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Original Article

Melatonin promoted renal regeneration in folic acid-induced acute kidney injury via inhibiting nucleocytoplasmic translocation of HMGB1 in tubular epithelial cells

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Abstract: Melatonin (N-acetyl-5-methoxytryptamine), a circadian-regulating hormone, has been reported to exert a protective role during acute kidney injury (AKI) induced by renal ischemia-reperfusion injury (I/R). High-mobility group box 1 (HMGB1) is a novel member of the damage-associated molecular pattern (DAMP) family, and has been verified to be an inflammatory cytokine mediating AKI induced by I/R and cisplatin. However, the effect of melatonin on HMGB1, as well as the relationship of these two with folic acid induced AKI are elusive. In this study, we sought to identify the role of melatonin on folic acid induced AKI and its association with HMGB1. Pretreatment with melatonin significantly attenuated folic acid-induced increase in serum creatinine and BUN levels, renal tubular epithelial cell (TEC) apoptosis, and the infiltration of inflammatory cells and secretion of cytokines. Moreover, melatonin pretreatment promoted renal tubular proliferation and improved cell cycle arrest of TECs after folic acid-induced renal damage. This protective role of melatonin was closely related to the inhibition of nucleocytoplasmic translocation of HMGB1 in TECs. These data provide a strong proof that administering melatonin prior to folic acid insult may shed light on a potential treatment for AKI.

Keywords: Acute kidney injury, melatonin, regeneration, HMGB1, nucleocytoplasmic translocation

Introduction

AKI confers high morbidity and mortality, however specific therapeutic interventions are still elusive [1, 2]. AKI encompasses a complex pathophysiological process, in which a variety of factors and cells are involved. It is often accompanied by acute tubular epithelial cell damage, including apoptosis and necrosis; infiltration of interstitial inflammatory cells, secretion of inflammatory cytokines and chemokines; acute vascular dysfunction [3, 4], etc. Injured kidneys usually cannot be fully recovered and may easily progress to chronic kidney disease (CKD), or end-stage renal disease (ESRD) [5]. Although there are several clinical symptomatic supportive treatments, there is still no effective way to prevent the above pathological damage and its progression to CKD or even ESRD [6, 7].

HMGB1 is a type of non-histone chromosome-binding protein that exists in the nucleus [8, 9]. Recent studies demonstrate that it is also a member of the damage-associated molecular pattern (DAMP) family, which can be released by necrotic and apoptotic cells and activated inflammatory cells [10], mediating acute injury process in the liver [11], heart [12], brain [13], kidney [14] and lungs [15]. In our previous study, we showed that inhibiting nucleocytoplasmic shuttling and release of HMGB1 by carbon monoxide-releasing molecule-2 (CORM-2) attenuated AKI, however, administration of exogenous HMGB1 significantly reversed the protective effect of CORM-2 in AKI induced by

I/R [16]. Based on this research, we speculate that drugs that can inhibit translocation of nucleocytoplasmic and release of HMGB1 will exert a good protective effect on AKI.

Melatonin is an amine hormone which is mainly secreted by the pineal gland [17, 18]. Recent studies have shown that melatonin has a variety of biological functions, including anti-oxidative stress [19], anti-inflammatory [20], anti-apoptosis [21] and anti-tumor [22] properties, in addition to regulating circadian rhythm. It has been reported that melatonin can reduce kidney damage induced by I/R [23], severe burns [20] and unilateral ureteral obstruction (UUO) [21] mainly through the potential of antioxidant and antiapoptosis. Whether it is beneficial in folic acid induced AKI is unknown. A previous study showed that melatonin protected the liver against ischemia and reperfusion injury by inhibiting the release of HMGB1, blocking toll-like receptor signaling pathways [24]. However, the relationship between melatonin and HMGB1 in folic acid induced AKI is still unclear.

Based on previous research, we speculate that melatonin may play a beneficial role on folic acid induced AKI and this effect may be associated with the inhibition of intracellular localization changes of HMGB1. In this study, we tested this hypothesis with the model of AKI induced by folic acid in mice.

Materials and methods

Animals

Male C57BL/6 mice (8-10 weeks old and weighing 20-25 g) were purchased from HuaFukang Company (Beijing, China). All animals were given free access to water and food and provided 12 hours of light and 12 hours of darkness at Tongji Medical School. All experimental procedures were approved and performed according to the institutional guidelines for animal care. Mice were intraperitoneally injected with vehicle (150 mM $NaHCO_3$) or folic acid (150 mg/kg, Sigma-Aldrich, USA) for 3 consecutive days after adaptive feeding for 1 week. Melatonin (Sigma-Aldrich, USA) was dissolved in 1% ethanol, which was diluted with physiological saline, and was administrated intraperitoneally (20 mg/kg) before continuous folic acid injection for 1 week. The mice were sacrificed on day 3, and kidney and blood specimens were collected for further investigation.

Kidney function

Blood samples were obtained and centrifuged at 3000 rpm for 10 minutes to gain serum. The levels of serum BUN and creatinine were investigated using colorimetric assays according to the manufacturer's instructions (BioAssay Systems, USA).

Histology

Kidneys were obtained after heart perfusion and fixed in 4% paraformaldehyde for 24 hours, embedded in paraffin and sliced into sections (2-3 um). HE staining was performed to assess pathological damage and the degree of general infiltration of inflammatory cells. PAS staining was used to evaluate kidney pathological injury according to our previous report [25]. The tubular damage score was evaluated based on our previous research [25].

Immunohistochemistry and immunofluorescence

Renal paraffin sections were subjected to heat antigen retrieval. Endogenous peroxidase was blocked with 10% H_2O_2 for 20 minutes and then the nonspecific antigens were blocked with serum for 30 minutes at room temperature. The slides were then incubated with specific primary antibodies against Kim-1 (1:800, R&D Systems, USA), LTL (1:50, Vector Laboratories, USA), Ly6G (1:100, Abcam, UK), CD3 (1:100, Dako, USA), F4/80 (1:50, Abcam, UK), HMGB1 (1:200, Abcam, UK), PH3 (1:300, Abcam, UK), and Ki67 (1:200, Abcam, UK) at 4°C overnight after being washed in phosphate-buffered saline. These sections were incubated with HRP-conjugated secondary antibodies for IHC and fluorescent labeled secondary antibodies for IF. TUNEL was stained using a kit from Roche Diagnostics (Indianapolis, IN, USA) according to the manufacturer's instructions. Staining was carefully quantified in each slide by capturing 8 randomly chosen fields in a blind manner by 2 experienced renal pathologists and the data were analyzed using Image Pro Plus software (Media Cybernetics, USA).

