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Calcitriol Ameliorates Cognitive Impairment in CKD-CI Rats: Potential Involvement of Hippocampal mTOR/Beclin1/LC3B-II Signaling and Neuroinflammation

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Abstract

Background and Purpose: Cognitive decline frequently accompanies chronic kidney disease (CKD), yet effective interventions remain limited. This study aimed to investigate the effects of calcitriol (Cal) on cognitive impairment in rats with CKD-associated cognitive impairment (CKD-CI) and to explore the potential involvement of hippocampal autophagy-related markers and neuroinflammation. **Methods:** Forty male Sprague-Dawley (SD) rats were randomly divided into Sham, Model, Model + Cal, and Model + Cal + 3-MA groups using a random number table, with 10 rats per group. Except for the Sham group, rats underwent left nephrectomy combined with adenine gavage (250 mg/kg, once every other day) for 4 weeks. CKD-CI models were then screened using the Morris water maze (MWM), and 8 rats per group were retained. The Model + Cal group received intraperitoneal calcitriol at 10 ng/(kg·d); the Model + Cal + 3-MA group received additional 3-MA at 30 mg/(kg·d); the Sham and Model groups received an equal volume of normal saline. The intervention lasted 4 weeks. Spatial learning and memory were assessed by the MWM. Twenty-four-hour urinary protein, blood urea nitrogen (BUN), and serum creatinine (SCr) levels were measured, and histopathological changes in the kidney and hippocampus were observed. Hippocampal interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), NF-κB, and p-NF-κB levels were measured by enzyme-linked immunosorbent assay (ELISA). The protein expression of hippocampal mTOR, p-mTOR, Beclin1, and LC3B-II was determined by Western blotting. The mRNA expression of LC3, Beclin1, and mTOR was measured by quantitative real-time PCR (RT-qPCR). **Results:** Compared with the Sham group, the Model group exhibited prolonged escape

latency in the place navigation test, decreased platform crossings and the time spent in the target quadrant in the spatial probe test, increased hippocampal IL-6 and TNF-α levels and p-NF-κB/NF-κB and p-mTOR/mTOR ratios, and downregulated Beclin1 and LC3B-II protein and mRNA expression ($P < 0.05$). Compared with the Model group, the aforementioned behavioral, inflammatory, and autophagy-related marker alterations were significantly attenuated in the Model + Cal group ($P < 0.05$), whereas conventional renal function indices were not significantly improved ($P > 0.05$). Compared with the Model + Cal group, the Model + Cal + 3-MA group showed markedly attenuated improvements in cognitive, inflammatory, and autophagy-related marker parameters ($P < 0.05$). **Conclusion:** Calcitriol alleviates cognitive impairment in CKD-CI rats. These benefits were accompanied by reduced hippocampal mTOR phosphorylation, increased Beclin1 and LC3B-II expression, and suppressed NF-κB phosphorylation with lower IL-6 and TNF-α levels. These findings suggest that calcitriol may modulate autophagy-related and neuroinflammatory pathways; however, these associations do not establish causality, and a specific causal role for autophagy requires further verification with autophagic flux assays and appropriate controls.

Keywords

Chronic Kidney Disease, Cognitive Impairment, CKD-CI, Calcitriol, Autophagy, Neruon Inflammation, mTOR Signal Pathway

1. Introduction

Chronic kidney disease (CKD) is characterized by structural or functional abnormalities of the kidneys caused by various etiologies that persist for more than 3 months [1]. The disease is progressive, and some patients eventually progress to end-stage renal failure. Against the backdrop of an aging population, the prevalence of CKD continues to rise; approximately 10% of adults worldwide are affected by CKD, making it a major global public health challenge and one of the leading causes of death globally [2]. Cognitive impairment (CI) is a common neurological complication of CKD; approximately 16% - 18% of CKD patients experience varying degrees of cognitive dysfunction, manifested as memory deficits, impaired executive function, and slowed information processing. Cognitive impairment associated with chronic kidney disease (CKD-CI) is particularly prevalent among dialysis patients: the prevalence of CI among patients on maintenance hemodialysis can reach 85% [3], and approximately 75% of peritoneal dialysis patients exhibit varying degrees of cognitive impairment [4]. Without timely intervention, cognitive impairment can progress to dementia, which not only severely reduces patients' quality of life but also significantly increases the risk of rehospitalization, disability, and death [5]. Currently, there are no specific medications for treating CKD-CI. Clinically, treatment strategies for dementia are often adopted, primarily involving cholinesterase inhibitors; however, evi-

dence regarding their efficacy, safety, and long-term outcomes in the CKD population remains insufficient [6]. Therefore, there is an urgent need to explore new intervention targets and therapeutic agents.

The exact mechanisms underlying CKD-CI have not yet been fully elucidated. Comorbidities of CKD—such as renal anemia, secondary hyperparathyroidism, hemodynamic abnormalities, and dialysis-related imbalances—have all been reported to be associated with cognitive impairment [7]. Furthermore, declining renal function leads to impaired clearance of metabolic waste products; the retention of uremic toxins is considered a key link between renal damage and central nervous system injury [8]. Uremic toxins can induce inflammatory responses and oxidative stress, thereby further impairing cognitive function by affecting the integrity of the blood-brain barrier and neuronal survival [9]-[12]. Recent studies have also found that uremic toxins, together with the chronic inflammation they cause, can inhibit autophagy in neurons and glial cells [13]. Autophagy is a lysosome-dependent intracellular degradation pathway capable of clearing damaged organelles, misfolded proteins, and protein aggregates; it is crucial for maintaining neuronal homeostasis and synaptic function [8] [14]. Impaired autophagy can lead to the accumulation of abnormal proteins and damaged mitochondria, thereby exacerbating neuroinflammation and neuronal damage, suggesting that it may be an important pathophysiological basis of CKD-CI [8] [14].

