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Inhibition of Pyroptosis of Renal Tubular Epithelial Cells by Puerarin via Regulation of lncRNA NEAT1 Ameliorating Chronic Renal Failure

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Original Paper

Keywords. chronic renal failure, puerarin, lncRNA NEAT1, pyroptosis

Introduction. Chronic kidney disease (CKD) is one of the major chronic human diseases worldwide. Puerarin, extensively used in traditional Chinese medicine, has shown favorable clinical effects in treating CKD. Here, we aimed to elucidate the mechanism by which puerarin alleviates CKD.

Methods. We constructed an animal model of CKD and intragastrically administered 400 mg/kg puerarin to the rat models. The extent of kidney injury was evaluated by performing hematoxylin and eosin staining. Then, we quantified the renal function indicators, inflammatory cytokines, apoptosis-related factors, and pyroptosis-related factors. HK-2 cells were treated with lipopolysaccharide (400 ng/mL) in H_2O_2 (200 μM) to induce oxidative stress. Then, the cells were treated with puerarin and transfected with overexpressed lncRNA NEAT1 vectors. Finally, the regulatory functions of lncRNA NEAT1 in cell apoptosis and pyroptosis were investigated.

Results. Puerarin treatment alleviated kidney damage and suppressed inflammation and apoptosis in the CKD rat model. Puerarin ameliorated pyroptosis in the CKD model by inhibiting caspase-1 and GSDMD-N expression. LncRNA NEAT1 was down-regulated in the CKD model after puerarin treatment. Puerarin enhanced cell viability when lncRNA NEAT1 was overexpressed, and the inhibition of apoptosis was reversed in the LPS/H_2O_2-stimulated HK-2 cells. Furthermore, lncRNA NEAT1 overexpression blocked the anti-pyroptosis effect of Puerarin in the CKD model.

Conclusion. Puerarin inhibits pyroptosis and inflammation by regulating lncRNA NEAT1, thereby ameliorating CKD.

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INTRODUCTION

The term chronic kidney disease (CKD) describes a state in which there is a progressive loss of kidney function and uremia, which is brought on by a variety of diseases and finally necessitates renal replacement therapy.[1] The pathogenesis of CKD is complicated and involves several biological processes, including renal tubular epithelial cell death, inflammatory response, and fibrosis.[2] Pyroptosis is a type of programmed cell death that occurs when cells are stimulated by infectious or endogenous injury-related signals, manifested as an excessive inflammatory response.[3,4] Pyroptosis occurs through the caspase-1-dependent classical pathway or the caspase-4/5/11-dependent non-classical pathway.[5,6] Cell contents released by

pyroptosis activate an inflammatory response, which participates in the progression of CKD.[7] Therefore, inhibition of pyroptosis may be a potential treatment for CKD.

Conventional treatment of CKD includes dialysis replacement therapy and kidney transplantation and diet therapy.[8,9] However, long treatment cycles and poor prognoses seriously affect their therapeutic efficacy.[10] Traditional Chinese medicine has achieved remarkable results by delaying the progression of CKD.[11] Puerarin, an isoflavone compound isolated from *Pueraria loata*, has diverse pharmacologic effects, such as vasodilation, antioxidant, anti-cancer, anti-inflammation, and anti-fibrosis effects. [12, 13] Li *et al.* reported that Puerarin reduced diabetic kidney injury by inhibiting the expression of NOX4 in podocytes.[14] Moreover, Zhou *et al.* indicated that puerarin mitigated renal fibrosis by lowering oxidative stress-triggered apoptosis of HK-2 cells via the MAPK pathway.[15] Puerarin could have protective effects on CKD; however, the regulatory mechanism remains unclear.

Long non-coding RNAs (lncRNAs) are considered key modulators of the biological processes.[16] They are associated with the occurrence and evolution of renal diseases and can be targeted for their prevention and treatment.[17, 18] Nuclear paraspeckle assembly transcript 1 (NEAT1) is a novel lncRNA discovered by Hutchinson *et al.* in 2007, and has been probed in multiple diseases including kidney diseases.[19, 20] Jiang *et al.* revealed that lncRNA NEAT1 facilitated the hypoxia-triggered apoptosis of renal tubular epithelial cells by down-regulating microRNA-27a-3p. [21] Ma *et al.* revealed that lncRNA NEAT1 accelerated acute kidney injury in CKD by facilitating the apoptosis of renal tubular epithelial cells.[22] However, the regulatory functions of lncRNA NEAT1 in the progression of CKD are still unclear.

