Development of A Chinese Medicine Formula for Treatment of Ketamine-induced Cognitive Deficits and Cystitis: Preclinical Evaluations on Its Efficacy, Safety and Molecular Mechanisms

1. Background

Drug addiction is a major public health problem that affects an increasing number of people and causes serious burdens to society and the economy. The burden of mental, neurological, and substance use disorders is estimated to increase by 10% in China between 2013 and 2025 [1]. Drug-seeking and -taking behaviors are the main characteristics of drug addiction [2]. In Hong Kong, there were 499 cases of ketamine abuser in 2020. Although various therapies for drug abuse and dependence have been developed, including pharmacological, psychological, and sociological interventions, their efficacy is still limited. In addition, long-term ketamine use causes damages to various organs, including the brain, heart, liver, gastrointestinal tract, and genitourinary system [3]. Ketamine alters numerous functions in the brain including color perception, memory, attention, cognition, reaction time and sense of time [4]. Moreover, chronic use of ketamine is associated with impairments in various cognitive functions [5]. Chronic abuse often leads to bladder contracture and severe lower urinary tract symptoms, clinically called ketamine-induced cystitis (KIC) with the interstitial cystitis like symptoms, such as bladder pain, dysuria, urinary frequency and urgency, and hematuria [6,7]. Treating patients with KIC is a medical dilemma. Cessation of ketamine abuse is the mainstay of treatment and results in some degree of improvement. Nevertheless, urinary symptoms may last for 1 year or more even after abstinence from ketamine [8]. In addition, patients with KIC who quit and relapse usually experience recurrence of symptoms [9]. In severe cases of KIC, augmentation enterocystoplasty might be beneficial in relieving refractory KIC pain and urinary symptoms but with a high risk of perioperative morbidity and long-term complications [10]. Cessation of ketamine use is the only effective treatment for preventing deterioration of organ function. However, there is currently no effective pharmacotherapy to improve the side effects such as cognitive deficits and cystitis in ketamine abuser. A single medication would not adequately resolve such complex conditions. By contrast, Chinese medicine formula, might be effective in

managing the complexity of KIC or as a complementary therapy.

Chinese herbal medicines have been used in China for more than 2000 years as the main therapeutic tool, and such treatments for dementia and urinary symptoms have been used for more than 1000 years [11,12]. In the last two decades, an increasing number of clinical trials and animal studies have shown that Chinese herbal medicines have efficacy against cognitive deficits or cystitis. A clinical empirical Chinese medicine formula (CECMF) is an empirical formula of Prof. Zhi-Xiu Lin, a Registered Chinese Medicine Practitioner with more than 30 years of clinical experience currently working at the School of Chinese Medicine, The Chinese University of Hong Kong. The reasons for this study focus on investigating CECMF for treatment of ketamine-cognitive deficits and cystitis can be divided into three parts. Firstly, CECMF has been prescribed in clinic in Hong Kong for many years and observed to be effective in improving patients' cognitive impairment and urinary tract symptoms clinical manifestations. CECMF contains 12 Chinese herbal medicines, including *Talcum* (Huashi in Chinese), *Lysimachiae Herba* (Jinqiancao in Chinese), *Salviae Milti Radix et Rhizoma* (Danshen in Chinese), *Puerariae Lobatae Radix* (Gegen in Chinese), *Ramulus Uncariae cum Uncis* (Gouteng in Chinese), *Corydalis Rhizoma* (Yanhusuo in Chinese), *Polygoni Cuspidati Rhizoma et Radix* (Huzhang in Chinese), *Plantaginis Semen* (Cheqianzi in Chinese), *Akebiae Caulis* (Mutong in Chinese), *Dianthi Herba* (Qumai in Chinese), *Polygoni Avicularis Herba* (Bianxu in Chinese), *Gardeniae Fructus* (Zhizi in Chinese) at the ratio of 5:5:5:5:4:4:4:4:3:3:3:3. Among the constituents of the CECMF, rhynchophylline (RN), the major ingredient isolated from *Ramulus Uncariae cum Uncis*, significantly inhibited zebrafish methamphetamine (METH) dependence [13]. RN (30-60 mg/kg) was found to reverse the reward effect induced by ketamine in rats [14,15]. RN can remarkably eliminate the rewarding effect and decrease the NR2B level in the brain of amphetamine-induced conditioned place preference (CPP) rats [16], mice [17] and zebrafish [13]. These lines of evidence suggest *Ramulus Uncariae cum Uncis*-derived alkaloids have good potential to be developed into therapeutics for the ketamine- or METH-associated addiction. *Corydalis Rhizoma* and its active constituent L-tetrahydropalmatine (L-THP), could inhibit heroin-induced self-administration and cue-induced relapse [18], and prevent morphine- and METH-induced CPP [19-21]. *Salviae Milti Radix et Rhizoma* and *Puerariae Lobatae Radix* be beneficial for the treatment of

alcohol abuse [22,23]. All these findings indicate that some constituents of the CECMF have good potential effects to improve the drug addiction.

Secondly, most of the constituents of CECMF have been reported to have potential neuroprotective effects against cognitive deficits in animal models or in patients. Among the constituents of CECMF, *Ramulus Uncariae cum Uncis*, the major component herb of CECMF, and its major ingredients such as rhynchophylline and isorhynchophylline, have been reported to improve the cognitive deficits in several animal models of cognitive deficits in our previous studies [24-29]. Tanshinone IIA, isolated from *Salviae Milti Radix et Rhizoma*, could reduce the risk of cognitive impairment in rat models of Alzheimer's disease [30]. Geniposide, isolated from *Gardeniae Fructus*, ameliorated the behavioral impairment in a mouse model of Alzheimer's disease [31]. *Acori Tatarinowii Rhizoma* extract has been reported to ameliorate Alzheimer's pathological syndromes by repairing myelin injury and lowering tau phosphorylation in mice [32]. Puerarin, isolated from *Puerariae Lobatae Radix*, has been reported to improve learning and memory functions in chronic ischemia rats [33]. Above findings suggest that CECMF has good potential effects against cognitive deficits.

Thirdly, some constituents of CECMF including *Talcum*, *Polygoni Cuspidati Rhizoma et Radix, Lysimachiae Herba*, *Plantaginis Semen*, *Dianthi Herba*, *Dianthi Herba*, *Polygoni Avicularis Herba* and *Gardeniae Fructus* are widely used to treat acute cystitis in clinic [34-36]. *Talcum*, *Plantaginis Semen*, *Dianthi Herba*, *Polygoni Avicularis Herba*, and *Akebiae Caulis* with the functions of clearing toxicity to quench inflammation, and inducing diuresis to treat gonorrhea are frequently used to treat urinary inflammation-related diseases including gonorrhea and urinary infection [37]. All these studies indicate that CECMF has good potential effects against cystitis.