Calcitriol is the active form of vitamin D3 and is commonly used in clinical practice to regulate mineral metabolism in patients with CKD. In addition to regulating calcium and phosphorus metabolism, calcitriol also exerts immunomodulatory, antioxidant, and neuroprotective effects, and can downregulate the expression of pro-inflammatory factors such as IL-6 and TNF-α [15]. Recent studies suggest that calcitriol can influence the signaling activity of the mammalian target of rapamycin (mTOR), thereby participating in the regulation of autophagy [16] [17]. However, there is currently a lack of direct evidence to determine whether calcitriol can inhibit neuroinflammation in the central nervous system by regulating autophagy and ultimately alleviate CKD-CI. Therefore, the present study aims to establish a CKD-CI rat model to evaluate the effects of calcitriol on cognitive function and to explore its potential mechanisms from the perspectives of the mTOR/Beclin1/LC3B-II autophagy pathway and the downstream NF-κB inflammatory pathway, with the goal of providing new experimental evidence for the treatment of CKD-CI.

2. Materials and Methods

2.1. Reagents and Main Instruments

Calcitriol injection was purchased from Chengdu Guowei Biopharmaceutical Co., Ltd (Chengdu, China). 3-Methyladenine (3-MA) was obtained from Shanghai Titan Scientific Co., Ltd. (Shanghai, China). ELISA kits for IL-6, TNF-α, NF-κB, and p-NF-κB were obtained from Quanzhou Ruixin Biotechnology Co., Ltd. (Fujian, China). Rabbit primary antibodies against mTOR, p-mTOR, Beclin1, and LC3B-

II were acquired from Taizhou Baijia Biotechnology Co., Ltd. (Shandong, China). Anti-β-actin antibody and HRP-conjugated goat anti-rabbit IgG were purchased from Servicebio (Wuhan, China). Trizol reagent was purchased from Invitrogen (USA), while the SuperRT III reverse transcription kit and SYBR Green qPCR Mix were obtained from Biosharp (Beijing, China). The Morris water maze and video behavioral analysis system (DigBehv-MWMG) were purchased from Shanghai Jiliang Software Technology Co., Ltd. (Shanghai, China).

2.2. Construction of the Animal Model

For this research, forty healthy adult male SD rats, aged 6 weeks and weighing 210 ± 10 g, were acquired from Guangdong Vital River Laboratory Animal Technology Co., Ltd. [License No.: SCXK (Yue) 2022-0063]. All experimental procedures were approved by the Animal Ethics Committee of Youjiang Medical University for Nationalities (Approval No. 2023120101) and followed the relevant principles for the care and use of laboratory animals. After 1 week of adaptive feeding under controlled conditions (22˚C ± 3˚C, 50% ± 10% humidity, and a 12-h light/dark cycle), the experimental animals were randomly divided into 4 groups: Sham group, Model group, Model + Cal group, and Model + Cal + 3-MA group, with 10 rats in each group. According to the previous research methods with modifications, left unilateral nephrectomy combined with adenine gavage was employed to establish the CKD rat model [18]. Following 8 h of pre-operative fasting, rats were anesthetized using avertin, secured in a prone position, and a 1.5 cm oblique incision was made 1.5 cm below the left costal margin to sequentially access the abdominal cavity and expose the left kidney. After separating the perirenal fat and renal capsule, the renal pedicle was doubly ligated, and the left kidney was completely resected while preserving the adrenal gland. Following hemostasis by compression, the incision was sutured and disinfected, and penicillin (60,000 U) was intramuscularly injected to prevent infection. In the sham group, rats underwent solely left kidney exposure and capsule stripping without any vascular ligation or renal tissue resection, with all other procedures identical to the model group. Post-surgery, each rat was fasted for 24 h with free access to water. After the recovery period, adenine (250 mg/kg) was intragastrically administered every other day for 4 consecutive weeks. Cognitive function was assessed 4 weeks post-intervention using the Morris water maze (MWM) test. Animals displaying marked impairments in visual acuity, motor endurance, or swimming performance were excluded prior to testing. Successful modeling (cognitive impairment) was confirmed if an animal's escape latency on day 4 exceeded the Sham group's mean by >20%. Other rats excluded from each group (0 - 2 per group), supplemented as needed with 0 - 2 randomly selected rats to make up for two animals per group, were euthanized to evaluate preliminary histopathological changes associated with CKD model induction. The remaining eight rats per group were included in subsequent analyses.

2.3. Morris Water Maze (MWM) Test

Four weeks after maintaining the CKD model rats on a specific diet, MWM testing was conducted to assess cognitive function and confirm the successful establishment of the cognitive impairment (CI) model. According to previous research methods [19], the apparatus consisted of a circular stainless-steel tank (200 cm in diameter, 50 cm deep) with the water temperature maintained at 25˚C ± 1˚C. The pool was divided into four equal quadrants (I-IV). An escape platform (10 cm in diameter) was fixed in Quadrant IV (the target quadrant) and submerged 1 cm below the water surface, while spatial reference markers of distinct geometric shapes (circle, pentagram, square, and triangle) were suspended around the pool perimeter. The rats' swimming trajectories were captured in real time by a video tracking system for parameter analysis.

Prior to formal testing, a one-day visual platform training session was conducted, during which the platform was raised 1 cm above the water surface. Rats were placed into the water facing the wall from the quadrant opposite the target quadrant. The time required for the rat to climb onto the platform was recorded as the escape latency (EL), with a longer EL indicating poorer spatial learning ability. Each rat underwent 4 training sessions with 20-min intervals. If a rat failed to locate the platform within 60 s, it was manually guided to the platform and allowed to remain there for 15 s, and its EL was recorded as 60 s.

The formal experiment comprised a 4-day spatial navigation test and a 2-day spatial probe test. During the spatial navigation test (designed to evaluate spatial learning and executive functions), the platform remained concealed 1 cm underwater. Each rat was tested 4 times daily to obtain the average EL. Subsequently, a 2-day spatial probe test was conducted to assess spatial memory retention. The tank was filled with water to a depth of 40 cm, and the escape platform was removed. Rats were released into the water from Quadrant II facing the tank wall and allowed to explore freely for 60 s. The time spent in the target quadrant, the percentage of the total swimming distance covered in that quadrant, and the number of platform crossings were recorded. Each rat was tested only once during this phase.

