models of chronic renal failure model and renal ischemia reperfusion model [13,32]. In summary, it is appropriate to suppose that melatonin may reduce acute inflammatory response caused by BUO and BUO-R via regulation of angiotensin II signaling cascade, changing the inflammatory scenario of the kidney, thus decreasing renal structural and functional deterioration. Moreover, Nørregaard et al. showed that downregulation of AQP2 and AQP3 was regulated via angiotensin II induction of COX-2 in both BUO [18] and BUO-R models [5]. In the present study, we found that COX-2 expression increased significantly in response to BUO and relief of BUO, but decreased markedly after melatonin treatment (Fig. 7). We speculate that BUO or BUO-R triggers the improved level of angiotensin II induction of COX-2, thereby reducing AQPs proteins expression and leading to polyuria. Interestingly, a recent study observed that overexpression of angiotensinogen downregulated AQP1 expression via modulation of Nrf2-HO-1 pathway in renal proximal tubular cells [33].

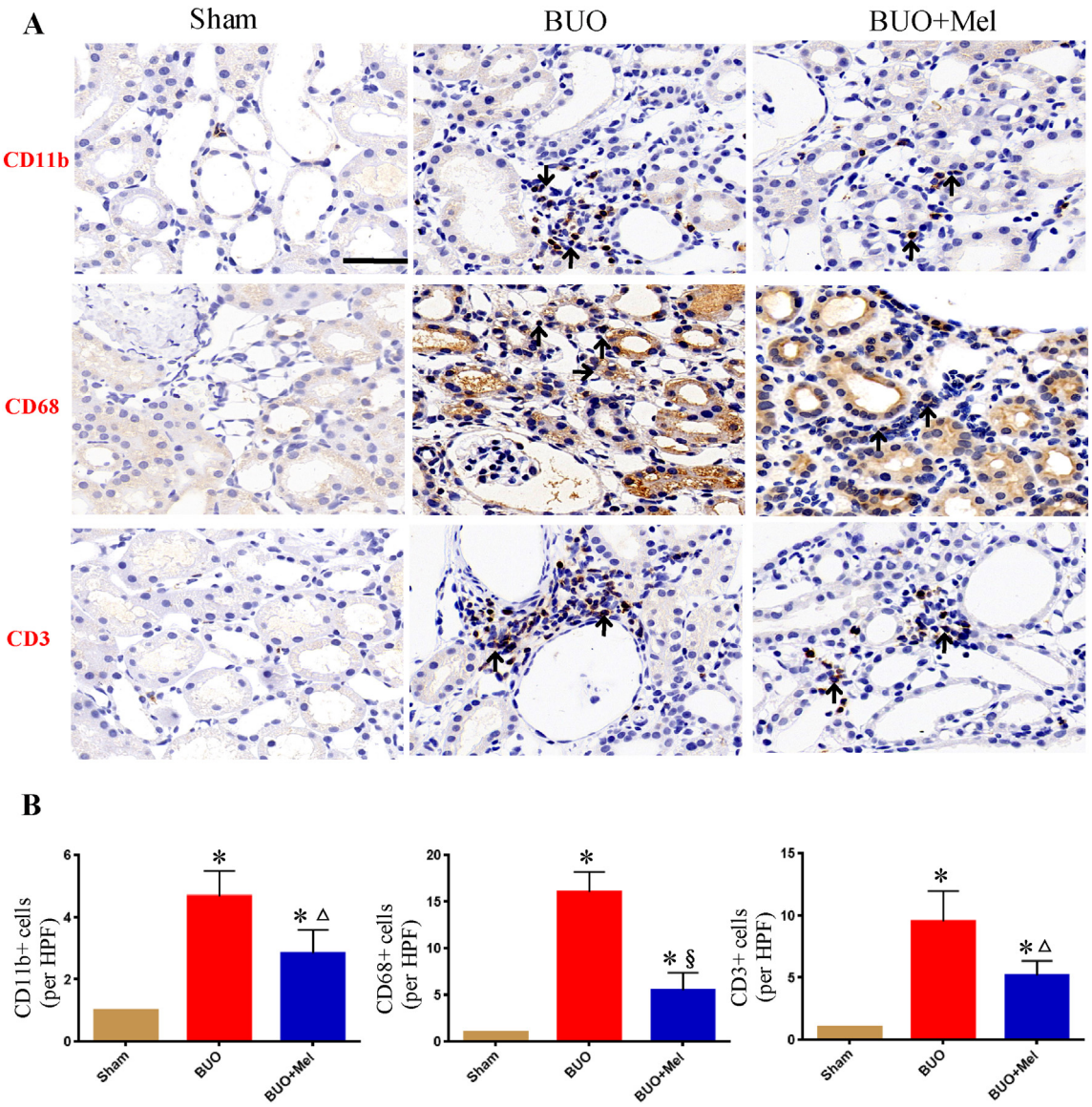

Fig. 5. Melatonin treatment reduced infiltration of inflammatory cells in bilateral ureteral obstruction (BUO) rats.
(A) Kidney sections were immunolabeled with primary antibodies against CD11b, CD68 and CD3 (magnification ×400). Scale bar = 50 μm. Arrowhead indicated neutrophils (CD11b), lymphocytes (CD3) and macrophages (CD68), respectively.
(B) The number of neutrophils, lymphocytes and macrophages infiltrated into kidneys were analyzed in 8 visual fields. $^{*}P < 0.001$ compared with Sham group, $^{\triangle}P < 0.01$, $^{\S}P < 0.001$ compared with BUO group.
Bars in B represent means ± standard deviation (SD), n = 10/group.