Importantly, our recent preliminary study revealed that CECMF could ameliorate cognitive deficits the induced by ketamine in rats, and reduce the urinary frequency and bladder damage induced by ketamine in rats, suggesting that CECMF can simultaneously resolve the cognitive deficits and cystitis induced by ketamine. Based on the promising preliminary data, literature studies and the clinical use experience by Prof. Zhi-Xiu Lin, we hypothesized that CECMF may improve the cognitive deficits and cystitis induced by ketamine. The testing of this hypothesis and elucidation of the underlying molecular

mechanisms would further advance our knowledge about the potential of CECMF as an innovative Chinese medicine-based for treatment of cognitive deficits and cystitis induced by ketamine.

2. Objectives

1) To explore the improving effects of CECMF the cognitive deficits induced by ketamine in neonatal and adult rats.

2) To investigate the effects of CECMF on cystitis induced by ketamine in rats.

3) To evaluate of the toxicological profile of the CECMF.

3. Materials and methods

3.1. To prepare the extract and establish the quality control of the clinical empirical Chinese medicine formula (CECMF)

3.1.1. Authentication of raw herbal materials: The crude decoction pieces (中藥飲片) of *Talcum* (滑石), *Lysimachiae Herba* (金錢草), *Salviae Milti Radix et Rhizoma* (丹參), *Puerariae Lobatae Radix* (葛根), *Ramulus Uncariae cum Uncis* (鉤藤), *Corydalis Rhizoma* (延胡索), *Polygoni Cuspidati Rhizoma et Radix* (虎杖), *Plantaginis Semen* (車前子), *Akebiae Caulis* (木通), *Dianthi Herba* (瞿麥), *Polygoni Avicularis Herba* (萹蓄), *Gardeniae Fructus* (梔子) were purchased from a reliable Chinese herbal supplier with GMP accreditation, and their identities were authenticated by Ms. Yu-ying Zong, a seasoned Pharmacognost at the School of Chinese Medicine, CUHK, where voucher specimens were deposited according to the guidelines of the Chinese Pharmacopoeia 2020.

3.1.2. Extraction and fractions of CECMF: The above 12 herbs were ground to powder or pieces and mixed in the ratio 5:5:5:5:4:4:4:4:3:3:3:3, and the ratio so adopted is based on the clinical experience of the Co-PI (Prof. Zhi-Xiu Lin, who is a highly experienced Registered Chinese Medicine Practitioner in Hong Kong) and conformed to the doses of individual herbs in the Chinese Pharmacopeia (2020). The mixture was extracted with boiling 70% aqueous ethanol for 1 hour, and the extract subsequently filtered and the residue further extracted twice as before. All three filtrates were combined, and concentrated in a rotary evaporator under negative pressure, and finally dried in a lyophilizer to get a freeze-dried power. The yield of CECMF extraction was 18.17%, and

the resultant CECMF extract was stored at -20 ºC for subsequent study.

3.2. Liquid chromatography - mass spectrometer (LC-MS) analysis

In this study, LC-MS was used to assessment of the quality control of CECMF. The CECMF sample (50 mg), was mixed with 500 μL of 70% methanol containing mixed internal standard (2-Chloro-L-phenylalanine, succinic acid-d4, L-Valine-d8, Cholic Acid-d4 and D-Luciferin, 4 μg/ml). The mixture was incubated at -40 °C for 2 min, and centrifuged with 12,000 rpm at 4°C for 10 min. The supernatant was diluted five times with 70% methanol containing mixed internal standard (2-Chloro-L-phenylalanine, succinic acid-d4, L-Valine-d8, Cholic Acid-d4 and D-Luciferin, 4 μg/ml) for analysis with LC-MS/MS.

The total components of the CECMF were determined by LC-MS. The LC-MS analysis was carried out with ACQUITY UPLC I-Class HPLC system (Waters, USA) equipped Thermo-Obritrap-QE HF mass spectrum (Thermo Fisher Scientific, USA). A Waters ACQUITY UPLC HSS T3 analytical column (1.8 um, 100 mm × 2.1 mm; Waters, USA) was selected for LC-MS metabolomics analysis. A linear gradient mobile phase composed of 0.1% formic acid water (solvent A) and acetonitrile (solvent B) was used mixed with a 16 min gradient program: 0-2.0 min (5% B); 2.0-4.0 min (5-30% B); 4.0-8.0 min (30-50% B); 8.0-10.0 min (50-80% B); 10.0-14.0 min (80-100% B); 14.0-15.0 min (100% B); 15-15.1 min (100-5% B); 15.1-16.0 min (5% B). The injection volume was 2 μL and flow rate was 0.35 ml/min. Multiple Reaction Monitoring (MRM) detection mode was used for detection with electro spray source (ESI) in both positive and negative ion modes

3.3. To explore the effects of CECMF the cognitive deficits induced by ketamine in neonatal and adult rats

It has been reported that long-term abuse of ketamine leads to cognitive impairment, especially within the domains of episodic and semantic memory [38-41]. In this present study, we aimed to explore the post-intervention effects of the clinical empirical Chinese medicine formula (CECMF) on improving the cognitive deficits induced by ketamine in neonatal and adult rats, as well as on reducing cystitis induced by ketamine in rats. We

mainly focused on investigating the post-intervention effects of CECMF but not the pre-intervention effects induced by long-term use of ketamine.

3.3.1. Animals

Adult male Sprague-Dawley (SD) rats weighing 230-250g were obtained from the Laboratory Animal Services Centre, The Chinese University of Hong Kong. Rats were housed in controlled conditions (5 rats per cage, 12 h light/dark cycle with a constant room temperature of 20-24 °C and relative humidity of 40-60%) and had free access to food and water. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health (NIH Publication No. 85-23, revised 2011). Approval for the experimental protocol was obtained from the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong (Ref. No.21/307/BDF).

3.3.2. Ketamine-induced cognitive impairment in rats and drug treatment:

Male SD rats (8-week-old) (available from LASEC, CUHK) were randomly assigned into six groups of ten animals each: control, ketamine + vehicle (ketamine-treated control), ketamine + CECMF (1.25 g/kg), ketamine + CECMF (2.5 g/kg), ketamine + CECMF (5.0 g/kg), and ketamine + donepezil (5 mg/kg). In this study, we would like to explore whether CECMF improved the cognitive deficits induced by ketamine in a dose-dependent manner. Therefore, we designed three different doses of CECMF. Ketamine was injected *i.p.* to rats at the dose of 80 mg/kg once daily for 49 consecutive days. The rats were orally administered by gavage once daily with CECMF or vehicle from day 15 to 49 at 30 min after ketamine administration. The dose of ketamine was selected according to previous studies [42,43]. CECMF was orally given to the rats 30 min after ketamine administration. In the clinical practice, CECMF is usually prescribed at a daily dose of 144 g of raw herbal material for adult. We converted this human dose into an animal dose using the following parameters (an extraction yield of 17.59%, a person of 60 kg, and a conversion factor of 6 between human and rats) and selected a dose equivalent to the middle dose (2.5 g/kg) for adult rats. At the end of drug treatment, they were assessed for spatial reference memory using behavioral tests, including a novel object recognition test (NORT) and the Morris water maze test (MWMT), as previously described before they are sacrificed. The body

weight, coat characteristics, and in-cage behaviors were monitored and recorded every other day throughout the study. The timeline of the drug administration and behavioral tests is shown in **Fig. 1A**.