Here, we investigated the effect of puerarin in the CKD model *in vivo* and *in vitro* by evaluating the inflammatory response and pyroptosis. Moreover, we identified whether lncRNA NEAT1 participated in regulating the effect of puerarin in CKD. Our results may provide a baseline for developing novel treatment strategies for CKD.

MATERIALS AND METHODS

Construction of the CKD Model In Vivo

We purchased 42 male Sprague–Dawley rats (7- to 8-week old, 250 to 300 g) from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). These rats were circled in a rearing cage under standard conditions with a 12-h/12-h dark/light cycle. After adaptive feeding for one-week, experimental rats were fed the chow containing 0.5% adenine for three weeks. Subsequently, model rats were fed the chow containing 0.3% adenine for two weeks. Finally, the chow with 0.15% adenine was fed to maintain the progress of CKD until the rats were sacrificed.[23] Adenine administration was stopped at the end of the 10th week. After modeling, the rats were randomly divided into three groups, namely the Control group (n = 14), CKD model group (model group, n = 14), and puerarin interference group (puerarin group; n = 14). Puerarin (purity > 99%) was purchased from Meilunbio (Dalian, China). Puerarin (400 mg/kg) was intragastrically administered to the rats in the Puerarin group.[24] The rats in the control group were fed the same chow without adenine. When the experiment was finished, the experimental rats were jejunitis for 4 hours, and the blood samples were collected from the tail vein of the rats to determine the concentrations of blood urea nitrogen (BUN) and creatinine (Cr). The animals were euthanized, and the renal tissues were collected and stored at −80 ℃. The animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Kunming Medical University (No. kmmu20221002).

Cell Treatment

HK-2 cells were procured from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were treated with lipopolysaccharide (LPS; 400 ng/mL) in H_2O_2 (200 μM) for 12 h to induce oxidative stress.[25] The different concentrations of puerarin (50, 100, 150, and 200 μM) were added to LPS-treated HK-2 cells to determine the optimal puerarin concentration for subsequent experiments.

Hematoxylin and Eosin (H&E) Staining

The renal tissues were fixed in 10% neutral formalin (Sigma,USA), soaked in EDTA, embedded in paraffin, cut into slices, and stained by using H&E.[26]

Assessment of Renal Function

The renal function indicators (BUN and

Creatinine) were quantified by using a biochemical analyzer (Hitachi, Tokyo, Japan).

ELISA

Renal tissue suspensions were centrifuged at 500 g and 4 °C for 10 min to collect the homogenate and supernatant. The concentrations of inflammatory cytokines (IL-1β and IL-18) were determined by using the respective detection kits (Abcam, MA, USA).

Cell Transfection

The lncRNA NEAT1 overexpression transfection system was established by using pcDNA3.1. The lncRNA NEAT1 overexpression and control vectors were synthesized by GenePharma (Shanghai, China). Lipofectamine 3000 (Invitrogen, California, USA) was used to transfect the HK-2 cells.

Cell Viability

Cell suspensions from each group were collected. Cell counting kit-8 (CCK-8; AbMole BioScience, Shanghai, China) was used to determine cell viability. CCK-8 solution (10 µL) was added to the cell suspensions in the culture medium followed by 1-hr incubation at 37 ℃. The absorbance was recorded at 450 nm using a microplate reader (Bio-Tek, VT, USA).

Cell Apoptosis

After cell stimulation and transfection, the culture medium was removed, and HK-2 cells were washed twice with PBS (Sigma). Then, HK-2 cells were immobilized in 70% ethanol, washed with PBS, and stained with 5 µL propidium iodide (PI) / fluorescein isothiocyanate–Annexin V (Sigma) for 15 min at the 25 ℃ in the dark. The apoptotic cells were observed by using flow cytometry (Guava Technologies, CA, USA) and analyzed by using a Synergy LX Multi-Mode Reader (BioTek).

RT-qPCR

After treatment with Puerarin and transfection with lncRNA NEAT1 overexpression vectors, RNA-spin™ total RNA extraction kit (iNtRON Biotechnology, Korea) was used to isolate total RNA from renal tissues and cells. RNA was reverse transcribed into cDNA using the PrimeScript Reverse Transcriptase Kit (Qiagen, CA, USA). RT-qPCR was performed by using SYBR1 Premix Ex TaqTM II (TaKaRa) and an ABI 7500 Sequencing Detection System (Applied Biosystems, Foster City, CA, United States). Expression of lncRNA NEAT1 was estimated by using the $2^{-\Delta\Delta Ct}$ method, and GAPDH served as an internal control. The primer sequences were: lncRNA NEAT1: forward primer (5′-3′) CTT CCT CCC TTT AAC TTA TCC ATT CAC and reverse primer (5′-3′) CTC TTC CTC CAC CAT TAC CAA CAA TAC and GAPDH: forward primer (5′-3′) TAA CCC TTC AGC GTT CAG CC and reverse primer (5′-3′) TAT AGG TGG TTT CGT GGA TGC C.