2.4. Drug Administration

According to the previous research methods [20], to maintain the CKD condition following successful establishment of the CKD-associated cognitive impairment (CKD-CI) model, adenine (250 mg/kg) was intragastrically administered every other day to all modeling groups. To explore the effect of calcitriol and 3-MA on CKD-CI rats, rats in the Model + Cal group received daily intraperitoneal (i.p.) injections of calcitriol (10 ng/kg). For the Model + Cal + 3-MA group, rats were administered 3-MA 30 mg/kg, i.p. daily in addition to calcitriol. In the sham and model groups, rats underwent solely daily i.p. injections of an equivalent volume of normal saline. The injections were performed once a day for 4 consecutive weeks. After 4 weeks of intervention, the MWM test was performed again to eval-

uate cognitive function.

2.5. Specimen Collection and Histological Examination

After the final MWM test, rats were housed individually in metabolic cages to collect 24-h urine samples, and the urine volume was recorded. Subsequently, the urine supernatant was collected by centrifugation to determine 24-h urinary protein levels. Rats were anesthetized using avertin, and blood was collected via left ventricular puncture. Blood samples were left to stand at room temperature and centrifuged to separate serum for determining blood urea nitrogen (BUN) and serum creatinine (SCr) levels.

The rats were sacrificed after blood collection, and then the right kidney was rapidly harvested and dissected free of perirenal tissue. A portion of the renal tissue was fixed in 4% paraformaldehyde for 24 h, dehydrated through a graded ethanol series, cleared in xylene, embedded in paraffin, and sectioned. The sections were subjected to hematoxylin-eosin (H&E) and Masson's trichrome staining to evaluate renal pathological alterations under a light microscope. Additionally, the skull was opened via the foramen magnum to completely harvest the whole brain, which was longitudinally bisected along the sagittal suture. One half was fixed using the same procedure for hippocampal H&E and Nissl staining; the other half was subjected to blunt dissection on ice to isolate the cortex and intact hippocampal tissue, which was immediately stored at −80˚C to detect relevant indicators.

2.6. Determination of Hippocampal Inflammatory Cytokines by ELISA

Hippocampal tissue samples were accurately weighed, homogenized in pre-cooled normal saline, and centrifuged at 4˚C to collect the supernatant. Strictly following the manufacturer's instructions, the concentrations of IL-6, TNF-α, NF-κB, and p-NF-κB were determined by a microplate reader at their respective optical density (OD) wavelengths, and the ratio of p-NF-κB to NF-κB was calculated.

2.7. Western Blot Analysis of Autophagy-Related Proteins in Hippocampal Tissue

Hippocampal tissues were lysed in RIPA lysis buffer containing protease and phosphatase inhibitors under ice-cold conditions, and then centrifuged at 4˚C to harvest the supernatant. Total protein concentration was determined using the BCA assay. A total of 50 μg of protein per lane was loaded onto SDS-PAGE gels for electrophoresis and transferred onto PVDF membranes. Subsequently, membranes were blocked with 5% non-fat milk at room temperature for 2 h and incubated overnight at 4˚C with primary antibodies against mTOR, p-mTOR, Beclin1, LC3B-II, and β-actin. After washing with TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:500) for 1 h at room temperature and visualized using ECL reagents. Finally, densitometric analysis of protein bands was performed using ImageJ software, the relative protein expres-

sion level was verified by calculating the ratio of target protein to β-actin optical density, and the ratio of p-mTOR to total mTOR was calculated.

2.8. Quantitative Real-Time PCR (RT-qPCR) for Autophagy-Related mRNA Expression

Total RNA from hippocampal tissue was extracted using the TRIzol method, and its concentration and purity were determined. cDNA was synthesized following the reverse transcription kit instructions and amplified via qPCR. The thermal cycling conditions were as follows: initial denaturation at 95˚C for 30 s, followed by 40 cycles of 95˚C for 15 s and 60˚C for 30 s. With β-actin as the internal reference, the relative mRNA expression levels of LC3B, Beclin1, and mTOR were calculated using the $2^{-\Delta\Delta Ct}$ method. Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), and their sequence details are shown in Table 1.

Table 1. RT-qPCR primer sequences.

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
β-actin	TACAACCTCCTTGCAGCTCC	GGATCTTCATGAGGTAGTCAGTC
mTOR	TTGGAGTGGCTGGGTGCTGA	AAGGGCTGAACTTGCTGGAA
LC3B	CCATGCCGTCCGAGAAGACCTTC	CTCTAGTGCCAGCTCCTTTAGC
Beclin1	CTGGACACTCAGCTCAACGTCA	CTCTAGTGCCAGCTCCTTTAGC

2.9. Statistical Analysis

Statistical analysis was performed using SPSS software (version 27.0). For the Morris water maze (MWM), the primary escape-latency endpoint was the mean escape latency on Day 4 of the 4-day place-navigation phase, calculated as the average of the four daily trials for each rat; this value was used for between-group comparisons and for the model-inclusion criterion. Normally distributed quantitative data are presented as mean ± standard deviation ($\overline{x} \pm s$). Multigroup comparisons were analyzed using one-way analysis of variance (ANOVA), followed by the Tukey test for pairwise comparisons. Non-normally distributed data are expressed as median (interquartile range) [M (IQR)] and were analyzed using the Kruskal-Wallis H test, followed by Mann-Whitney U tests with Bonferroni correction for pairwise comparisons. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Calcitriol Ameliorates Cognitive Impairment in CKD Rats via Autophagy Activation

We first evaluated the cognitive performance of rats in each group using the Morris water maze (MWM) test. As shown in Figures 1(a)-(c), relative to the Sham group, rats in the Model group exhibited a significant increase in escape latency (EL) from 26.93 ± 5.36 s to 39.06 ± 7.98 s, whereas the number of platform cross-

ings (TCP) and the time spent in the target quadrant (TSPQ) were markedly reduced ($P < 0.05$), indicating the successful establishment of the cognitive impairment (CI) model. Representative swimming trajectories during the place-navigation test and spatial probe test are illustrated in **Figure 1(d)** and **Figure 1(e)**.