3.3.3. Ketamine-induced cognitive impairment in neonatal rats and drug treatment:

Male Sprague-Dawley (SD) rats at postnatal day 7 (P7) were purchased from LASEC, CUHK. The neonatal rats were randomly divided into six groups (n=10): control, ketamine + vehicle (ketamine-treated control), ketamine + CECMF (0.75 g/kg), ketamine + CECMF (1.5 g/kg), ketamine + CECMF (3.0 g/kg), and ketamine + donepezil (5 mg/kg). In this study, we would like to explore whether CECMF improved the cognitive deficits induced by ketamine in a dose-dependent manner. Therefore, we designed three different doses of CECMF. Control rats were treated with saline once daily through intraperitoneal injection (i.p.) from P7 to P49. Ketamine was dissolved in saline and injected i.p. to rats at the dose of 40-80 mg/kg once daily from P7 to P49. CECMF and donepezil were dissolved in distilled water and given orally to the rats 30 min after ketamine administration from P28 to P49. At 50 days of age, the rats were subjected to behavioral tests including NORT and MWMT. The timeline of the drug administration and behavioral tests is shown in **Fig. 1B**.

3.3.4. Behavioral tests

3.3.4.1. NORT

The object recognition test is based on the natural tendency of rodents to investigate a novel object instead of a familiar one, as well as their innate tendency to restart exploring when they are presented with a novel environment. The choice to explore the novel object, as well as the reactivation of exploration after object displacement, reflects the use of learning and recognition memory processes. The NORT was conducted in an open-field arena (60 × 60 × 60 cm for rats) constructed with polyvinyl chloride, plywood and acrylic. The task includes training and retention over two consecutive days. On day 1, the mice were explored two identical objects (5 × 5 × 5 cm, black plastic cubes) for 5 min in training session. On day 2, one of the objects was replaced with a new shape and color (5 × 5 × 7 cm, a gray plastic square pyramid), and the mice were acclimated to the area for 5 min in recognition session. The field was decontaminated with 10% ethanol solution between the

tests. The animals were explored the test area by touching or sniffing the objects with their forepaws and/or noses at a distance of less than 2 cm. Total exploration time refers to the amount of time devoted to locating the two objects. The cognitive function was determined using a recognition index, which was the exploration time involved with either of the two objects (training session) or the novel object (recognition session) divided by the total exploration time in exploring both objects. The research team has rich experience in conducting NORT [44].

3.3.4.2. MWMT.

MWMT is a sensitive method commonly used for revealing the impairment of spatial learning and memory [45]. After drugs treatment, rats were assessed for spatial reference memory using MWMT as per our previous description [46-49]. The experimental apparatus consists of a blank circular water tank (160 cm in diameter, 40 cm in height). A platform is submerged 2 cm below the water surface and placed at the midpoint of one quadrant. Spatial training to find the hidden platform in the water maze was conducted for four consecutive days. The rats were given 60 sec to find the platform and allowed to stay on it for 20 sec. Each rat performed four trials per day, with the inter-trial interval for 60 sec. The escape latency to the platform of each trial was recorded, and the average value of the four trials calculated. Twenty-four hours after the last day of spatial training, the probe test was carried out by removing the platform and allowing each rat to swim freely for 60 sec. The time each rat spending in swimming in the target quadrant (where the platform is once hidden) and the number of times (frequency) that it crosses over the platform site were recorded. The MWMT is frequently performed in the PI's laboratories [44, 46-49].

3.3.5. Determination of the effects of CECMF on synaptotoxicity in the brains of ketamine-treated rats.

To investigate whether CECMF is able to ameliorate synaptic deficits in the brains of ketamine-treated rats, we measured the synapse-related proteins by western blotting using antibodies against N-methyl D-aspartate receptor subtype 2A (GluN2A), N-methyl D-aspartate receptor subtype 2B (GluN2B), AMPA-selective glutamate receptor 1 (GluA1),

AMPA-selective glutamate receptor 2 (GluA2), postsynaptic density protein 93 (PSD93), postsynaptic density protein 95 (PSD95), synapsin 1 (SYN1), synaptotagmin (SYT) (Cell Signaling). These experiments helped reveal whether CECMF treatment can ameliorate the synaptotoxicity in ketamine-treated rats.

3.4. To investigate the effects of CECMF on cystitis induced by ketamine in rats.

3.4.1. Establishment of ketamine-induced cystitis in rats and drug treatment

Ketamine-induced cystitis in rats as described in previous study [50,51]. Female SD rats (8-week-old) were purchased from the LASEC, CUHK, and randomly assigned into six groups (n=10): control, ketamine + vehicle (ketamine-treated control), ketamine + CECMF (1.25 g/kg), ketamine + CECMF (2.5 g/kg), ketamine + CECMF (5.0 g/kg), and ketamine + parecoxib sodium (COX-2 inhibitor, 10 mg/kg, i.p). In this study, we would like to explore whether CECMF improved the cystitis induced by ketamine in a dose-dependent manner. Therefore, we designed three different doses of CECMF. Ketamine was dissolved in saline and given to rats by intraperitoneal (i.p.) injection at the dose of 80 mg/kg at 09:00 h each day for 49 consecutive days. CECMF was dissolved in physical saline and given orally to rats from day 15 to 49. At the same time, the rats in control group received intraperitoneal injection of saline whereas control group were untreated. All rats were weighed weekly to adjust the quantity of ketamine administrate. The timeline of the drug administration and behavioral tests is shown in **Fig. 1C**.