A possible decrease of angiotensinogen induced by melatonin may explain the increase of AQP1 expression in melatonin-treated BUO or BUO-R rats. In addition, we observed that iNOS was highly expressed in BUO and BUO-R groups, which was suppressed after melatonin treatment (Fig. 7). INOS is the main synthetase of nitric oxide (NO) and will express under inflammation stimulation [34]. NO has been reported to inhibit renal collecting duct water transport through reduction of AQP2 expression [35]. Thus we put forward a guess that melatonin might decrease NO expression through the inhibition of iNOS, which presents a possible mechanism of melatonin to promote AQP2 recovery in BUO and BUO-R rats. But until now, there have been few detailed research reports on the effect of melatonin treatment directly on renal AQPs expression. Therefore, further explorations, such as RNA interference or agonist of AQPs in vitro models, will be required for understanding the underlying mechanisms of melatonin mediated regulation of AQPs levels, along with the protective influence of melatonin treatment.

Besides the anti-downregulation of AQPs and anti-inflammation actions, involvements of other mechanisms in melatonin function that prevent renal function decline are unknown. As observed previously, melatonin supplements in ischemic reperfusion renal failure upgrade renal function rescue via reduction of endoplasmic reticulum stress and induction of the Akt pathway, suggesting that it has a direct effect on tubular injury [36]. Moreover, the present study found that melatonin treatment reduces morphological lesions from BUO, reinstating the “normal” morphology and cytoarchitecture of kidneys, along with well preserved and elongated mitochondria having well defined cristae, which is also consistent with findings of a renal ischemic-reperfusion injury model [37]. This study emphasizes the need for further research to explore possible mechanisms that result in favorable effects for melatonin post blockage in ureters. Moreover, previous studies have shown that melatonin can interact with two high-affinity G protein-coupled receptors, termed melatonin receptor-1 (MT1) and − 2 (MT2) [38]. Experimental evidence indicates that melatonin regulates renal tubular functions via MT1 located in the renal cortex (predominantly in