Western Blotting

After Puerarin treatment and transfection with lncRNA NEAT1 overexpression vectors, the total proteins were extracted from the cells using RIPA lysis Buffer (Beyotime, Shanghai, China). The supernatants were collected after centrifugation, and the BCA kit (Beyotime) was used to determine the protein concentrations in the samples. SDS-PAGE was performed to separate the proteins, which were transferred to the PVDF membranes. Then, the membranes were blocked with 5% defatted milk and incubated overnight at 4℃ with the following primary antibodies: Bcl-2 (1:2000; ab196495, Abcam), Bax (1:2000; ab182733, Abcam), cleaved caspase-3 (1:500; ab32042, Abcam), caspase-1 (1:1000, ab207802, Abcam), GSDMD-N (1:1000; DF13758, Affinity Biosciences, USA), and GAPDH (1:2000; ab8245, Abcam). Finally, the membranes were incubated with the anti-rabbit secondary antibody (1:2000; ab288151, Abcam) for 1 hour at 25 ℃. The protein bands were visualized by using a BeyoECL Plus Kit (Beyotime).

Statistical Analysis

Statistical analysis was performed by using Prism 7.0 software (GraphPad Software Inc., CA, USA). The data were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey's post hoc test were used for multigroup comparison. Statistically significant results were indicated as $P < .05$.

RESULTS

Puerarin Inhibits the Release of Inflammatory Factors and Apoptosis in the CKD Model In Vivo

The animal model was constructed by intragastric administration of puerarin (400 mg/kg). HE staining images revealed cavitation, blurred boundary, and

apoptosis in the renal tubular epithelial cells of the CKD model group compared to those in the control group (Figure 1A). However, the pathologic kidney injury was alleviated after puerarin treatment. The concentrations of renal function indicators (BUN and Cr) were increased in the CKD model group ($P < .001$; Figure 1B and 1C). The concentrations of BUN and Cr were lower in the puerarin treatment group than in the CKD model and control groups ($P < .01$ and $P < .01$; Figure 1B and 1C).

We explored the effect of puerarin on inflammatory response in the renal tissue by quantifying IL-1β and IL-18 using ELISA. IL-1β and IL-18 concentrations were higher in the CKD model group than in the Control group ($P < .001$; Figures 1D and 1E). Conversely, puerarin treatment inhibited CKD-triggered increase in IL-1β and IL-18 concentrations ($P < .01$ and $P < .01$). Furthermore, western blotting results revealed that the concentrations of apoptosis marker proteins (Bax and cleaved caspase-3) were significantly higher, whereas Bcl-2 protein concentration was lower in the CKD model group compared to the control group ($P < .001$; Figure 1F). CKD-induced changes in the Bax, Bcl-2, and cleaved caspase-3 concentrations were reversed in the puerarin treatment group ($P < .05$, $P < .01$; Figure 1F). Overall, puerarin ameliorated CKD-induced inflammatory response and apoptosis.

Puerarin Inhibits lncRNA NEAT1 Expression and Ameliorates Pyroptosis in CKD Model In Vivo

Next, we investigated whether lncRNA NEAT1 affected the function of puerarin in CKD. Compared to the control group, lncRNA NEAT1 was overexpressed in the CKD model group ($P < .001$, Figure 2A). LncRNA NEAT1 expression was