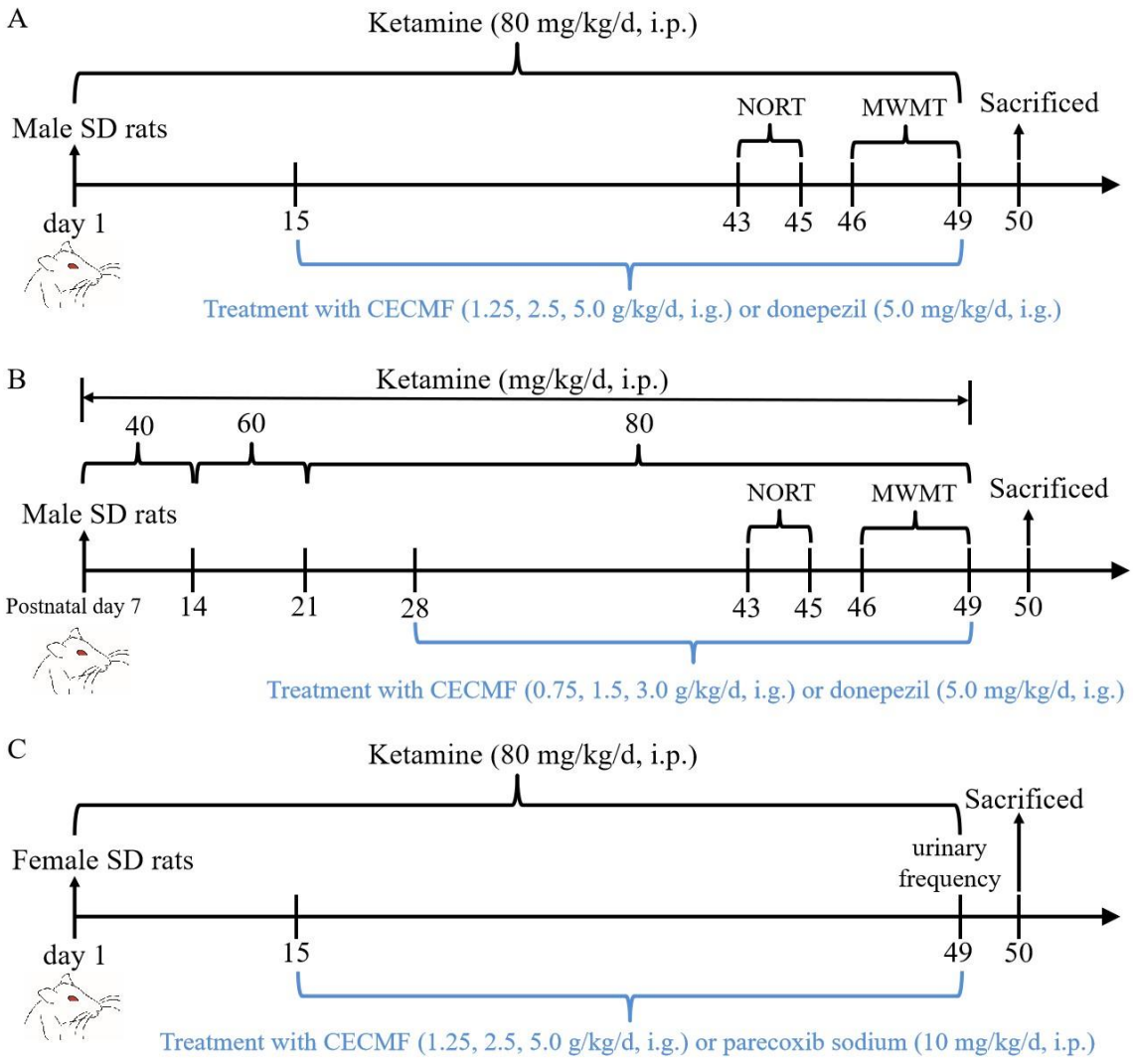


Fig. 1. Procedure of the establishment of the ketamine-treated rats and drug treatment. (A) Ketamine-induced cognitive deficits in adult rats and drug treatment. (B) Ketamine-induced cognitive deficits in neonatal rats and drug treatment. (C) Ketamine-induced cystitis in adult rats and drug treatment.

3.4.2. Determination of urinary frequency of ketamine-treated rats

Urinary frequency was determined by housing the rats individually in modified metabolic cages that had a fine stainless steel mesh, which retained feces but allowed urine to pass, through its base. Paper, impregnated with saturated copper sulfate solution (CuSO4 $5H_2O$) and dehydrated at 200 ℃ for 1 h, was placed under the base of the cage. The urine falling onto this paper, rehydrated the anhydrous CuSO4, turning it blue. This method provided a

sensitive system for estimating even small amounts of urine since the CuSO4 $5H_2O$ does not dehydrate at room temperature. Micturition was recorded for 24 h for three consecutive days to minimize the effect of the circadian cycle on urinary frequency. The copper sulfate papers were changed every 6 h during the measurement. The used papers were removed from the cages, and the number of urinations was determined by counting the number of blue spots observed. Overlapped urine spots with legible different edges were considered as separate urination. Baseline urinary frequency was measured 3 days before ketamine administration.

3.4.3. Histological analysis

At the end of treatment period, the female rats were sacrificed by cervical dislocation. The bladder tissues were harvested, and fixed in 10% (v/v) neutral buffered formalin for 24 h. The tissue samples are then embedded in paraffin and sectioned at 4 μm thickness. The sections were then stained with hematoxylin and eosin (H&E) solution and Masson's trichrome solution to verify the leukocyte infiltration and collagen deposition in rats' bladders.

3.4.4. Urine markers and cytokines determination

Urine samples were collected from bladders after the female rats were sacrificed. The urine markers (antiproliferative factor (APF) and glycoprotein 51(GP51)) were assessed by using the enzyme-linked immunosorbent assay kits (ELISA). NO levels were estimated using Griess reaction. The levels of interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and cyclooxygenase-2 (COX-2) in the bladders were determined using ELISA kit according to the manufacturer's instructions.

3.4.5. Western blot analysis

Hippocampus tissues from the male rats or bladder tissues from the female rats were disrupted by homogenization in RIPA buffer containing 1% protease inhibitor cocktail. After centrifugation (12,000 rpm, 15 min) at 4 ° C, the supernatants were collected. Protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific, USA). The protein lysates were separated by 10% SDS-PAGE and