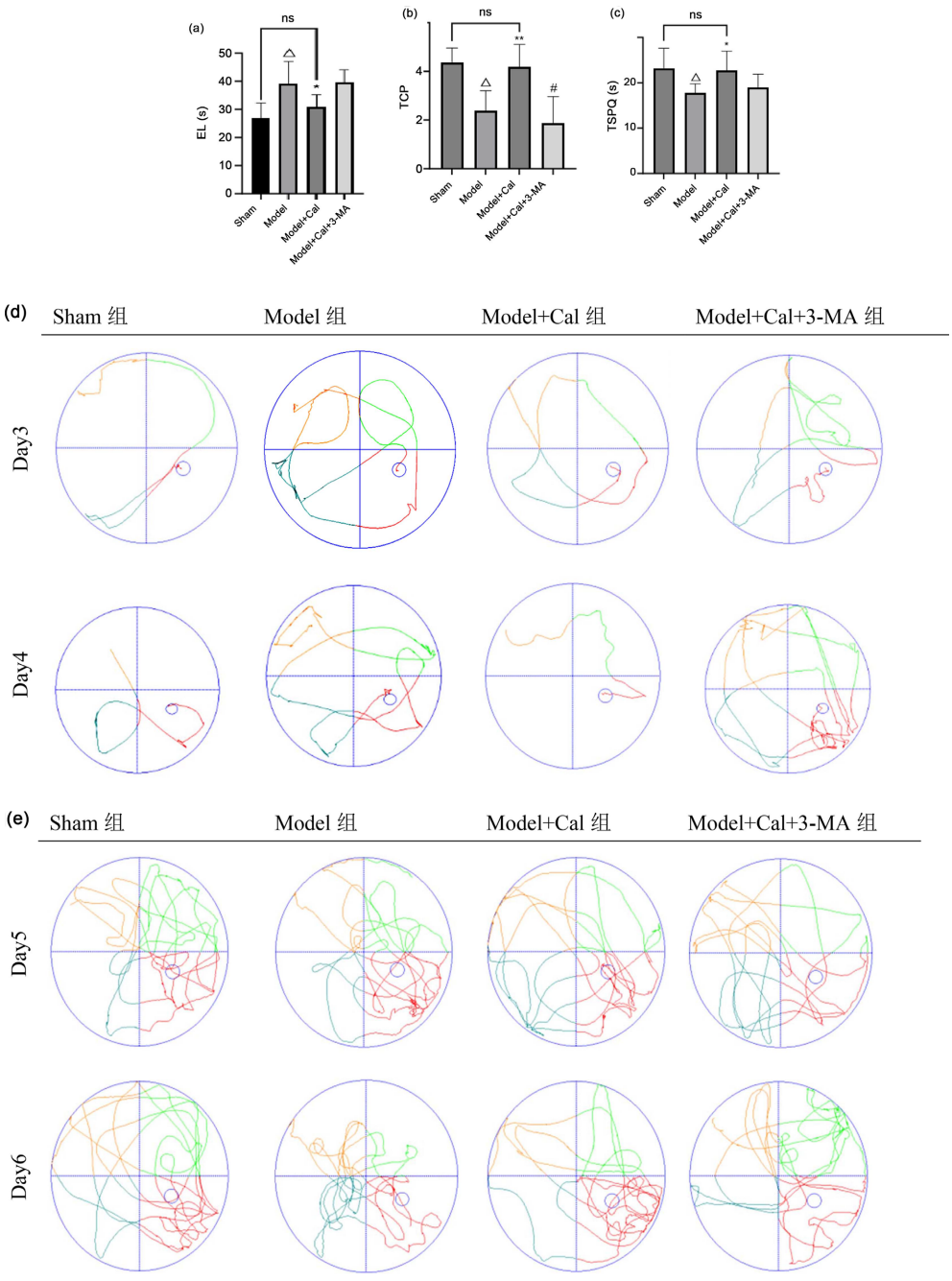


Figure 1. Morris water maze (MWM) test outcomes in each group of rats. (a) Escape latency of rats in each group; (b) Number of target platform crossings of rats in each group; (c) Time spent in the target quadrant of rats in each group; (d) Representative swimming trajectories of rats during the MWM place-navigation test; (e) Representative swimming trajectories of rats during the MWM spatial probe test ($\Delta P < 0.05$ vs Sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs Model group; $^{\#}P < 0.05$ vs Model + Cal group. EL: escape latency; TCP: target platform crossings; TSPQ: time spent in target quadrant).

Compared with the Model group, treatment with calcitriol in the Model + Cal group significantly decreased EL (30.91 ± 4.26 s vs 39.06 ± 7.98 s, $P < 0.05$), accompanied by a notable increase in TCP (4.50 [2.00] vs 2.00 [1.75], $P < 0.05$) and TSPQ (22.74 ± 4.26 s vs 17.73 ± 2.03 s, $P < 0.05$). In contrast to the Model + Cal group, co-administration of 3-MA in the Model + Cal + 3-MA group significantly prolonged EL (39.66 ± 4.42 s vs 30.91 ± 4.26 s, $P < 0.05$) and reduced TCP (1.50 [2.13] vs 4.50 [2.00], $P < 0.05$) as well as TSPQ (18.99 ± 2.92 s vs 22.74 ± 4.26 s, $P < 0.05$).

The above results demonstrated that calcitriol could attenuate cognitive impairment in CKD rats, and this protective effect was significantly blunted by the autophagy inhibitor 3-MA, suggesting that the neurocognitive improvement induced by calcitriol depends on the activation of the autophagy pathway.