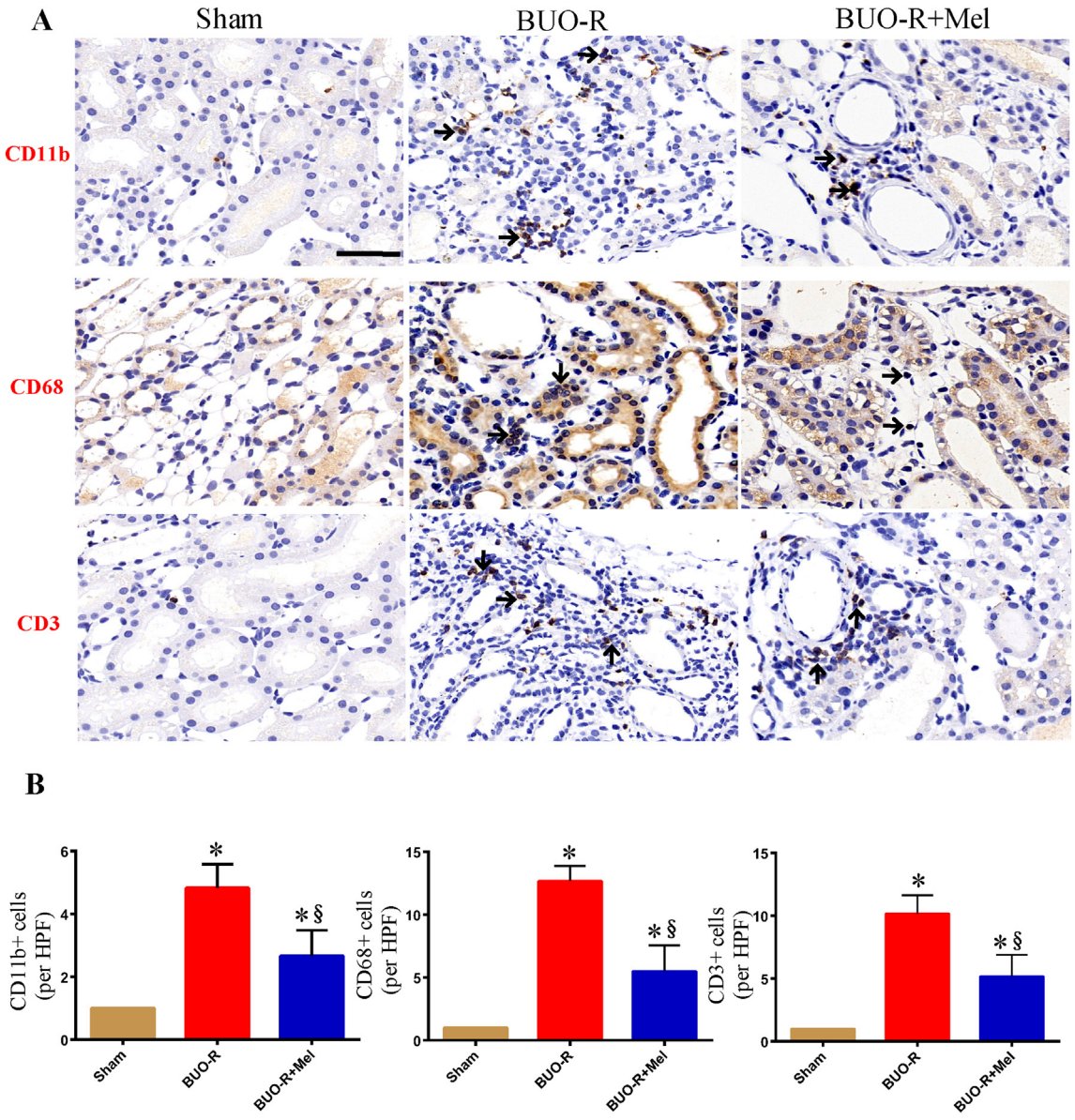

Fig. 6. Melatonin treatment reduced infiltration of inflammatory cells in bilateral ureteral obstruction (BUO) followed by release for 48 h (BUO-R) rats.
(A) Kidney sections were immunolabeled with primary antibodies against CD11b, CD68 and CD3 (magnification ×400). Scale bar = 50 μm. Arrowhead indicated neutrophils (CD11b), lymphocytes (CD3) and macrophages (CD68), respectively.
(B) The number of neutrophils, lymphocytes and macrophages infiltrated into kidneys were analyzed in 8 visual fields. *$P < 0.001$ compared with Sham group, §$P < 0.001$ compared with BUO-R group.
Bars in B represent means ± standard deviation (SD), n = 10 for Sham; n = 9 for BUO-R group; n = 8 for BUO-R treated with melatonin group.

renal tubules) [39,40]. MT1 is linked to the inhibition of adenylyl cyclase and subsequent decrease in cyclic adenosine monophosphate (cAMP) levels and cAMP-responsive element-binding protein (CREB) phosphorylation [41]. Activation of MT1 also activates other signaling pathways, such as the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway [38]. MT1 plays a role in the rhythmic regulation of clock gene expression via the cAMP-CREB or ERK pathways [42]. Dysregulation of circadian rhythms is associated with accelerated progression of chronic kidney disease [43]. These data imply that MT1 may be implicated in the regulation of kidney homestasis. It remains to be seen if MT1 plays a potential role in BUO and BUO-R, but the idea certainly warrants further investigation.

5. Conclusions

Melatonin administration significantly opposes the deterioration of kidney function in BUO and BUO-R rats, to a great extent, inhibiting the suppression of renal AQP-1, -2, -3 expression, as well as inflammatory infiltration and secretion of cytokines. Further, melatonin use preserves kidney ultrastructural integrity after ureteral obstruction. Hence, the beneficial mechanisms of melatonin may be extensive and multifactorial, including renal hemodynamic regulation, retaining renal structure and tubular function integrality, as well as changing inflammatory milieu. Grouped together, the results obtained shed light on the fact that melatonin treatment potentially plays a protective role before and after release of BUO. The findings of this study highlight its potential clinical relevance in obstructive nephropathy.