electrophoretically transferred onto PVDF membranes (Roche Applied Science, Germany). After blocking with 5% nonfat milk in TBS-T for 1 h, membranes were incubated with rabbit anti-B cell lymphoma-2 (Bcl-2, 1:1000, ABclonal), rabbit anti-Bcl-2-associated X (Bax, 1:1000, Cell Signaling Technology), rabbit anti-cleaved caspase 3 (1:1000, Cell Signaling Technology), rabbit anti-N-methyl D-aspartate receptor subtype 2A (GluN2A, 1:1000, Cell Signaling Technology), rabbit anti-N-methyl D-aspartate receptor subtype 2B (GluN2B, 1:1000, Cell Signaling Technology), rabbit anti-AMPA-selective glutamate receptor 1 (GluA1, 1:1000, Cell Signaling Technology), rabbit anti-AMPA-selective glutamate receptor 2 (GluA2, 1:1000, Cell Signaling Technology), rabbit anti-postsynaptic density protein 93 (PSD93, 1:1000, Cell Signaling Technology), rabbit anti-postsynaptic density protein 95 (PSD95, 1:1000, Cell Signaling Technology), rabbit anti-synapsin 1 (SYN1, 1:1000, Cell Signaling Technology), rabbit anti-synaptotagmin (SYT, 1:1000, Cell Signaling Technology), rabbit anti-p-CaMKIIβ (T287) (1:1000, Abcam), rabbit anti-CaMKIIβ (1:1000, Abcam), rabbit anti-phospho-extracellular signal-regulated kinase (T202/Y204) (p-ERK (T202/Y204), 1:1000, Cell Signaling Technology), rabbit anti-ERK (1:1000, Cell Signaling Technology), rabbit anti-phospho-cAMP-response element-binding protein (p-CREB, 1:1000, Cell Signaling Technology), rabbit anti-phospho-nuclear factor kappa-B (p-NF-κB, p-p65, 1:1000, Cell Signaling Technology), rabbit anti-NF-κB p65 (1:1000, Cell Signaling Technology), rabbit anti-β-actin (1:1000, Cell Signaling Technology), rabbit anti-intercellular cell adhesion molecule-1 (ICAM-1, 1:1000, Proteintech), rabbit anti-transforming growth factor-β1(TGF-β1, 1:1000, Invitrogen), mouse anti-type I collagen (Collagen I, 1:1000, Abcam), mouse anti-type III collagen (Collagen III, 1:1000, Invitrogen), mouse anti-fibronectin (Fibronectin, 1:1000, Invitrogen), rabbit anti-transient receptor potential vanilloid 1 (TRPV1, 1:1000, ABclonal), rabbit anti-muscarinic acetylcholine receptor M2 (M2-mAChR, 1:1000, ABclonal), rabbit anti-M3-mAChR (1:1000, ABclonal), rabbit anti-P2X ligand-gated ion channel 1 (P2X1, 1:1000, Invitrogen), rabbit anti- P2X2 (1:1000, ABclonal), rabbit anti- P2X3 (1:1000, ABclonal), rabbit anti-heme oxygenase-1 (HO-1, 1:1000, Cell Signaling Technology), rabbit anti- nuclear factor-erythroid 2-related factor 2 (Nrf2, 1:1000, Abcam), rabbit anti-phospho-NF-κB inhibitor α (p-IκBα, 1:1000, Cell Signaling Technology), rabbit anti-IκBα (1:1000, Cell Signaling Technology) and rabbit anti-GAPDH (1:10,000, Abcam)

antibodies overnight at 4 ° C, and then incubated with secondary antibodies for 1 h at room temperature. The protein bands were detected using an enhanced chemiluminescence (ECL) substrate reagent kit (Invitrogen, USA) and quantified by ImageJ software using β-actin or GAPDH as the internal control.

3.5. Evaluation of the toxicological profile of the CECMF.

Since any pharmacologic treatment for ketamine addiction would usually last for a long time, therefore it is necessary to determine the toxicological profiles of the formula for drug safety. We followed a previous study to evaluate the toxicology of the combination [52,53].

3.5.1. Acute toxicity study of CECMF.

ICR mice were randomly assigned to each of five groups of 20 mice each (10 females and 10 males). They were fasted overnight (12 h) with free access only to water prior to administration of single doses (0, 4, 8, 16, 32, 64, 128 g/kg) of CECMF dissolved in distilled water. Treatment was given by gavage of 10 mL/kg. The general behaviors of the mice were continuously monitored for 4 h after the treatment, intermittently (every 6 h) during a 24-h period, and thereafter daily up to 7 days. The median lethal dose (LD_{50}) was calculated as previously described [52,53].

3.5.2. Sub-chronic toxicity study of CECMF.

SD rats (weighing 140-180 g; both sexes) were used for the sub-chronic toxicity assessment. Rats were randomly divided into 4 groups of 20 each (10 females and 10 males): vehicle, CECMF (5 g/kg), CECMF (10 g/kg) and CECMF (20 g/kg). The CECMF was dissolved in distilled water, and given intragastrically once a day for 90 consecutive days, and the rats of the sham control were treated with the same volume of the vehicle for the same duration. The animals were observed for signs of toxicity and mortality throughout the experimental period. The body weight, water and food consumption were recorded weekly. At the end of the 90-day experiment, 10 rats of each group (10 rats left for the withdrawal study) were sacrificed by decapitation under anaesthesia (thiopental 50 mg/kg). Blood was collected with and without anticoagulant (EDTA) for haematological and biochemical

studies, respectively, under anaesthesia. The organs (brain, thymus, heart, lungs, liver, spleen, kidneys, adrenal glands, testicles, epididymis, ovaries, pancreas and uterus) were weighed and the relative organ weight (weight of organ as proportional to the total body weight of each rat) was calculated and compared with the value of control. Organ samples (kidneys, pancreas, lungs and liver) were fixed in 10% formalin for histopathological examination.

In 30-day of post treatment, the animals were withdrawn from the CECMF and have free access to standard commercial diet and tap water. At the end of withdrawal study, rats were treated similar to the 90-day experiment described above.

3.6. Data analysis and sample size estimation

Data were expressed as mean ± standard error mean (SEM). Group differences in the escape latency in the MWMT training task and urinary frequency were analyzed using two ways analysis of variance (ANOVA) with repeated measures, with the factors being treatment and time. Group differences in other experiments were analyzed using one-way ANOVA, followed by Dunnett's test with GraphPad Prism (version 8.0). The $p < 0.05$ was considered statistically significant.

4. Results

During the experiment duration, 1-2 rats in each group died since the individual differences of the animal responded to high dose of ketamine (80 mg/kg). Therefore, there were 8-9 rats in each group at the end of the animal experiments of investigating the improving effect of CECMF on cognitive deficits and cystitis induced by ketamine. It had been reported that 8 rats in each group had reached the standard for statistical analysis [54,55]. Hence, we adjusted the sample size of each group to 8 rats after comprehensive consideration. However, no animal was found to die during the experiment duration of acute and sub-chronic toxicities assessments of CECMF extract.

4.1. The quality control of CECMF

The chemical compositions of CECMF were determined by LC-MS analysis, 13 components of which were identified as 3'-hydroxypuerarin, puerarin, 3'-methoxypuerarin,

neopuerarin B, polydatin, quercitrin, corydaline, resveratrol, salvianolic acid C, emodin, cryptotanshinone, tanshinone IIA, miltirone presented in **Table 1** and **Fig. 2**.