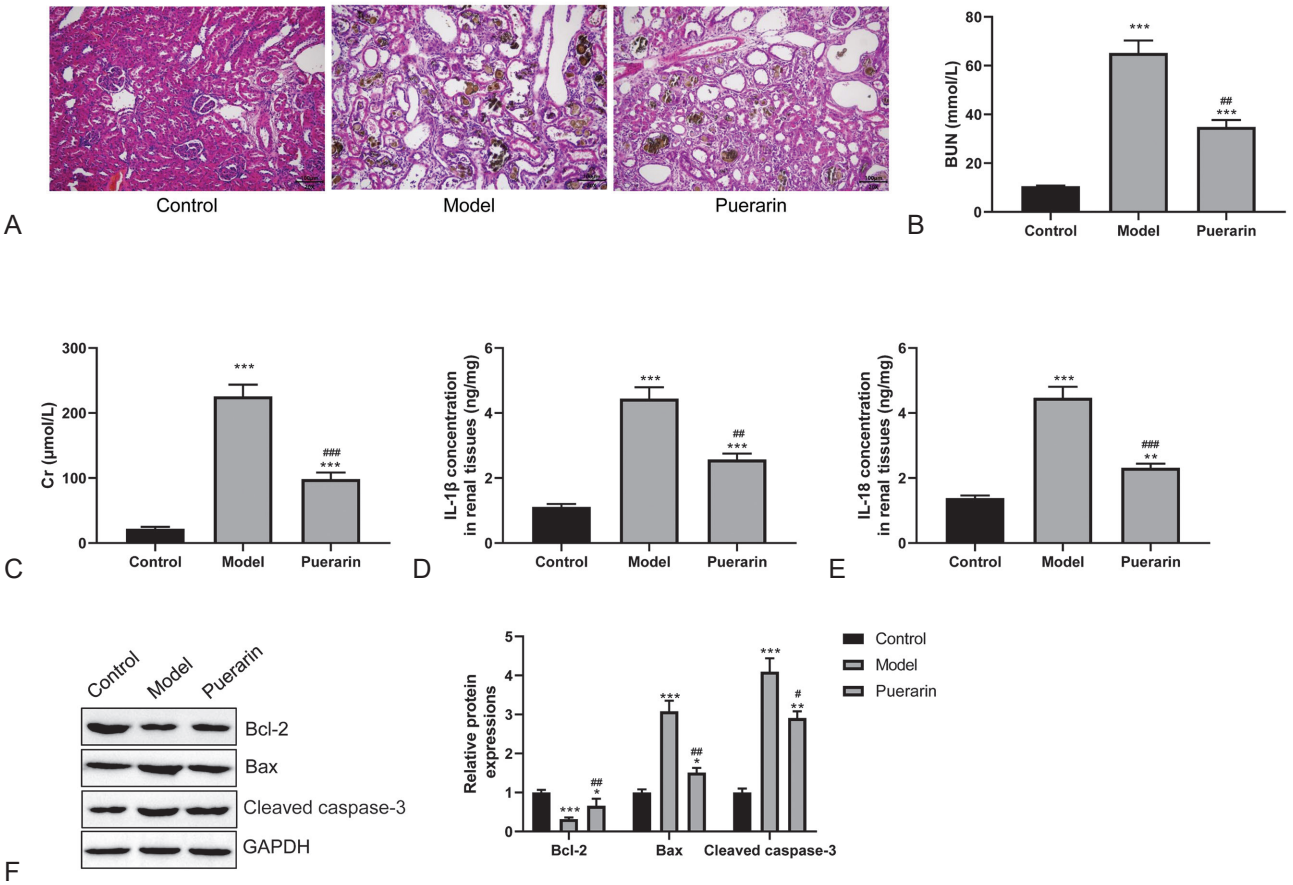

Figure 1. Puerarin Treatment Has Protective Effects in the CKD Rat Model
Adenine was used to construct an animal model of CKD, and 400 mg/kg puerarin was intragastrically administered. The model rats were divided into three groups, namely Control, Model, and Puerarin: A) Renal tissue damage was assessed by using HE staining, B and C) Renal function indicators (BUN and Cr) were quantified using the respective kits, D and E) Inflammatory cytokine (IL-1β and IL-18) concentrations in renal tissue homogenate were quantified using ELISA, and F) Concentrations of apoptosis-associated proteins (Bcl-2, Bax, and cleaved-caspase-3) were determined using western blotting (*$P < .05$, **$P < .01$, and ***$P < .001$ *vs.* Control group; #$P < .05$, ##$P < .01$, and ###$P < .001$ *vs.* Model group).