Western blotting

Fresh renal tissues were lysed and total proteins were extracted as shown in previous research [25]. Cytoplasmic and nuclear proteins were isolated using the Nuclear and

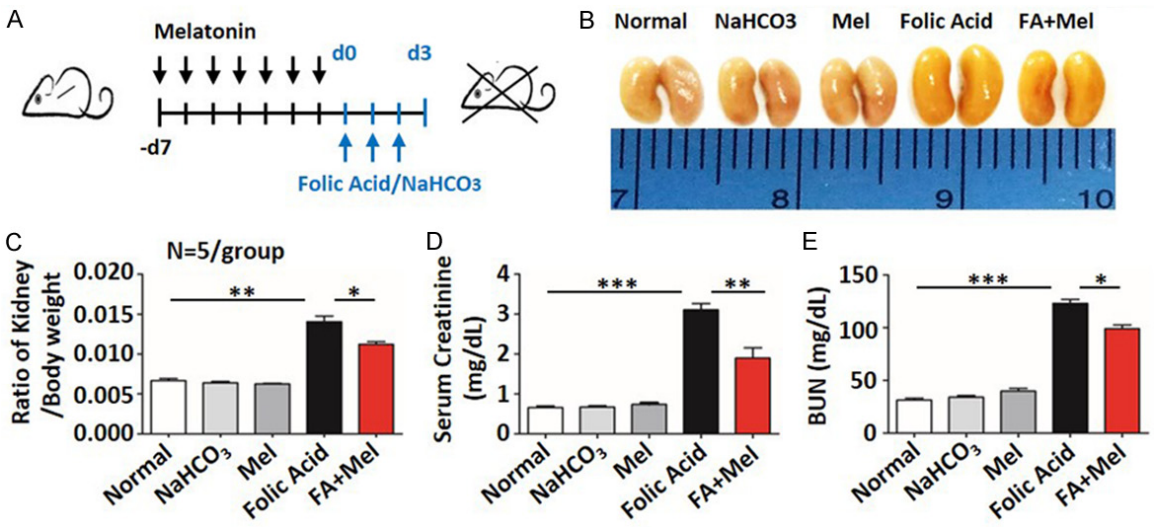

Figure 1. Melatonin pretreatment improved renal function in folic acid-induced AKI in mice. (A) Scheme of the experimental plan, which showed that mice were treated with folic acid or pretreated with melatonin. (B) Gross appearance of representative kidneys from mice in each group. (C) Statistical analysis of the ratio of the left kidney to body weight. Serum creatinine (D) and BUN (E) levels among different groups. Group Normal, mice without treatment; Group $NaHCO_3$, mice treated with vehicle; Group Mel, mice treated with melatonin alone; Group Folic Acid, mice treated with folic acid alone; Group FA+Mel, folic acid treated mice pretreated with melatonin for 1 week. N=5/group. Data were presented as means ± SEM. **P*<0.05, ***P*<0.01, ***P<0.001.

Cytoplasmic Protein Extraction Kit (KeyGen-Corporation of Biotechnology, Jiangsu, China) according to the manufacturer's instructions. Fifty micrograms of total protein or 20 micrograms of cytoplasmic protein were loaded and separated by SDS-PAGE. The gel was transferred onto PVDF membranes (Roche, Switzerland). The membranes were blocked with 5% skimmed milk in TBST for 1 hour at room temperature and were then incubated with appropriate primary antibodies against Bax (1:1000, CST, USA), Bcl-2 (1:1000, CST, USA) and HMGB1 (1:2000, Abcam, UK) at 4°C overnight. The membranes were incubated with HRP-conjugated secondary antibodies and were visualized by enhanced chemiluminescence (ECL, BioRad, USA). The relative expression levels were normalized to those of GAPDH (1:4000, Abbkine, China) and the signal intensity was analyzed using Image J.

Quantitative RT-PCR

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen, USA) and 1 μg of RNA was reverse transcribed into first-strand cDNA using the GoScript reverse transcription system (Promega, USA). Quantitative RT-PCR was conducted using the SYBR master-mix (Qiagen, Germany) on the Roche light 480II. Relative mRNA expression levels were calculated using the 2-ΔΔCt method and were normalized to the expression levels of GAPDH. The primer sequences were as follows (TsingKe Biological Technology, Beijing, China):

Genes	Forward and Reverse Primers
Mouse GAPDH	5'-AGGTCGGTGTGAACGGATTTG-3'
	5'-GGGGTCGTTGATGGCAACA-3'
Mouse Kim-1	5'-ACA TATCGTGGAATCACAACGAC-3'
	5'-ACTGCTCTTCTGATAGGTGACA-3'
Mouse IL-6	5'-GAGGATACCACTCCCAACAGACC-3'
	5'-AAGTGCATCATCGTTGTTCATACA-3'
Mouse IL-1β	5'-GAAATGCCACCTTTTGACAGTG-3'
	5'-TGGATGCTCTCATCAGGACAG-3'
Mouse TNF-α	5'-CCCTCACACTCAGATCATCTTCT-3'
	5'-GCTACGACGTGGGCTACAG-3'

Statistical analysis

All results were presented as means ± SEM from at least 3 separate experiments. Statistical differences between two groups were analyzed using the unpaired *t*-test or the Mann-Whitney *U* test by GraphPad Prism 5.0. The statistical significance of differences was set at P<0.05.