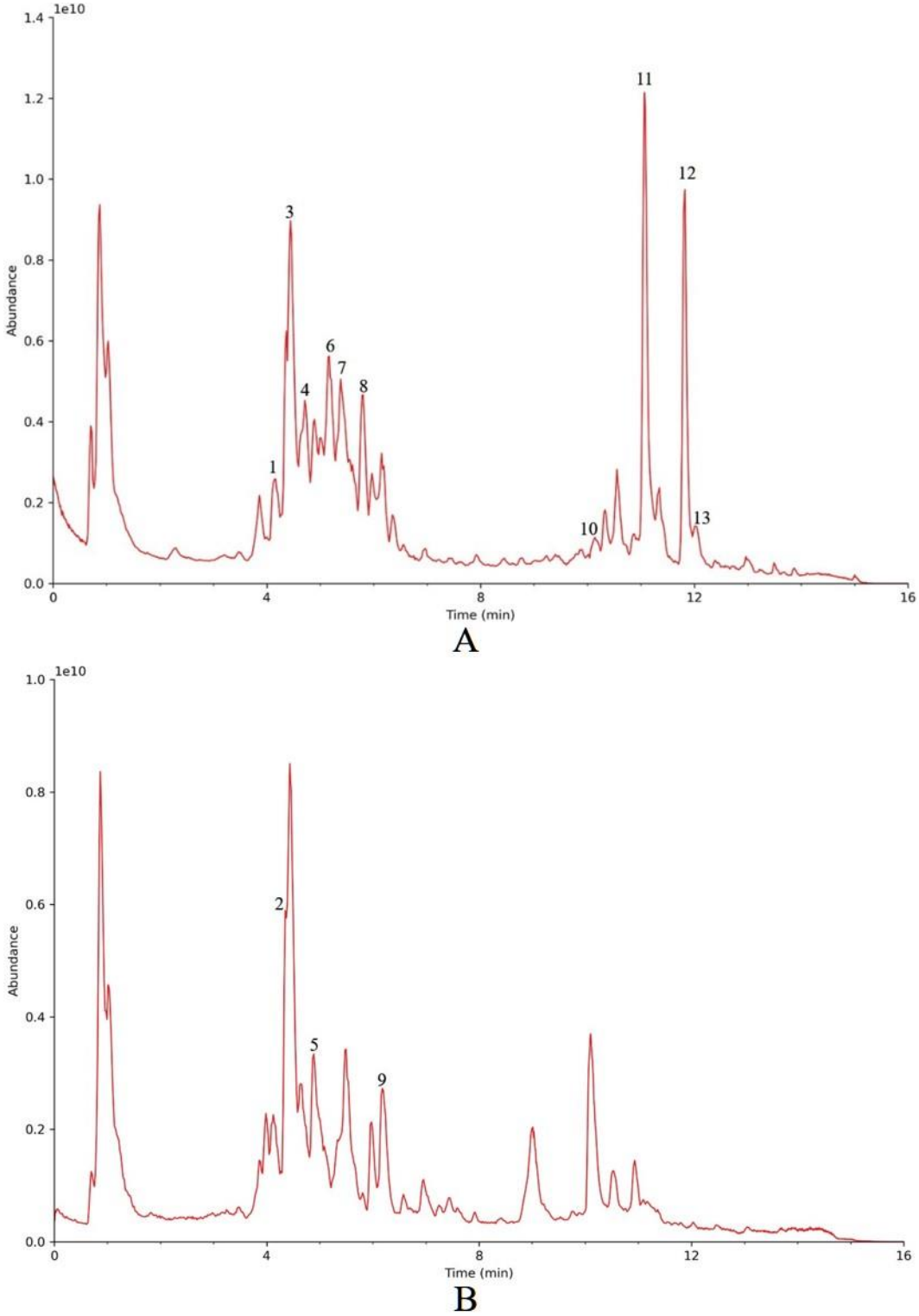


Fig. 2. Total ion chromatograms of CECMF by LC-MS analysis. (A) Positive ion mode.

(B) Negative ion mode.

Table 1. LC-MS analysis of CECMF.

No.	Retention time (min)	Molecular weight	Parent ion (m/z)		Product ion (m/z)	Compounds *
			$[M+H]^+$	$[M+H]^-$		
1	4.15	432.38	433.11	—	283, 313, 337, 367, 397, 415, 433	3'-Hydroxypuerarin
2	4.35	416.38	—	415.10	267, 295, 415	Puerarin
3	4.44	446.40	447.13	—	285, 297, 327, 337, 351, 381, 411, 429, 447	3'-Methoxypuerarin
4	4.71	416.38	417.12	—	255, 297, 399, 417	Neopuerarin B
5	4.89	390.39	—	389.12	227, 389	Polydatin
6	5.17	448.38	449.11	—	71, 85, 287, 303, 338	Quercitrin
7	5.38	369.46	370.20	—	165, 192, 370	Corydaline
8	5.80	228.24	229.09	—	91, 107, 119, 135, 229	Resveratrol
9	6.18	492.44	—	491.10	135, 245, 249, 265, 293, 311, 431, 491	Salvianolic acid C
10	10.14	270.24	271.06	—	229, 271	Emodin
11	11.07	296.36	297.15	—	251, 279, 297	Cryptotanshinone
12	11.83	294.33	295.13	—	249, 277, 295	Tanshinone IIA
13	12.03	282.38	283.17	—	223, 240, 241, 265, 283	Miltirone

*Analyzed by LC-ESI-MS analysis both in positive and in negative ionization modes.

4.2. To explore the effects of CECMF the cognitive deficits induced by ketamine in adult rats

4.2.1 Effects of CECMF on the recognition memory in ketamine-treated adult rats

The NORT was usually used to observe the recognition potential of the rats. It was designed to assess the spontaneous preference of rodents to explore an unfamiliar object rather than a familiar one. Eight rats from each group were exploited to conduct the NORT. As shown in **Fig. 3**, the ketamine + vehicle group had a markedly lower recognition index than the control group ($p < 0.001$), suggesting that rats received ketamine had an impaired ability to discriminate between familiar and novel objects as compared with the normal control rats. The administration of CECMF (1.25, 2.5 and 5.0 g/kg) effectively increased the recognition index in the rats ($p < 0.01$ for all), when compared with the ketamine vehicle group (**Fig. 3**). In addition, the ketamine + donepezil group also had a higher recognition

index than the ketamine + vehicle group ($p < 0.01$). All these experimental findings demonstrated that CECMF could improve the recognition memory impairments in adult rats induced by ketamine.

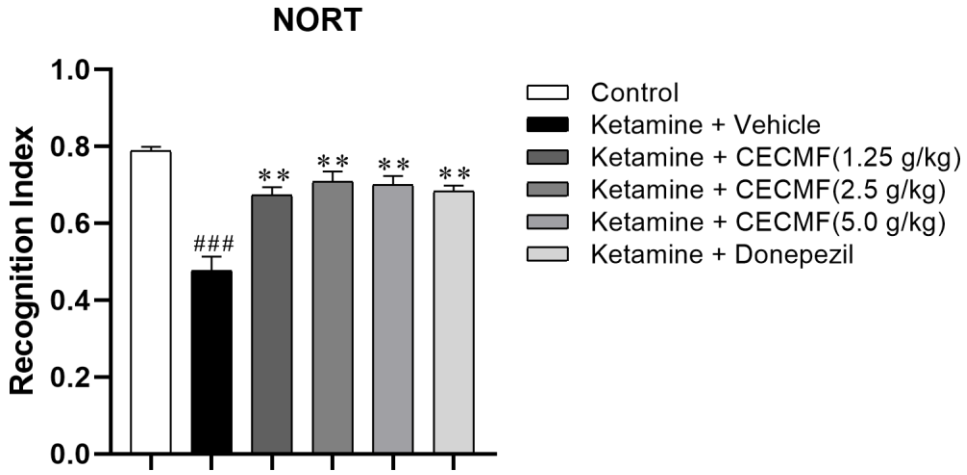


Fig. 3. Recognition Index in NORT. Data were expressed as the mean ± SEM (n = 8). $^{###}p < 0.001$ compared with the control group; $^{**}p < 0.01$ compared with the ketamine + vehicle group.