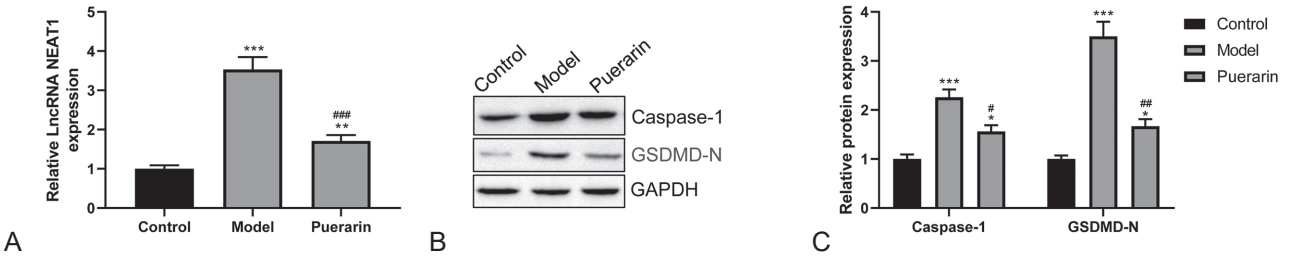

Figure 2. Puerarin Inhibits lncRNA NEAT1 Expression and Alleviates Pyroptosis in the CKD Rat Model
After the construction of the CKD model and puerarin (400 mg/kg) administration, the experimental rats were divided into three groups, namely Control, Model, and Puerarin: A) RT-qPCR assay was used to detect the lncRNA NEAT1 expression, B, C) Western blotting was used to quantify caspase-1 and GSDMD-N protein levels (*P < .05, **P < .01, and ***P < .001 *vs.* Control group; $^{\#}P$ < .05, $^{\#\#}P$ < .01, and $^{\#\#\#}P$ < .001, *vs.* Model group).

downregulated after puerarin treatment (P < .001, Figure 2A). Furthermore, we explored the effects of puerarin on pyroptosis in the CKD model. The concentrations of caspase-1 and GSDMD-N, the pyroptosis marker proteins, were higher in the CKD model group compared to the control group (P < .001, Figure 2B-C); however, these concentrations decreased after puerarin treatment (P < .05 and P < .01, Figure 2B-C). Overall, puerarin treatment inhibited lncRNA NEAT1 expression and CKD-triggered pyroptosis.

Overexpression of lncRNA NEAT1 Inhibits the Protective Function of Puerarin in LPS/H_2O_2-stimulated HK-2 Cells

Stimulated HK-2 cells were treated with different concentrations of puerarin to screen the optimal experimental concentration. The viability of HK-2 cells was significantly impaired after 150 and 200 μM puerarin treatment (P < .05 and P < .01, Figure 3A). HK-2 cell viability was similar after 50 and 100 μM puerarin treatment (Figure 3A), and 100 μM puerarin was selected for subsequent experiments. LncRNA NEAT1 expression was inhibited by 50 μM (P < .05) or 100 μM puerarin treatment (P < .001, Figure 3B). Subsequently, the lncRNA NEAT1 overexpression vector was constructed and transfected into HK-2 cells, and the effect of lncRNA NEAT1 on the protective function of puerarin in LPS/H_2O_2-stimulated HK-2 cells was determined. LncRNA NEAT1 expression was enhanced in HK-2 cells transfected with overexpression vectors (P < .001, Figure 3C). Moreover, the decrease in the concentrations of lncRNA NEAT1 triggered by Puerarin in LPS/H_2O_2-stimulated HK-2 cells was reversed after transfecting the cells with lncRNA NEAT1 overexpression vectors (P < .001, Figure 3D). Furthermore, the increase in cell viability and decrease in apoptosis of HK-2 cells induced by puerarin were reversed by the overexpression of lncRNA NEAT1 (P < .05 and P < .01, Figure 3E-3G). These results indicated that lncRNA NEAT1 overexpression impaired the protective function of puerarin in LPS/H_2O_2-stimulated HK-2 cells.

Overexpression of lncRNA NEAT1 Impairs the Anti-pyroptosis Effect of Puerarin in LPS/H_2O_2-stimulated HK-2 Cells

We transfected HK-2 cells with the overexpression vectors and explored the effects of lncRNA NEAT1 on the pyroptosis and inflammatory response. The concentrations of caspase-1 and GSDMD-N were lower in the Puerarin group than in the CKD model group (P < .05 and P < .01, Figure 4A and 4B). However, overexpression of lncRNA NEAT1 significantly reversed the effect of puerarin on caspase-1 and GSDMD-N protein concentrations in LPS/H_2O_2-stimulated HK-2 cells (P < .05 and P < .01, Figure 4A and 4B). Similarly, the concentrations of IL-1β and IL-18 were decreased by puerarin intervention in the LPS/H_2O_2-stimulated HK-2 cells (P < .01, Figure 4C and 4D). The decline in the IL-1β and IL-18 concentrations was reversed after transfection with lncRNA NEAT1 overexpression vectors (P < .01, Figure 4C and 4D). Overall, lncRNA NEAT1 overexpression hampered the anti-pyroptosis effect of puerarin in LPS/H_2O_2-stimulated HK-2 cells.