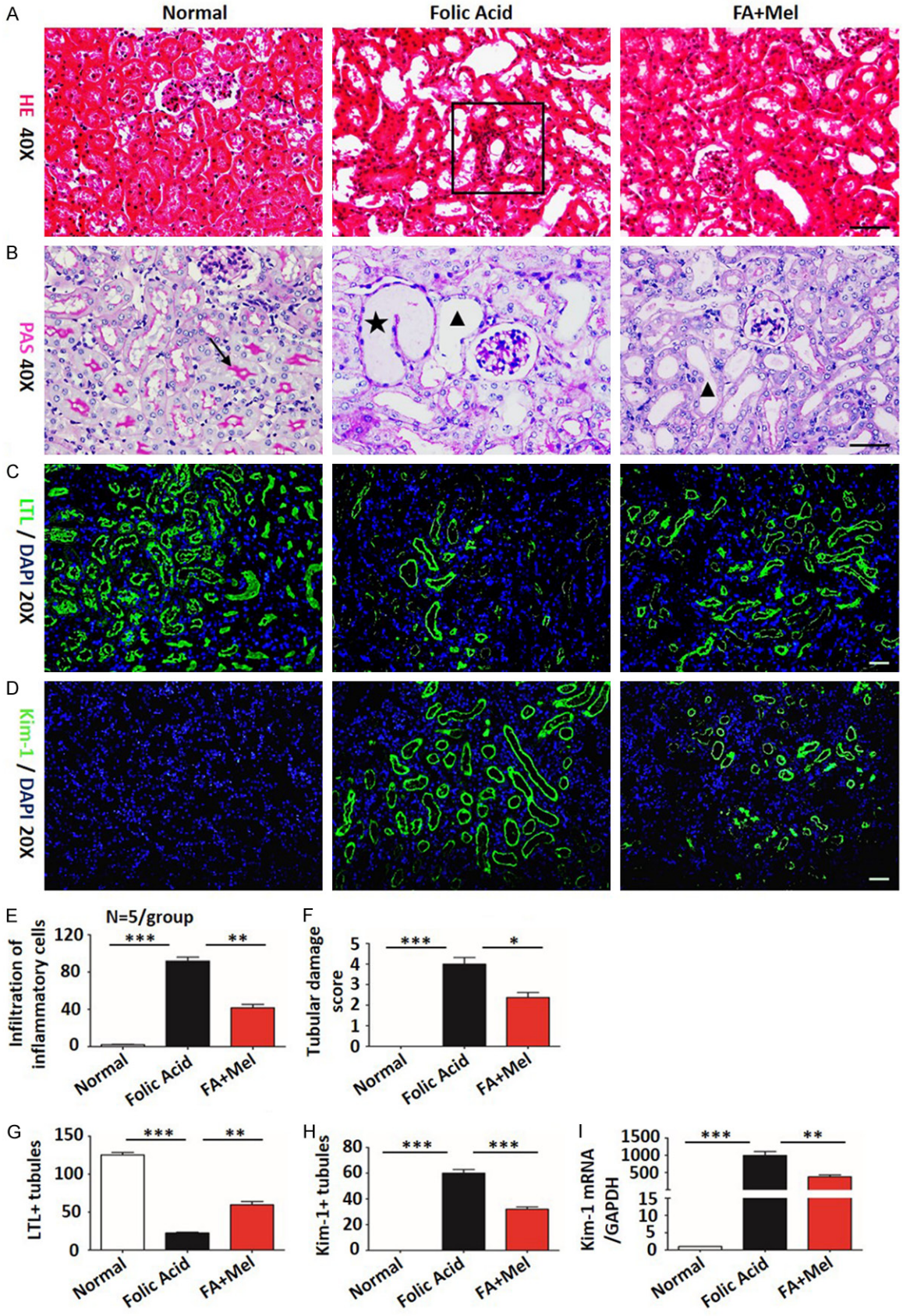
A
Normal
Folic Acid
FA+Mel
HE 40X
B
PAS 40X
C
LTL / DAPI 20X
D
Kim-1 / DAPI 20X
E
N=5/group
Infiltration of inflammatory cells
120
80
40
0
***
**
Normal
Folic Acid
FA+Mel
F
Tubular damage score
5
4
3
2
1
0
***
*
Normal
Folic Acid
FA+Mel
G
LTL+ tubules
150
100
50
0
***
**
Normal
Folic Acid
FA+Mel
H
Kim-1+ tubules
80
60
40
20
0
***
***
Normal
Folic Acid
FA+Mel
I
Kim-1 mRNA /GAPDH
1500
1000
500
15
10
5
0
***
**
Normal
Folic Acid
FA+Mel

Figure 2. Melatonin pretreatment attenuated renal pathological injury induced by folic acid. Representative photomicrographs of hematoxylin and eosin (HE) (A) and periodic acid-Schiff (PAS) (B) stained kidney sections. Nucleated cells in the black rectangle in (A) represented inflammatory cells. Black arrows in (B): represented the proximal tubule brush border; Triangles in (B): represented the expansion of tubules; Pentagon in (B): represented casts. Kidney sections immunolabeled with primary antibodies against LTL (C) and Kim-1 (D) were shown. LTL: lotus tetraglonolobus lectin; Kim-1: kidney injury molecule-1. (E) The general infiltration of inflammatory cells were quantified in 8 visual fields from HE stained sections. (F) Tubular damage was scored semiquantitatively using PAS-stained kidney sections. The remaining tubules were analysed by LTL staining (G). Injured tubules were assessed by Kim-1 staining (H). The gene expression level of intrarenal Kim-1 was assessed by RT-PCR (I). N=5/group. Scale bars =50 μm. Data were presented as the means ± SEM. **P*<0.05, ***P*<0.01, ***P<0.001.

Results

Melatonin pretreatment improved renal function

We first observed the effect of melatonin on renal function in AKI induced by folic acid. As shown in **Figure 1A**, AKI was induced by intraperitoneal injection of folic acid into male C57BL/6 mice at a dose of 150 mg/kg for 3 consecutive days. The mice were sacrificed on day 3. Melatonin was administrated intraperitoneally before folic acid injection at a dose of 20 mg/kg for 1 week. Folic acid treatment significantly increased the volume of kidney compared to the control group based on gross observations, while the kidney volume in the melatonin group decreased slightly (**Figure 1B**). We calculated the ratio of kidney weight to body weight, and the result was in line with gross observations (**Figure 1C**). Pretreatment with melatonin before folic acid insult significantly decreased the levels of serum creatinine and blood urea nitrogen (BUN) compared to folic acid treatment alone (**Figure 1D** and **1E**). Sodium bicarbonate (vehicle) or melatonin treatment alone had no effect on kidney weight and morphology. The above data suggested that melatonin pretreatment can effectively improve renal function.