3.2. Renal Function Parameters in Rats

Compared with the Sham group, rats in the Model group exhibited significantly elevated levels of 24-h urinary protein (201.21 ± 36.74 mg), blood urea nitrogen (BUN, 23.64 ± 5.07 mmol/L), and serum creatinine (SCr, 160.34 ± 26.07 μmol/L) ($P < 0.001$), indicating severe proteinuria and a decreased glomerular filtration rate, which confirmed the successful establishment of the CKD model. Following 4 weeks of calcitriol intervention, no significant improvements in renal function parameters were observed in the Model + Cal group compared with the Model group ($P > 0.05$), with a reduction of only 4.6% in 24-h urinary protein ($P = 0.654$), 16.4% in BUN ($P = 0.086$), and 1.5% in SCr ($P = 0.854$). Furthermore, no statistically significant differences were detected between the Model + Cal + 3-MA group and the Model + Cal group ($P > 0.05$) (Figure 2). These results demonstrated that calcitriol exerted no obvious restorative effect on renal function in CKD rats within the observation period.

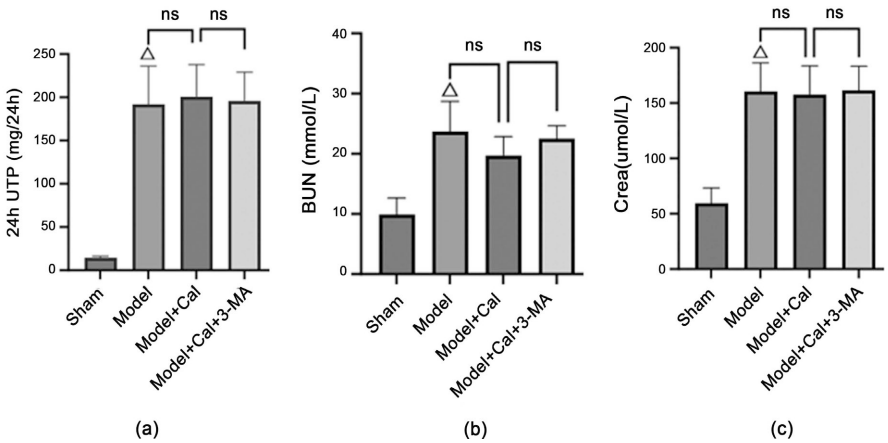


Figure 2. Comparison of 24-h urinary protein, blood urea nitrogen (BUN), and serum creatinine (SCr) levels in rats among different groups ($\overline{x} \pm s$). (a) 24-h urinary protein quantification; (b) Blood urea nitrogen (BUN); (c) Serum creatinine (SCr). ($\Delta P < 0.05$ vs Sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs Model group; $^{\#}P < 0.05$ vs Model + Cal group. N = 8).

3.3. Histopathological Evaluation of Renal and Hippocampal Tissues

As illustrated in Figure 3(a) and Figure 3(b), H&E staining of renal tissue in the Model group revealed irregular tubular morphology with variable lumen sizes, lumen dilation, and interstitial fibrosis accompanied by predominant lymphocytic infiltration. Glomerular structural abnormalities, volume shrinkage, irregular basement membrane thickening, and Bowman's capsule wall thickening with capsular expansion were observed, indicating nephron loss and progressive fibrosis. Masson's trichrome staining showed extensive blue-stained collagen deposition in the renal interstitium, encircling atrophic glomeruli. Compared with the Model group, collagen fiber deposition was slightly reduced in the Model + Cal group, suggesting a mild protective tendency of calcitriol against renal fibrosis; however, organic renal damage showed no marked attenuation during calcitriol intervention.

Histomorphological analysis of hippocampal tissue (Figure 3(c) and Figure 3(d)) revealed intact architecture, densely arranged and evenly distributed neurons,

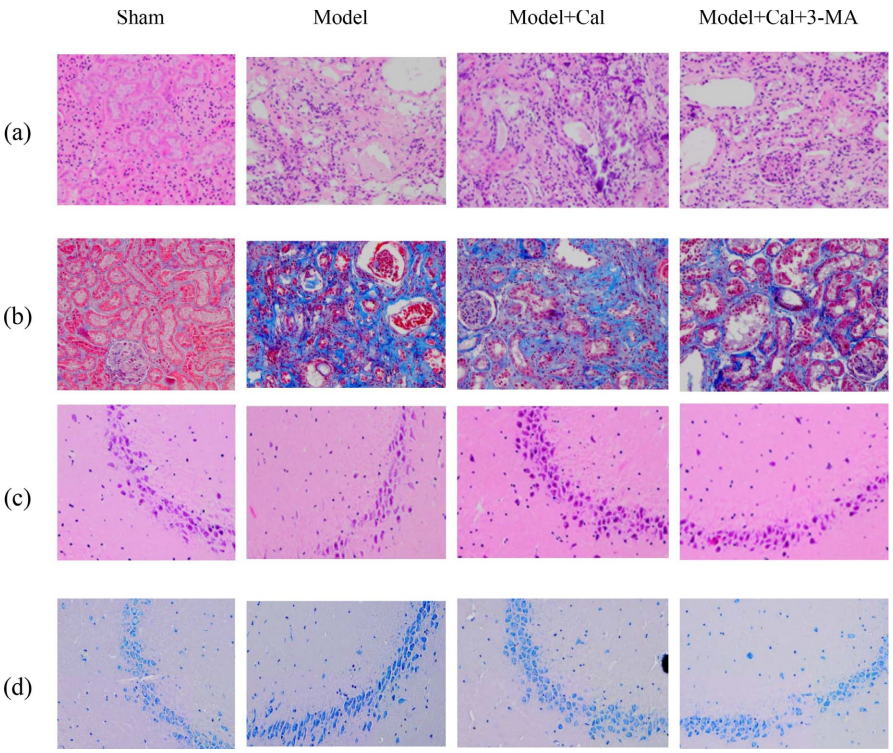


Figure 3. Pathological evaluation of renal and hippocampal tissue by H&E, Masson, and Nissl's staining (200×). (a) Pathological evaluation of kidney tissue by H&E staining: Sham group, Model group, Model + Cal group, and Model + Cal + 3-MA group; (b) Pathological evaluation of kidney tissue by Masson's Trichrome staining: Sham group, Model group, Model + Cal group, and Model + Cal + 3-MA group; (c) Pathological evaluation of hippocampal tissue by H&E staining.: Sham group, Model group, Model + Cal group, and Model + Cal + 3-MA group; (d) Pathological evaluation of hippocampal tissue by Nissl's staining: Sham group, Model group, Model + Cal group, and Model + Cal + 3-MA group. Magnifications: 200×.