DISCUSSION

CKD is a general term for variety of kidney diseases, including primary and secondary glomerulonephritis, tubular injury, and vascular disease.[27,28] The incidence of CKD in China exceeds

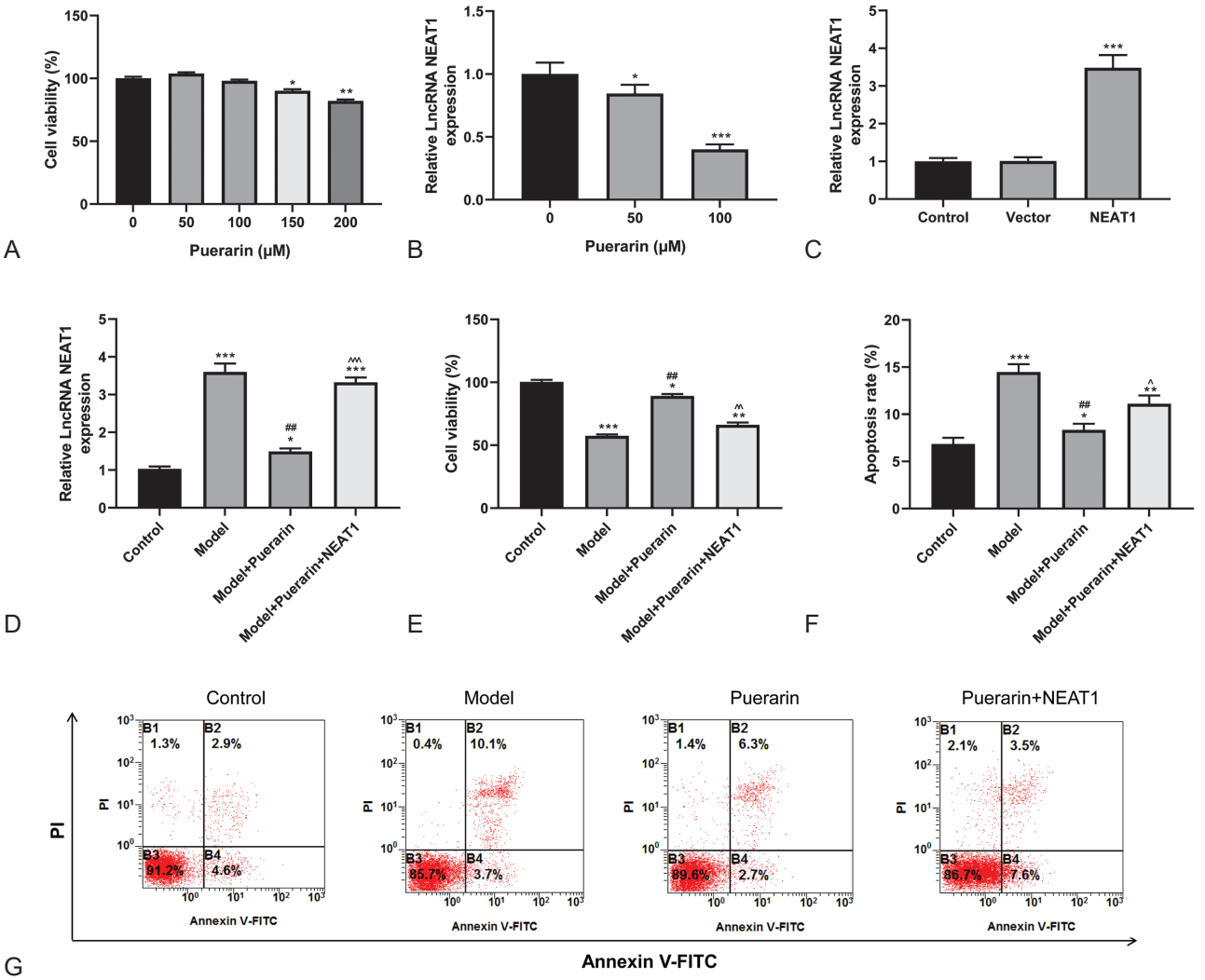

Figure 3. LncRNA NEAT1 Overexpression Reverses the Protective Function of Puerarin in LPS/H_2O_2-stimulated HK-2 Cells
A) HK2 cells were treated with different concentrations of Puerarin (0, 50, 100, 150, and 200 μM), and cell viability was determined using the CCK-8 assay, B) Expression of lncRNA NEAT1 in HK2 cells was evaluated using RT-qPCR after treatment with puerarin (50 and 100 μM), C) After transfection with lncRNA NEAT1 overexpression vectors, RT-qPCR was used to determine the lncRNA NEAT1 expression in HK-2 cells. HK-2 cells were treated with LPS (400 ng/mL) in H_2O_2 (200 μM) to induce oxidative stress. After treatment with puerarin (100 μM) and transfection with lncRNA NEAT1 overexpression vectors, D) Expression of lncRNA NEAT1 was determined using RT-qPCR, E) Cell viability was examined using CCK-8 assay *in vitro*, F and G) Cell apoptosis was examined using flow cytometry (*P < .05, **P < .01, and ***P < .001, *vs.* Control group; $^{\#}P$ < .05, $^{\#\#}P$ < .01, and $^{\#\#\#}P$ < .001, *vs.* Model group; $^{\wedge}P$ < .05, $^{\wedge\wedge}P$ < .01, and $^{\wedge\wedge\wedge}P$ < .001 *vs.* Model + Puerarin).