Melatonin pretreatment attenuated tubulointerstitial injury induced by folic acid

We next conducted pathological and immunofluorescent staining to confirm the alleviation of kidney injury after melatonin pretreatment. Histopathological examination showed that folic acid induced severe lesions in the TECs and interstitium on day 3 through hematoxylin and eosin (HE) staining (**Figure 2A**) and periodic acid-schiff (PAS) staining (**Figure 2B**), including severe tubular epithelial cell edema and necrosis, significant tubular dilation (the triangle marked tubules in **Figure 2B**), lots of casts formation (the pentagon marked tubules in **Figure 2B**), brush border loss (the LTL-positive tubules in **Figure 2C**), and a large number of inflammatory cells infiltration into the interstitium (the nucleated cells in the yellow rectangle in **Figure 2A**). In contrast, the lesions were significantly alleviated after melatonin pretreatment, which was presented by the obviously improved structure of the TECs, reduced infiltration of inflammatory cells in the interstitium, as evidenced by semi-quantitative analysis of the tubular damage score and infiltration of the inflammatory cells (**Figure 2F** and **2E**). Immunofluorescent staining showed that folic acid induced an obvious increase in Kim-1 expression (**Figure 2D** and **2H**), an important indicator of early renal injury, and a significant decrease in LTL-positive tubules (**Figure 2C** and **2G**), a marker presented the existence of tubular brush border. However, melatonin pretreatment reduced expression of Kim-1 and increased LTL positive tubules, the result of RT-PCR for Kim-1 was in line with this, as shown in **Figure 2I**. Vehicle or melatonin treatment alone had no effect on kidney pathology (data not shown). In summary, our data verified that melatonin pretreatment attenuated tubulointerstitial injury induced by folic acid.

Melatonin pretreatment reduced infiltration of inflammatory cells and secretion of inflammatory cytokines

Infiltration of inflammatory cells has long been recognized as an important pathogenesis of AKI [3, 4, 26]. To explore the effect of melatonin on the inflammatory response in folic acid-induced AKI, immunofluorescence was performed to evaluate the infiltration of inflammatory cells and RT-PCR was conducted to assess the secretion of inflammatory cytokines. As described in **Figure 3A-C**, Ly6G, CD3 and F4/80-positive cells were rarely detectable in

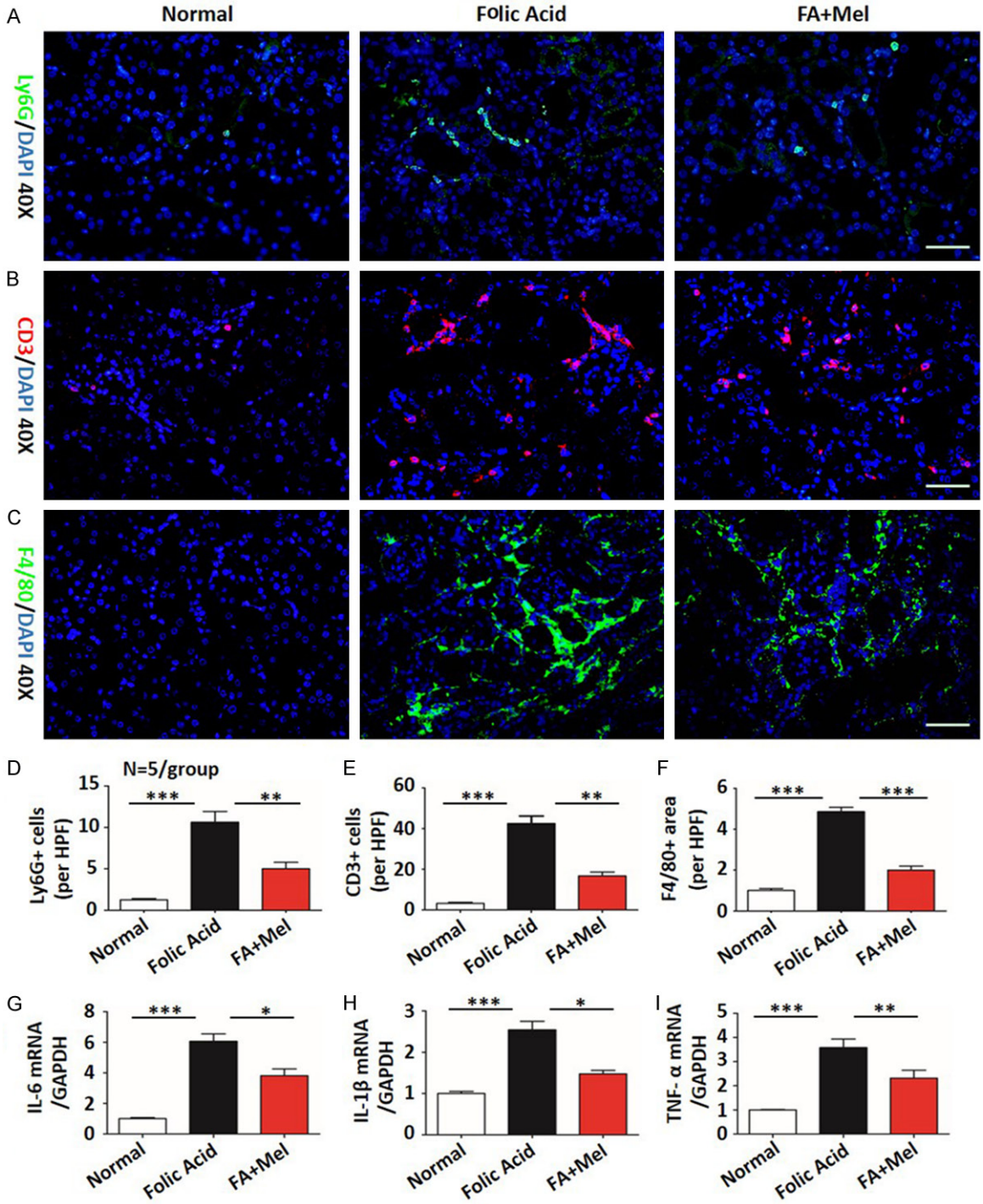

Figure 3. Melatonin pretreatment reduced infiltration of inflammatory cells and secretion of inflammatory cytokines. Kidney sections were immunolabeled with primary antibodies against Ly6G (A), CD3 (B) and F4/80 (C). The number of neutrophils (D), lymphocytes (E) and macrophages (F) infiltrated into kidneys were analysed in 8 visual fields using IF staining. (G-I) Real-time PCR of IL-6, IL-1β and TNF-α was performed to evaluate the expression levels of inflammatory cytokines in the kidney. N=5/group. Scale bars =50 μm. Data were presented as the means ± SEM. **P*<0.05, ***P*<0.01, ***P<0.001.