4.2.2. Effects of CECMF on cognitive and learning functions in ketamine-treated adult rats

MWMT was applied to evaluate spatial learning and memory in ketamine-treated rats. As shown in **Fig. 4B**, during the training days, the rats in the ketamine + vehicle group spent significantly longer time to find the hidden platform than the normal control group (from day 2 to day 3, $p < 0.01$ for 2 days). As compared with the ketamine vehicle group, the rats in the three ketamine + CECMF (1.25, 2.5, 5.0 g/kg) groups had a shorter escape latency finding the hidden platform during the training days (CECMF at the dose of 1.25 g/kg: $p < 0.05$ for day 2 and $p < 0.01$ for day 3; CECMF at the dose of 2.5 g/kg: $p < 0.001$ for day 2 and day 3; CECMF at the dose of 5.0 g/kg: $p < 0.01$ for day 2 and $p < 0.05$ for day 3). In the probe trial, when compared with the control group, there were conspicuous reductions in the number of the target quadrant crossing and the time spent in the target quadrant in the ketamine + vehicle group ($p < 0.001$ for both) (**Fig. 4C & D**), demonstrating that ketamine treated rats had cognitive and memory impairments. The administration of three treatments, including CECMF (2.5 and 5.0 g/kg) and donepezil, effectively increased the

number of the target quadrant crossing in the ketamine treated-rats ($p < 0.001$ for all), when compared with the ketamine vehicle group (**Fig. 4C**). The ketamine treated-rats with CECMF (1.25, 2.5 and 5.0 g/kg) and donepezil administration spent significantly more time in the target quadrant than the rats in the ketamine + vehicle group ($p < 0.01$, $p < 0.001$, $p < 0.001$ and $p < 0.01$, respectively) (**Fig. 4D**). There were no statistical differences in the swimming speed among all groups (**Fig. 4E**). These experimental results indicated that CECMF could ameliorate learning and memory impairments in ketamine treated-rats.

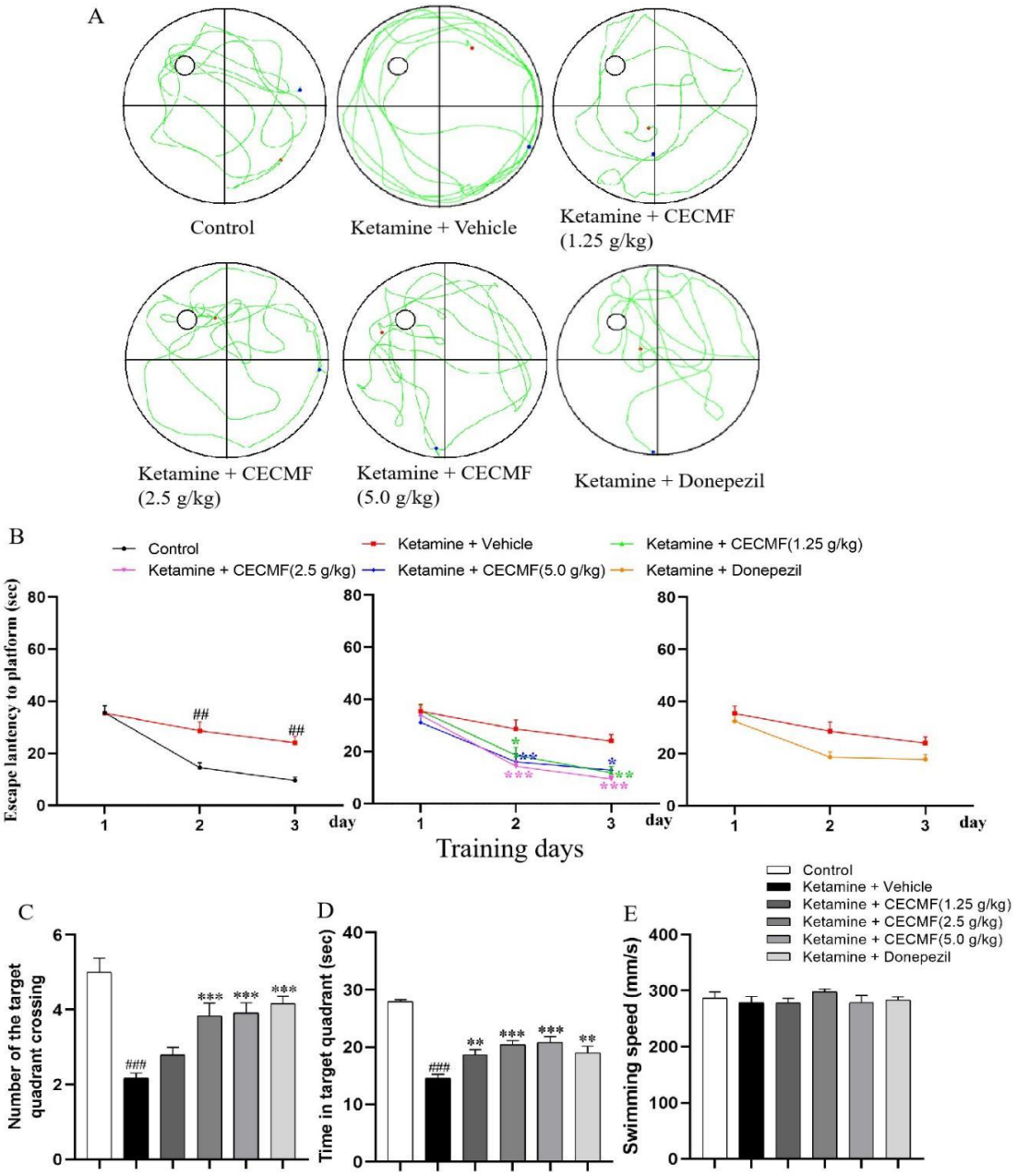


Fig. 4. Effects of CECMF on cognitive and learning functions in ketamine-treated adult rats. (A) Representative images of the swimming paths of rats in the MWMT probe test. (B) escape latency to platform during training days in MWMT. (C) numbers of the target crossing in the MWMT probe test. (D) the time spent in the target quadrant in the MWMT probe test. (E) swimming speed in the target quadrant in the MWMT probe test. Data were expressed as the mean ± SEM (n = 8). $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared with the control group; $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared with the ketamine + vehicle group.