10%; it progresses to CKD and then the end-stage renal disease, which poses a serious threat to human health. [29, 30] The limited availability of donated kidneys and the high costs of transplantation are the limiting factors for most patients with end-stage kidney diseases. Therefore, the prevention and treatment of CKD are of great significance. Here, we explored the effect of puerarin in CKD *in vivo* and *in vitro* and found that puerarin ameliorated CKD by suppressing cell apoptosis, reducing the release of inflammatory factors (IL-1β and IL-18), and decreasing the concentrations of pyroptosis-associated proteins (caspase-1 and GSDMD-N). Notably, the protective function of puerarin in CKD was reversed by lncRNA NEAT1 overexpression.

Traditional Chinese Medicine has a long history of use in the treatment of kidney diseases with remarkable efficacy in long-term clinical practice. *Ophiocordyceps lanpingensis* polysaccharides improved renal function by decreasing the concentrations of Cr and BUN in a CKD model.[31] Juzentaihoto alleviated adenine-triggered CKD

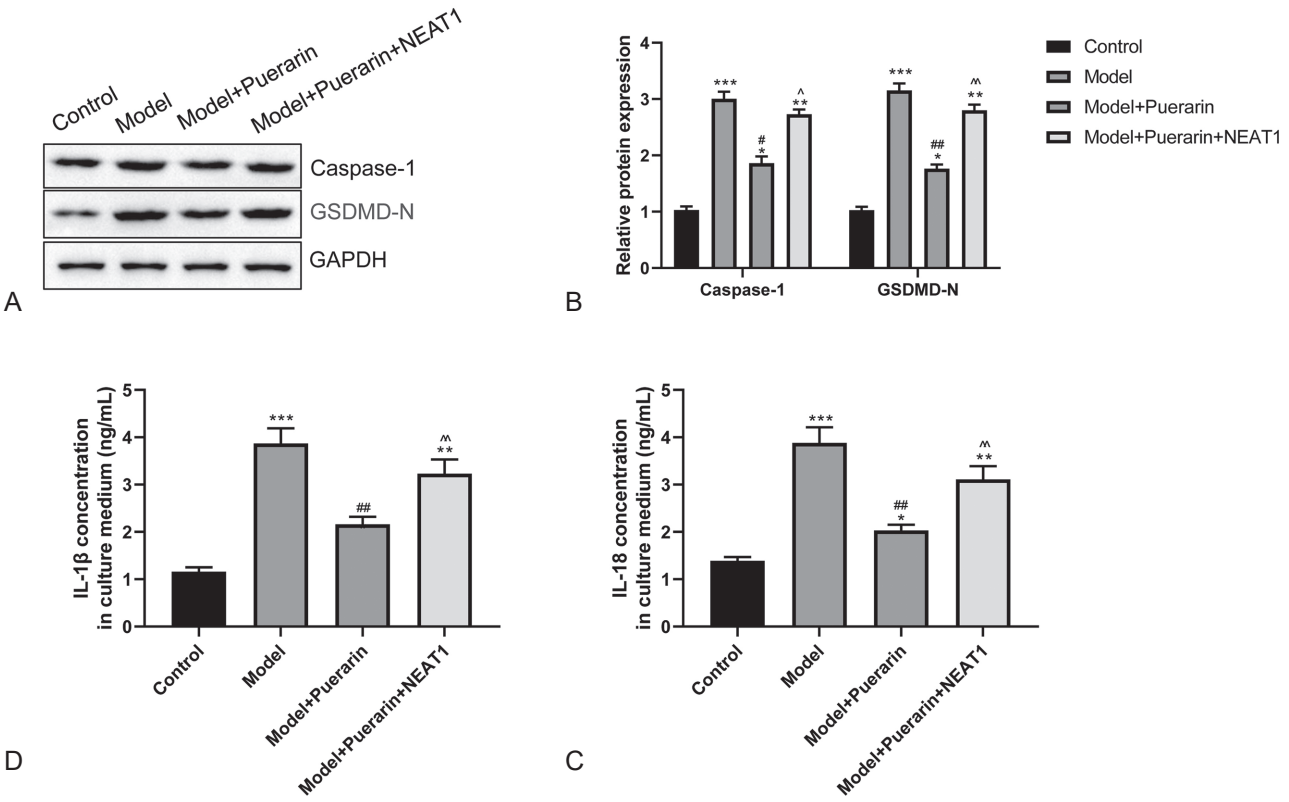

Figure 4. LncRNA NEAT1 Overexpression Suppresses the Anti-pyroptosis Function of Puerarin in LPS/H_2O_2-stimulated HK-2 Cells
HK-2 cells were treated with LPS (400 ng/mL) in H_2O_2 (200 μM) to induce oxidative stress. After treatment with Puerarin (100 μM) and transfection with lncRNA NEAT1 overexpression vectors: A and B) protein levels of caspase-1 and GSDMD-N were determined using western blotting, C and D) IL-1β and IL-18 concentrations were determined using ELISA (**P* < .05, ***P* < .01, and ****P* < .001, *vs.* Control group; #*P* < .05, ##*P* < .01, and ###*P* < .001, *vs.* Model group; ^*P* < .05, ^^*P* < .01, and ^^^*P* < .001 *vs.* Model + Puerarin).

in BALB/c mice by inhibiting renal fibrosis and inflammation.[32] Puerarin is a natural flavonoid compound with multiple biological activities and pharmacologic functions in several disorders, such as central nervous system diseases, ophthalmology, and cardiovascular diseases.[33-35] Gong *et al.* revealed that puerarin improved renal function and was linked to the mitochondrial homeostasis-involved pathways in diabetic nephropathy [36]. However, the effect of puerarin on CKD is still unclear. Here, we observed that puerarin treatment ameliorated kidney injury in the CKD model by regulating renal function, inflammatory response, and apoptosis. Overall, puerarin had a protective effect on CKD.