the control kidneys, while a large number of positive cells were observed in the interstitium after folic acid injection, suggesting that the infiltration of neutrophils, lymphocytes and

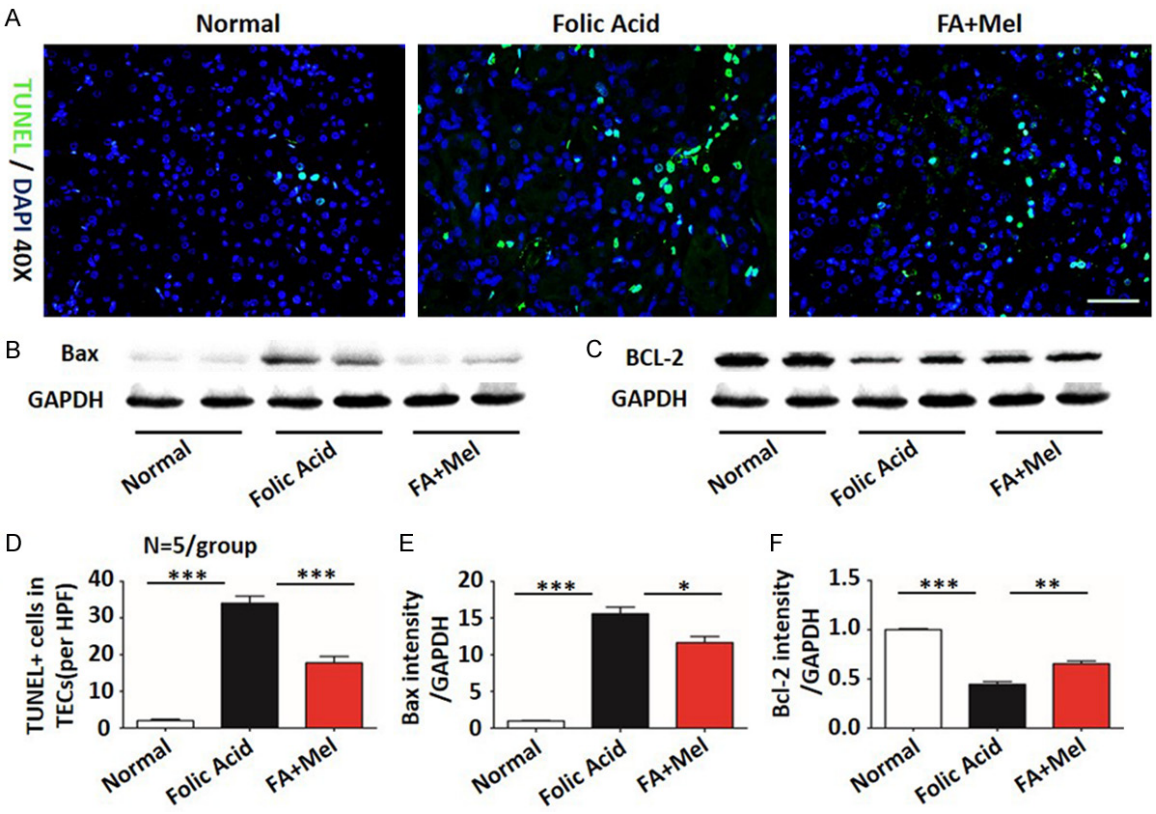

Figure 4. Melatonin pretreatment mitigated the apoptosis of TECs. Kidney sections were immunolabeled with primary antibodies against TUNEL (A). The number of apoptotic tubules was analysed in 8 visual fields using TUNEL staining (D). The expression of Bax (B) and Bcl-2 (C) was assessed using immunoblotting. Quantitation of Bax (E) and Bcl-2 (F) was normalized to GAPDH. Statistics analyzed using unpaired *t* test (Mann-Whitney *U* test). N=5/group. Scale bars =50 μm. **P*<0.05, ***P*<0.01, ***P<0.001. Values were means ± SEM.

macrophages into injured kidneys was increased (**Figure 3D-F**). Pretreatment with melatonin significantly decreased the number of Ly6G, CD3 and F4/80-positive inflammatory cells in folic acid-insult kidneys. Indeed, after folic acid injection, the expression of inflammatory cytokines such as IL-6, IL-1β, and TNF-α was upregulated significantly. As illustrated in **Figure 3G-I**, melatonin pretreatment strongly suppressed the expression of those inflammatory cytokines induced by folic acid. Vehicle or melatonin treatment alone had no effect on the infiltration of inflammatory cells (data not shown). In short, melatonin pretreatment reduced infiltration of inflammatory cells and inhibited secretion of inflammatory cytokines.