and abundant Nissl bodies in the Sham group. Conversely, the Model group exhibited neuronal degeneration in the hippocampal CA3 region, characterized by blurred structural boundaries, dispersed alignment, reduced cell density, pyramidal cell atrophy, and a marked depletion of Nissl bodies. Following calcitriol intervention, neurons in the CA3 region of the Model + Cal and Model + Cal + 3-MA groups were more tightly arranged, with fewer shrunken pyramidal cells and attenuated loss of Nissl bodies compared to the Model group.

3.4. Expression of Inflammatory Cytokines and Signaling Proteins in Hippocampal Tissue

The levels of TNF-α, IL-6, NF-κB, and the p-NF-κB/NF-κB ratio in hippocampal tissue measured by ELISA are shown in **Figures 4(a)-(d)**. Relative to the Sham group, TNF-α and IL-6 levels were significantly upregulated in the Model group ($P < 0.001$). Although total NF-κB protein expression showed no significant differences among groups ($P > 0.05$), the p-NF-κB/NF-κB phosphorylation ratio was markedly elevated in the Model group ($P < 0.001$), indicating abnormal activation of the hippocampal NF-κB pathway in CKD-CI rats. Compared with the Model group, calcitriol treatment in the Model + Cal group significantly decreased TNF-α and IL-6 levels ($P < 0.05$) and suppressed the elevation of the p-NF-κB/NF-κB ratio ($P < 0.001$). However, co-administration of 3-MA in the Model + Cal + 3-MA group significantly blunted these anti-inflammatory effects, leading to a notable rebound in TNF-α, IL-6 levels, and the p-NF-κB/NF-κB ratio ($P < 0.05$).

Western blot analysis was performed to evaluate the expression of autophagy-related proteins (mTOR, p-mTOR, Beclin1, and LC3B-II) in hippocampal tissue (**Figures 4(e)-(h)**). Compared with the Sham group, total mTOR protein expression remained unchanged in the Model group ($P > 0.05$), whereas the p-mTOR/mTOR ratio was abnormally elevated, and LC3B-II and Beclin1 protein levels were markedly downregulated ($P < 0.001$). Relative to the Model group, the p-mTOR/mTOR ratio was significantly reduced, while LC3B-II and Beclin1 expression was markedly upregulated in the Model + Cal group ($P < 0.001$). In contrast, 3-MA administration in the Model + Cal + 3-MA group significantly blunted these effects, resulting in a distinct rebound in the p-mTOR/mTOR ratio and a significant downregulation of LC3B-II and Beclin1 ($P < 0.001$).

3.5. Autophagy-Related mRNA Expression in Hippocampal Tissue

RT-qPCR results were consistent with the Western blot findings (**Figure 5**). Compared with the Sham group, hippocampal mRNA expression levels of LC3B and Beclin1 were significantly decreased in the Model group ($P < 0.001$). Treatment with calcitriol in the Model + Cal group significantly upregulated LC3B and Beclin1 mRNA expression compared to the Model group ($P < 0.01$). Conversely, 3-MA co-treatment in the Model + Cal + 3-MA group markedly reduced LC3B and Beclin1 mRNA levels compared with the Model + Cal group ($P < 0.05$). No significant differences in mTOR mRNA expression were observed across groups ($P > 0.05$).