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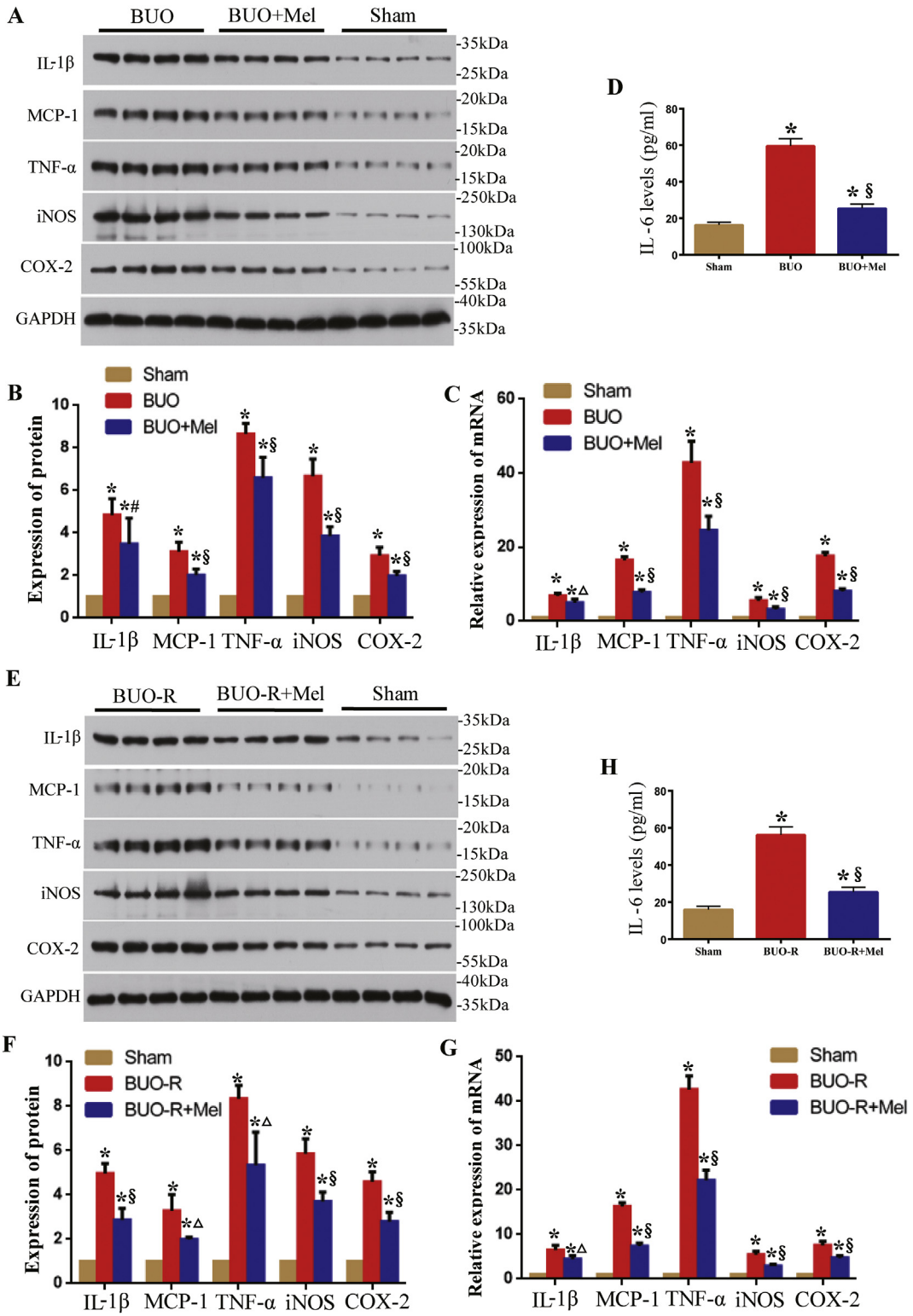

Fig. 7. Melatonin treatment reduced secretion of inflammatory cytokines and production of inducible-NOS (iNOS) in bilateral ureteral obstruction (BUO) rats and BUO followed by release for 48 h (BUO-R) rats.

Kidney tissues were analyzed by Western blot and quantitative RT-PCR for IL-1β, MCP-1, TNF-α, COX-2 and iNOS expression in Sham, BUO and BUO treated with melatonin groups (A-C). ELISA analyses for IL-6 levels in Sham, BUO and BUO treated with melatonin groups (D).

*$P < 0.001$ compared with Sham group, #$P < 0.05$, △$P < 0.01$, §$P < 0.001$ compared with BUO group.

Bars in A-D represent means ± standard deviation (SD), n = 10/group.

Kidney tissues were analyzed by Western blot and quantitative RT-PCR for IL-1β, MCP-1, TNF-α, COX-2 and iNOS expression in Sham, BUO-R and BUO-R treated with melatonin groups (*E*-G). ELISA analyses for IL-6 levels in Sham, BUO-R and BUO-R treated with melatonin groups (H).

*$P < 0.001$ compared with Sham group, △$P < 0.01$, §$P < 0.001$ compared with BUO-R group.

Bars in E-H represent means ± standard deviation (SD), n = 10 for Sham; n = 9 for BUO-R group; n = 8 for BUO-R treated with melatonin group.

Declaration of Competing Interest

All authors have contributed significantly to the manuscript and declared that the work is original and has not been submitted or published elsewhere. None of the authors have any financial disclosure or conflict of interest.

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