Melatonin mitigated apoptosis of TECs in injured kidneys

Previous findings have shown that necrosis and apoptosis of TECs act as another important mechanism of AKI pathogenesis [3]. We next aimed to explore the influence of melatonin on apoptosis in folic acid-induced kidney injury, TUNEL staining was performed on kidney sections and western blotting was conducted from the total protein extracted from kidneys of each group. As shown in **Figure 4A** and **4D**, TUNEL-positive nuclei were almost undetectable in the control kidneys, while the number of TUNEL-positive nuclei was significantly increased after folic acid insult. In contrast, melatonin pretreatment reduced the number of TUNEL-positive nuclei on day 3. In line with this, the expression of Bax, another indicator of apoptosis was increased in the group of folic acid insult, and melatonin pretreatment prominently reversed this effect (**Figure 4B** and **4E**). In contrast, folic acid treatment reduced the expression of Bcl-2, an important anti-apoptotic and pro-survival factor, whereas melatonin pretreatment restored its expression (**Figure 4C** and **4F**). Vehicle or melatonin treatment alone had no

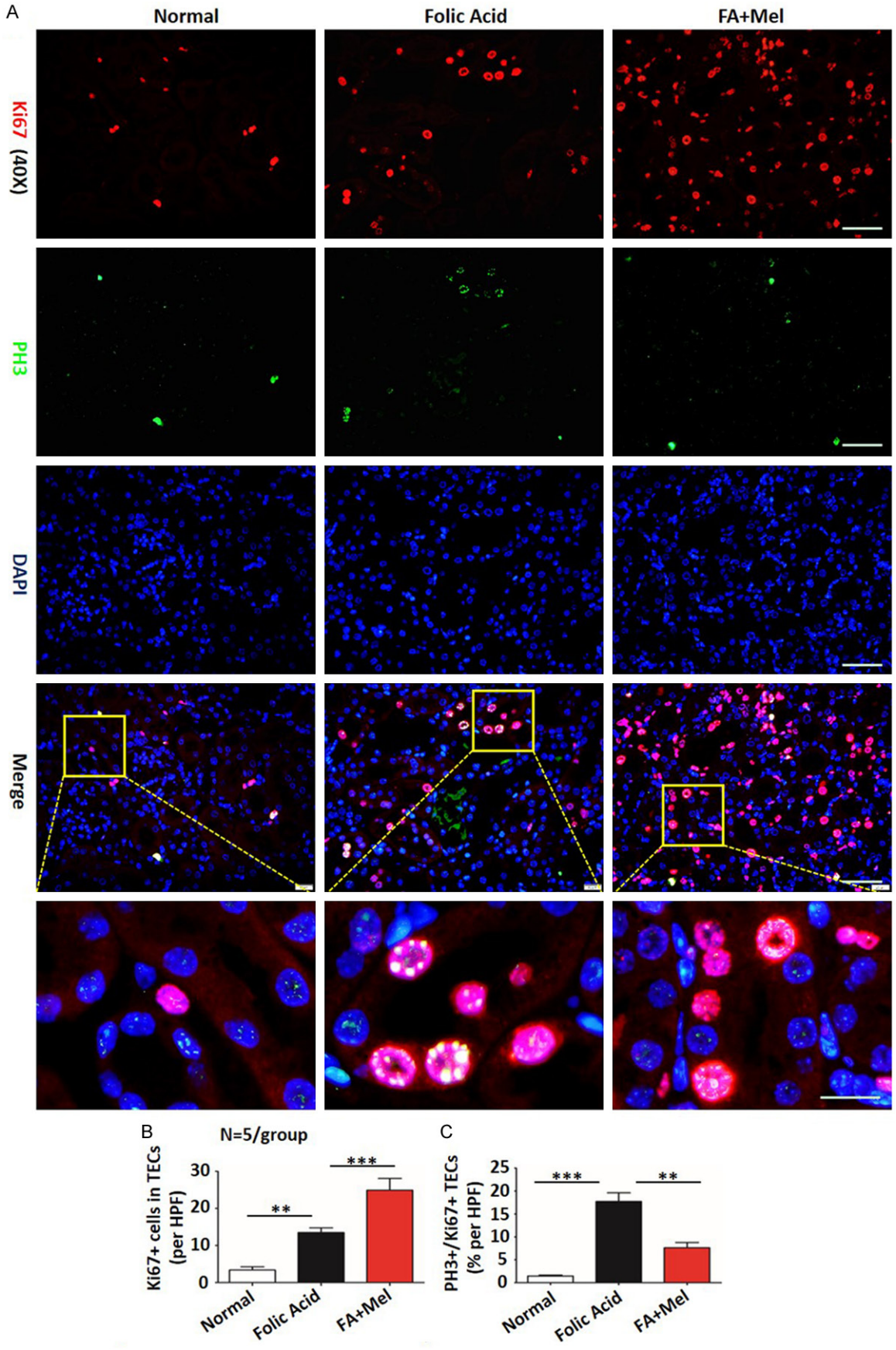
A
Normal
Folic Acid
FA+Mel
Ki67 (40X)
PH3
DAPI
Merge
B
N=5/group
Ki67+ cells in TECs (per HPF)
30
20
10
0
**
***
Normal
Folic Acid
FA+Mel
C
PH3+/Ki67+ TECs (% per HPF)
25
20
15
10
5
0
***
**
Normal
Folic Acid
FA+Mel

Figure 5. Melatonin pretreatment promoted the proliferation and improved cell cycle arrest of TECs. Representative photographs of kidney sections immunolabeled with primary antibodies against Ki67, and double-stained with Ki67 and pH3 were shown (A). The proliferation of epithelial cells was assessed in 8 visual fields using Ki67 staining (B). Cells arrested in G2/M phase were analysed in 8 visual fields using double-staining with Ki67 and PH3 (C). Statistics analyzed using unpaired *t* test (Mann-Whitney *U* test). N=5/group. Scale bars =50 μm. **P*<0.05, ***P*<0.01, ***P<0.001. Values were means ± SEM.