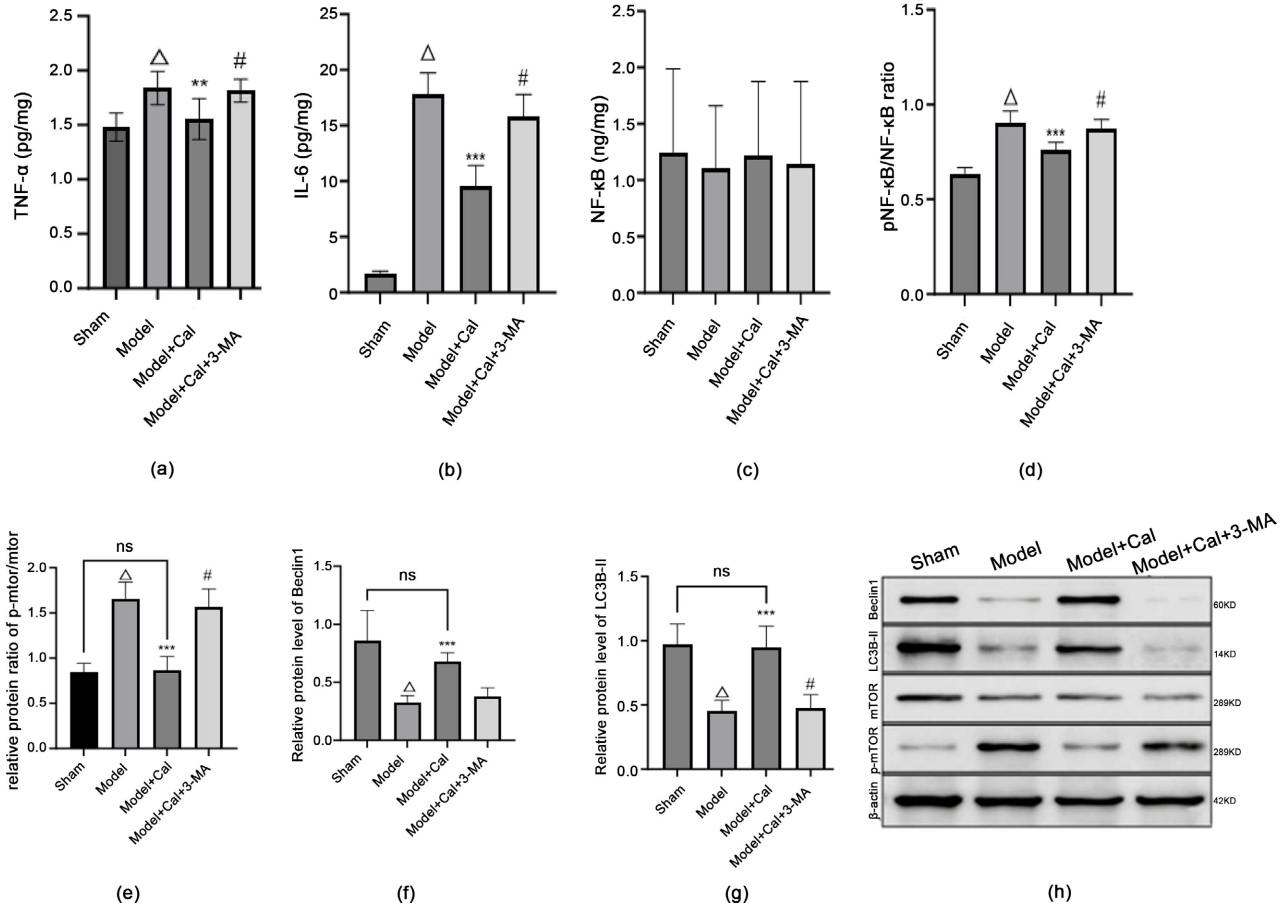


Figure 4. Expression levels of inflammatory cytokines, NF-κB signaling proteins and autophagy related proteins in hippocampal tissues ($\overline{x} \pm s$). (a) (b) Protein levels of TNF-α and IL-6; (c) Total NF-κB protein level; (d) p-NF-κB/NF-κB phosphorylation ratio. (e) Relative protein expression ratio of p-mTOR/mTOR; (f) Relative protein expression of LC3B-II; (g) Relative protein expression of Beclin1; (h) Representative Western blot images of Beclin1, LC3B-II, mTOR, and p-mTOR (internal control: β-actin); ($\Delta P < 0.05$ vs Sham group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Model group; $^{\#}P < 0.05$ vs Model + Cal group. N = 8).

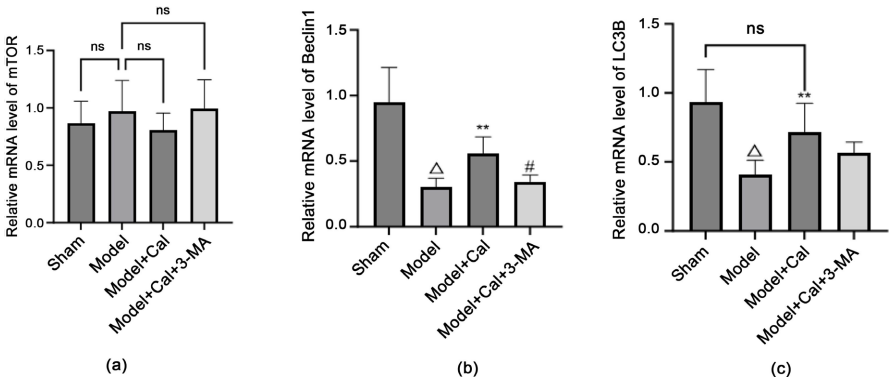


Figure 5. Autophagy-related mRNA expression levels in hippocampal tissues of rats in each group. (a)-(c) Relative mRNA expression of LC3B, Beclin1, and mTOR. ($\Delta P < 0.05$ vs Sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs Model group; $^{\#}P < 0.05$ vs Model + Cal group. n = 8).

4. Discussion

In addition to the progressive deterioration of renal function, the neurocognitive

complications associated with chronic kidney disease (CKD) are gaining increasing attention. Current studies suggest that the accumulation of uremic toxins and the subsequent chronic inflammatory state are critical drivers of CKD-associated cognitive impairment (CKD-CI) [8] [21]-[23]. The accumulation of uremic toxins disrupts the integrity of the blood-brain barrier, allowing toxins to enter cognition-related brain regions such as the hippocampus. This induces the overproduction of pro-inflammatory cytokines and reactive oxygen species, thereby damaging neurons and synaptic structures [8] [22]-[24]. Meanwhile, toxin exposure and the inflammatory microenvironment also interfere with the autophagic clearance functions of neurons and glial cells. As a crucial self-protective mechanism for maintaining cellular protein homeostasis and mitochondrial function, autophagy is responsible for clearing damaged organelles and abnormal protein aggregates. Its functional decline may be involved in the pathogenesis of CKD-CI [8] [14] [25]. In this research, a CKD rat model was established by unilateral nephrectomy combined with adenine gavage. In the Model group, 24-h urinary protein, BUN, and SCr were significantly elevated. Furthermore, H&E and Masson's trichrome staining of renal tissues revealed tubular damage, interstitial inflammatory cell infiltration, and collagen deposition, indicating the successful establishment of the CKD model. Concurrently, Model rats exhibited a prolonged escape latency, alongside a decreased number of platform crossings and reduced time spent in the target quadrant during the MWM test. Histopathological analysis showed neuronal degeneration, loose structural arrangement, and a marked depletion of Nissl bodies in the hippocampal CA3 region, consistent with hippocampus-dependent spatial learning and memory impairment.

Following calcitriol intervention, the escape latency of CKD rats was significantly shortened, and the number of platform crossings and the time spent in the target quadrant rebounded, suggesting that calcitriol could alleviate spatial learning and memory impairments. However, this behavioral improvement was significantly blunted by co-treatment with 3-MA, which is commonly used as an autophagy inhibitor but also affects PI3K signaling. Notably, calcitriol did not significantly improve 24-h urinary protein, BUN, or SCr levels, and renal pathology showed only a mild reduction in interstitial collagen deposition, indicating limited overall attenuation of organic renal damage. Although this pattern raises the possibility that the cognitive benefit may not be entirely secondary to improved renal excretory function or a reduced uremic toxin burden, the nonsignificant differences in conventional renal markers do not exclude a contribution of renal factors to the cognitive improvement. Because uremic toxin burden was not measured, these data should be interpreted as cognitive improvement without detectable improvement in conventional renal indices rather than as proof of renal independence. Previous studies have indicated that the vitamin D/VDR signaling pathway exerts anti-inflammatory, antioxidant, calcium-regulating, and autophagy-modulating effects in the central nervous system, and its neuroprotective effects may occur independently of mineral metabolism regulation; however, this possibility

requires direct verification.