Pyroptosis is involved in the occurrence and development of multiple renal diseases, such as acute kidney injury caused by ischemia/reperfusion, diabetic nephropathy, renal fibrosis, and crystal-associated nephropathy.[37,38] Pyroptosis can aggravate renal tubular damage.[39] However, the modulatory function of pyroptosis in CKD is unclear. The classical pathway of pyroptosis inflammasome activates the precursor of caspase-1, thereby activating downstream molecules of IL-1β and IL-18.[40] GSDMD is a key effector of pyroptosis, which is cleaved by caspase-1 to generate GSDMD-C and GSDMD-N. GSDMD-N forming cellular pores, which, in turn, leads to the release of inflammatory factors, resulting in an inflammatory cascade.[41] Puerarin inhibits the oxidative damage of human retinal endothelial cells through the pyroptosis signaling pathway.[42] Li *et al.* showed that baicalin relieved contrast-triggered acute kidney injury by acting on caspase-1/GSDMD pathway-regulated proptosis *in vitro*. Similar to these findings, we found that puerarin reduced caspase-1 and GSDMD-N expressions and decreased the accumulation of IL-1β and IL-18 in the CKD model *in vivo* and *in vitro*. Taken together, puerarin ameliorated CKD progression by obstructing pyroptosis.

LncRNA NEAT1 has been extensively researched in kidney diseases. Gao *et al.* suggested that

lncRNA NEAT1 ameliorated septicemia-induced renal damage and inflammation in HK-2 cells.[43] It also accelerated tubular epithelial cell damage in diabetic nephropathy by regulating mitophagy. Here, lncRNA NEAT1 was downregulated in the CKD model after administration of puerarin, and its overexpression reversed the protective function of puerarin in CKD. Therefore, lncRNA NEAT1 may participate in regulating the protective function of Puerarin in CKD. Puerarin inactivated NLRP3-regulated pyroptotic cell death to relieve cerebral ischemia/reperfusion damage through mediating lncRNA double homeobox A pseudogene 8.[44] Han *et al.* reported that puerarin protected the cardiomyocytes from ischemia/reperfusion damage through modulating lncRNA ANRIL.[45] Taken together, puerarin inhibited pyroptosis to ameliorate CKD by regulating lncRNA NEAT1.

CONCLUSION

Puerarin treatment ameliorated CKD in a rat model, which was linked to pyroptosis inhibition and lncRNA NEAT1 regulation. Our findings suggest a novel mechanism by which puerarin exerts therapeutic effects in CKD. However, further investigations are imperative to understand whether other regulators are involved in this protective mechanism.

ETHICAL CONSIDERATIONS

This animal experiment was approved by the Ethics Committee of the First Affiliated Hospital of Kunming Medical University (No. kmmu20221002).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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