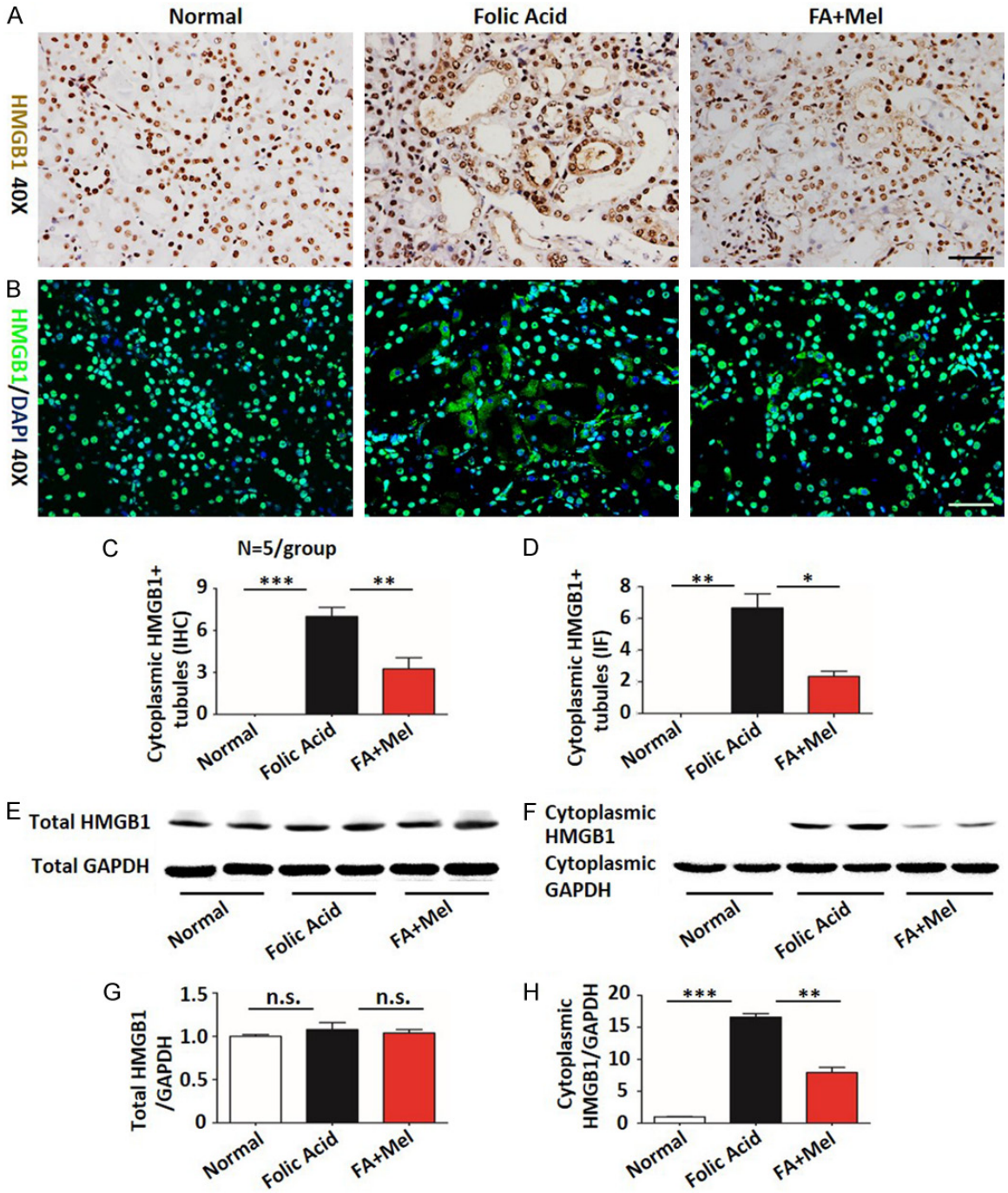

Figure 6. Melatonin pretreatment inhibited nucleocytoplasmic shuttling of HMGB1 in TECs. Representative photographs of kidney sections immunolabeled with primary antibodies against HMGB1 through IHC (A) and IF (B). Numbers of cytoplasmic HMGB1-positive tubules were calculated in (C) and (D). Total HMGB1 (E) in total protein and cytoplasmic HMGB1 (F) in cytoplastic protein were immunoblotted. The expression levels were normalized to GAPDH (G). (H) Statistics analyzed using unpaired *t* test (Mann-Whitney *U* test). N=5/group. Scale bars =50 μm. **P*<0.05, ***P*<0.01, ***P<0.001. Values were means ± SEM.

effect on the apoptosis of TECs (data not shown). In summary, these data demonstrated that melatonin could exert a beneficial role on AKI induced by folic acid through reducing the apoptosis of TECs as well as promoting its survival.

Melatonin pretreatment promoted proliferation and improved cell cycle arrest of TECs

Previous research have demonstrated that melatonin can promote regeneration of liver suffering from fulminant hepatitis of viral origin [27]. To substantiate the pro-regeneration effect of melatonin in kidney, we next aimed to detect renal tubular repopulation through immunofluorescence staining against Ki67. Folic acid insult induced a slight increase of Ki67-positive nuclei, while melatonin pretreatment significantly increased the number of Ki67-positive nuclei in TECs (**Figure 5A** and **5B**), suggesting that melatonin pretreatment could further promote the proliferation of TECs. Vehicle or melatonin treatment alone had no effect on the proliferation of TECs (data not shown).

As described previously, cell cycle arrest of TECs mediated pathological injury of AKI and progression into CKD [28, 29]. We next performed immunofluorescence double labeling against Ki67 and PH3 to assess the level of cell cycle arrest in TECs. Ki67 and PH3 double-positive nuclei were significantly increased upon folic acid insult, suggesting that the number of cells in G2/M arrest increased after folic acid injury. Conversely, the number of double labeled nuclei was reduced after melatonin pretreatment (**Figure 5A** and **5C**), suggesting that the number of cells in G2/M arrest decreased compared to folic acid insult. In summary, melatonin pretreatment promoted tubular proliferation and improved cell cycle arrest in TECs.

Melatonin pretreatment inhibited nucleocytoplasmic shuttling of HMGB1 in TECs

To determine whether melatonin could prevent nucleocytoplasmic shuttling of HMGB1, immunohistochemistry and immunofluorescence staining were performed to observe the changes in the intracellular location of HMGB1. In the control kidneys, HMGB1 is mainly present in the nucleus of renal parenchymal cells especially in TECs. However, cytoplasmic HMGB1 was significantly upregulated after folic acid insult. In contrast, melatonin pretreatment significantly reduced the expression of HMGB1 in cytoplasm as shown in **Figure 6A** and **6B**, which were quantified in **Figure 6C** and **6D**. There were no significant difference in total HMGB1 expression level among the control group, the folic acid treatment group and the melatonin pretreatment group by western blotting (**Figure 6E** and **6G**). However, the expression of cytoplasmic HMGB1 in the folic acid treatment group was significantly increased, which was significantly reduced in the melatonin pretreatment group (**Figure 6F** and **6H**). Vehicle or melatonin treatment alone had no effect on the location of HMGB1 in TECs (data not shown). In summary, our data suggested that melatonin can exert a renal protective effect via inhibiting the nucleocytoplasmic translocation of HMGB1.