The hippocampus is the core region for spatial learning and memory encoding and is particularly vulnerable to inflammatory stimuli. Pro-inflammatory cytokines such as IL-6 and TNF-α can impair synaptic plasticity, inhibit long-term potentiation, and induce neuronal damage, acting as essential mediators of cognitive decline [26]-[29]. NF-κB is a pivotal regulator of pro-inflammatory gene transcription, and the extent of its activation depends more on the phosphorylation and nuclear translocation of the p65 subunit than on its total protein expression [30] [31]. In this study, no significant difference in total NF-κB expression in the hippocampus was observed among the groups; however, the p-NF-κB/NF-κB ratio was significantly elevated in the Model group, accompanied by increased TNF-α and IL-6 levels. These findings indicate that the hippocampal NF-κB pathway was abnormally activated in CKD-CI rats. Calcitriol was associated with a lower p-NF-κB/NF-κB ratio and reduced levels of TNF-α and IL-6, suggesting that it may block NF-κB activation at the phosphorylation stage, thereby reducing the transcription of downstream inflammatory cytokines. Following co-treatment with 3-MA, these anti-inflammatory effects were attenuated, further suggesting that the neuroinflammation-inhibiting effect of calcitriol is associated with 3-MA-sensitive pathways; however, because 3-MA also affects PI3K signaling and no 3-MA-alone group was included, this does not establish autophagy-specific causality.

mTOR is a key negative regulatory kinase in autophagy initiation, Beclin1 participates in autophagosome formation, and LC3B-II is a commonly used autophagosome membrane marker. These three markers are widely used to assess autophagy-related processes [32]-[35]. In this research, total mTOR protein and mRNA levels in the hippocampus of the Model group showed no obvious changes, but the p-mTOR/mTOR ratio was significantly elevated, while Beclin1 and LC3B-II expression levels were markedly downregulated at both protein and mRNA levels. These static markers suggest that hippocampal autophagy-related signaling was altered under CKD-CI conditions, with changes appearing predominantly at the level of mTOR phosphorylation rather than total mTOR expression. However, static measurements of Beclin1 and LC3B-II cannot distinguish reduced autophagosome formation from impaired autophagic flux. After calcitriol intervention, the p-mTOR/mTOR ratio decreased, and the expression of Beclin1 and LC3B-II was upregulated. Upon the addition of 3-MA, these changes were significantly blunted, and the behavioral improvements were concurrently attenuated. These findings suggest that calcitriol may enhance autophagy-related signaling by reducing mTOR hyperphosphorylation and increasing Beclin1 and LC3B-II expression; however, restoration of autophagic flux was not demonstrated. If autophagic capacity were improved, enhanced clearance of damaged organelles and protein aggregates might reduce cellular stress and inflammasome activation, thereby attenuating NF-κB phosphorylation and downstream IL-6 and TNF-α expression. These observations are broadly consistent with previous reports demonstrating

that calcitriol can modulate mTOR activity and influence cell survival and apoptosis [36], but they should be regarded as associative rather than mechanistic proof.

The present study has several limitations relevant to renal independence and clinical translation. First, the lack of statistically significant differences in 24-h urinary protein, BUN, and SCr does not rule out a contribution of renal factors to the cognitive improvement, and uremic toxin burden was not measured; therefore, renal independence remains unproven. Second, no 3-MA-alone group was included, and 3-MA is not autophagy-specific because it also affects PI3K signaling; thus, the attenuation by 3-MA cannot establish an autophagy-specific causal role. Autophagic flux was not assessed by LC3 turnover, p62 measurement, or transmission electron microscopy, and static Beclin1 and LC3B-II measurements cannot confirm restoration of autophagic flux. Third, only a single calcitriol dose was tested, and hippocampal VDR expression and upstream uremic toxin-AhR signaling were not examined. Finally, the clinical relevance of these findings remains exploratory. Although CKD patients often have vitamin D deficiency and are at high risk for cognitive impairment, patient cognitive efficacy of calcitriol has not been established, and its calcium/phosphorus safety profile for cognitive indications is unknown; therefore, clinical use should not be inferred from the present animal data.

In summary, calcitriol may improve the spatial learning and memory capacity of CKD-CI rats. These benefits were accompanied by reduced hippocampal mTOR phosphorylation, increased Beclin1 and LC3B-II expression, and suppressed NF-κB phosphorylation with lower IL-6 and TNF-α levels. These findings suggest that calcitriol may modulate autophagy-related and neuroinflammatory pathways, but they do not establish autophagy-specific causality or renal independence. Given that CKD patients, especially those with renal failure undergoing dialysis, consistently suffer from hypocalcemia, abnormal calcium and phosphorus metabolism, and vitamin D deficiency, and are simultaneously at a high risk for cognitive impairment, careful correction of vitamin D deficiency may have potential multi-beneficial effects [37] [38]; however, the clinical significance remains exploratory because patient cognitive efficacy and calcium/phosphorus safety have not been established. This study provides preliminary experimental evidence for the potential use of calcitriol in CKD-CI; however, the mechanistic conclusions require further validation and clinical application should be regarded as exploratory. Subsequent studies should incorporate 3-MA-alone controls, autophagy agonists such as rapamycin for reciprocal validation, autophagic flux assays, uremic toxin measurements, and calcium/phosphorus safety assessments, and examine the dose-response relationship of calcitriol, thereby further elucidating the precise molecular mechanisms by which calcitriol may modulate autophagy-related pathways and alleviate cognitive impairment.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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