Discussion

Here, we have demonstrated that melatonin exerted a favourable renal protective role in folic acid induced AKI. The protective effects of melatonin on kidney injury included improving renal function, attenuating renal pathological damage, reducing infiltration of inflammatory cells and the secretion of inflammatory cytokines and decreasing the apoptosis of TECs. Moreover, melatonin pretreatment increased the proliferation of TECs and improved cell cycle arrest of TECs. The mechanism that melatonin protected against acute kidney injury was via inhibiting nucleocytoplasmic shuttling of HMGB1 in TECs.

Melatonin (N-acetyl-5-methoxytryptamine) has been reported to exert renoprotective effects in kidney injury induced by I/R [23], severe burns [20] and UUO [21], mainly focused on its potential to remove reactive oxygen species (ROS), reduce endoplasmic reticulum stress or inhibit apoptosis of TECs. However, melatonin also exerts anti-inflammatory properties in many other diseases, such as myocardial infarction [30], graft rejection [31] and the brain injury [32]. Thus, in this study, we pretreated C57BL/6 mice with successive injection of melatonin for 1 week before folic acid insult and evaluated its anti-inflammatory effects. The dosage, frequency and methods of administration of melatonin were consistent with previous research [33]. We observed that

melatonin pretreatment significantly mitigated renal pathological damage, reduced apoptosis of TECs after folic acid insult. In addition, our study also found that melatonin pretreatment attenuated infiltration of neutrophils, lymphocytes and macrophages and the production of inflammatory cytokines induced by folic acid insult. It was convincible to speculate that melatonin alleviated acute inflammation response induced by folic acid, changing the inflammatory milieu in the kidney, thereby reducing renal damage of function and structure.

Previous studies have shown that AKI is often accompanied by necrosis and apoptosis of TECs and the loss of parenchymal cells will inhibit the repair and regeneration of injured kidneys [4, 34]. Li Yang [28] and colleagues verified that TECs arrested in the G2/M phase mediated renal injury and prevented the repair and regeneration of TECs. Thus, drugs that can promote the proliferation of TECs and improve cell cycle arrest may contribute to the repair and regeneration of injured kidneys [29]. Almudena Laliena [27] and colleagues demonstrated that melatonin treatment promoted liver regeneration in rabbits with fulminant hepatitis of viral origin. In view of this, we performed immunofluorescence double staining against Ki67 and PH3 and we demonstrated that melatonin pretreatment promoted the proliferation and improved cell cycle arrest of TECs, which was an excellent complement to previous studies. It is conceivable to speculate that melatonin pretreatment may facilitate the repair and regeneration of TECs through advancing the time point of proliferation and repair and reducing the proportion of cells in the aberrant cell cycle phase.

HMGB1 acts as a DAMP molecule and serves as a critical extracellular cytokine, released by apoptotic and necrotic cells and activated inflammatory cells, mediated the immune response to acute infection and injury [10, 13, 17]. A large number of studies have shown that HMGB1 nucleocytoplasmic translocation is the key mechanism to play a proinflammatory effect in the acute phase of immune response [16, 35]. As had been demonstrated in previous research, HMGB1 migrates from the nucleus to the cytoplasm upon acute injury and activates inflammatory cells, promoting the secretion of inflammatory cytokines and chemokines to form inflammatory cascades, aggravating the progression of acute injury [14, 15, 35]. Our previous study showed that lethal ischemia-reperfusion injury significantly induced the nucleocytoplasmic shuttling and release of HMGB1, when dealt with neutralizing antibodies of HMGB1 reduced kidney damage but exogenous supplementation of HMGB1 aggravated kidney injury [16]. In the present study, we observed that folic acid-induced AKI was accompanied by the overexpression of HMGB1 in the cytoplasm in line with our observation in lethal I/R. However, melatonin pretreatment reversed this process, manifested by the decreased expression of HMGB1 in the cytoplasm, suggesting that melatonin pretreatment inhibited nucleocytoplasmic shuttling of HMGB1.

Previous study found that melatonin protected the liver against ischemia and reperfusion injury through inhibiting the release of HMGB1, thereby preventing toll-like receptor signaling pathways [24]. The results in the present study were consistent with this report, but there were also some highlights: we found that melatonin pretreatment induced a change in the intracellular localization of HMGB1 from the cytoplasm to the nucleus; we also observed that melatonin pretreatment promoted the proliferation of renal epithelial cells and improved cell cycle arrest. However, there are still some limitations in our research. One is regarding the mechanism by which melatonin inhibits the nucleocytoplasmic shuttling of HMGB1. Recent studies revealed that melatonin can exert numerous biological activities through receptor-mediated or receptor-independent pathways [33, 36]. Whether the mechanism by which melatonin inhibits nucleocytoplasmic translocation of HMGB1 is receptor-mediated or receptor-independent will be further clarified and confirmed in our future experiments. Another limitation is regarding the effect of melatonin on chronic kidney fibrosis. Novel studies revealed that melatonin can also exert a protective role in chronic organ fibrosis [37-39] and whether this protective role can be associated with the inhibition of nucleocytoplasmic shuttling of HMGB1 is still unclear. We will elucidate these 2 questions in subsequent studies.

In conclusion, we have provided evidence demonstrating that pretreatment with melatonin can promote renal regeneration in folic acid-induced AKI by inhibiting nucleocytoplasmic

translocation. Grouped together, our data supports the idea that administering melatonin prior to folic acid insult may shed light on a potential treatment for AKI.

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Disclosure of conflict of interest

None.

Authors' contribution

The experiments were designed by Ying Yao, Rui Zeng, Gang Xu and Fengming Zhu, and performed by Fengming Zhu, Octavia L.S. Chong Lee Shin, Zhi Zhao and Huzi Xu. Data analysis was carried out by Fengming Zhu, Guangchang Pei, Juan Yang, Yanchao Guo, Han Zhu, and Meng Wang. Reagents were contributed by Zhizhi Hu, Jingyi Mou, Jie Sun, Yuxi Wang, Qian Yang, and Wenhui Liao. The manuscript was written by Fengming Zhu and Octavia L.S. Chong Lee Shin. The manuscript was read and approved by all of the authors.

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