4.2.3. Effects of CECMF on neuronal apoptosis in hippocampus of ketamine-treated adult rats.

As illustrated in **Fig. 5**, Bcl-2, Bax and cleaved caspase 3 protein expression levels were quantified in hippocampus of ketamine-treated rats by western blotting. Our results showed that the protein expression of Bcl-2 was significantly reduced while Bax and cleaved caspase 3 were markedly elevated in the brains of ketamine + vehicle rats, as compared with the control group ($p < 0.001$ for all). However, CECMF treatment at the two doses (2.5 and 5.0 g/kg) could effectively promote the protein expressions of Bcl-2 ($p < 0.01$ and $p < 0.001$, respectively), and CECMF treatment at the three doses (1.25, 2.5 and 5.0 g/kg) could significantly decrease the expressions of Bax ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively) and cleaved caspase 3 ($p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively), in the hippocampus of ketamine treated rats as compared with the ketamine + vehicle group. Comparably, donepezil (5 mg/kg) evidently increased the expressions of Bcl-2 ($p < 0.01$), and decrease the expressions of Bax ($p < 0.001$) and cleaved caspase 3 ($p < 0.001$), in the hippocampus of ketamine treated adult rats, as compared with the ketamine + vehicle control group.

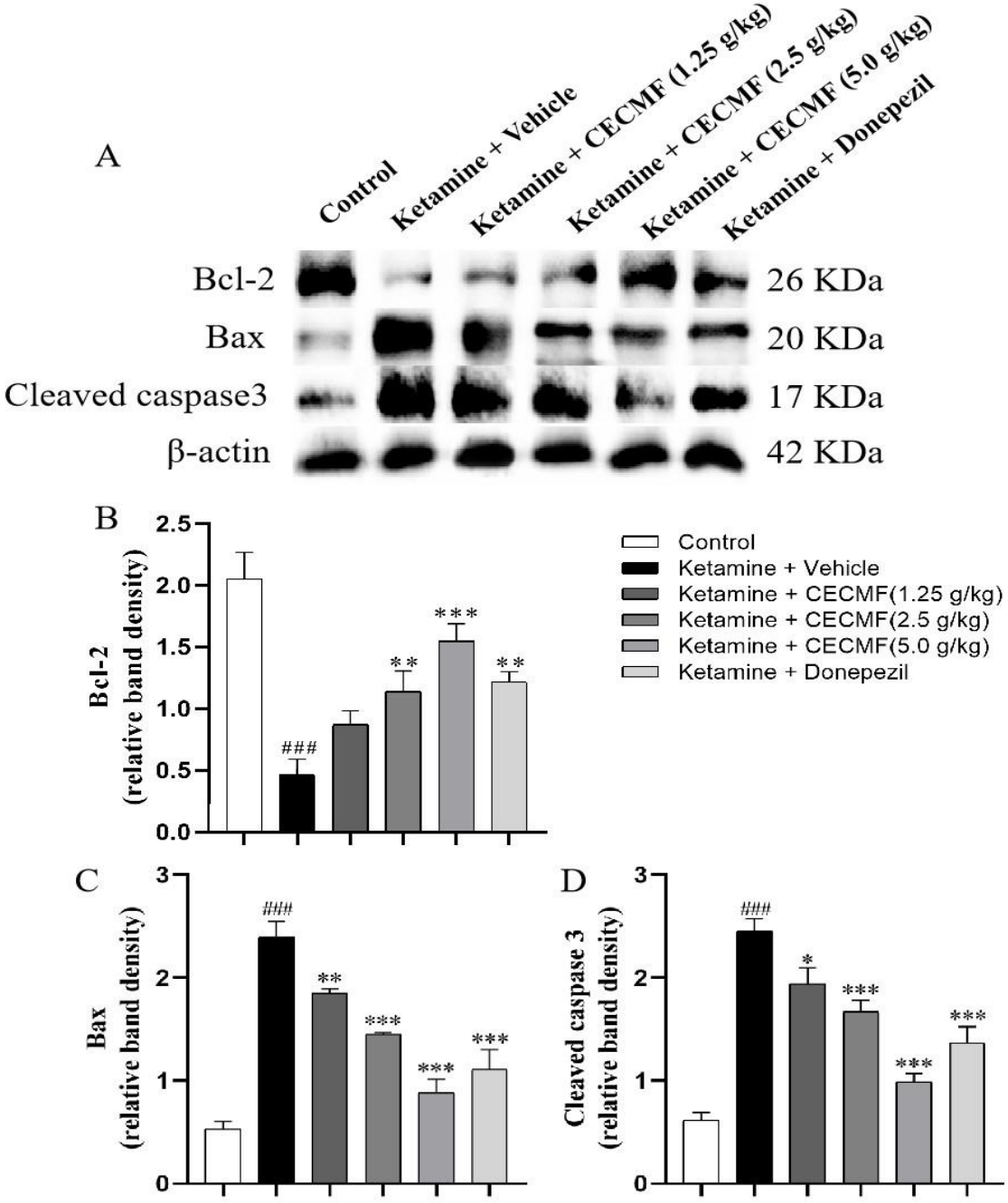


Fig. 5. Effects of CECMF on neuronal apoptosis in the brains of ketamine-treated adult rats. (A) Representative western blotting images. Quantitative analysis of the protein expressions of Bcl-2 (B), Bax (C), and cleaved caspase 3 (D). Data were expressed as the mean ± SEM (n = 3). $^{###}p < 0.001$ compared with the control group; $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared with the ketamine + vehicle group.

4.2.4. Effects of CECMF on synaptotoxicity in hippocampus of ketamine-treated adult rats.

To evaluate the effects of CECMF (1.25, 2.5 and 5.0 g/kg) on synaptic pathology, we examined the levels of GluN2A, GluN2B, GluA1, GluA2, PSD93, PSD95, SYN1 and SYT, in the brains of rats (**Fig. 6**). The results demonstrated that the protein levels of GluN2A, GluN2B, GluA1, GluA2, PSD93, PSD95, SYN1 and SYT were significantly decreased in the brains of ketamine + vehicle rats as compared with the control group ($p < 0.001$ for all). Treatment with CECMF (1.25 g/kg) markedly elevated the protein expressions of PSD93 ($p < 0.01$) and PSD95 ($p < 0.05$) in the brains of ketamine treated rats. Interestingly, CECMF (2.5 and 5.0 g/kg) significantly enhanced the protein expressions of GluN2A ($p < 0.001$ and $p < 0.01$, respectively), GluN2B ($p < 0.05$ for both), GluA1 ($p < 0.05$ and $p < 0.01$, respectively), GluA2 ($p < 0.05$ and $p < 0.01$, respectively), PSD93 ($p < 0.001$ for both), PSD95 ($p < 0.01$ and $p < 0.001$, respectively), SYN1 ($p < 0.05$ and $p < 0.01$, respectively), and SYT ($p < 0.01$ and $p < 0.05$, respectively), in the brains of ketamine treated rats as compared with the ketamine + vehicle group. Similarly, donepezil (5 mg/kg) obviously increased the protein expressions of GluN2A ($p < 0.05$), GluN2B ($p < 0.05$), GluA2 ($p < 0.05$), PSD93 ($p < 0.001$), PSD95 ($p < 0.001$), SYN1 ($p < 0.01$) and SYT ($p < 0.01$), in the brains of ketamine treated rats, as compared with the ketamine + vehicle control.

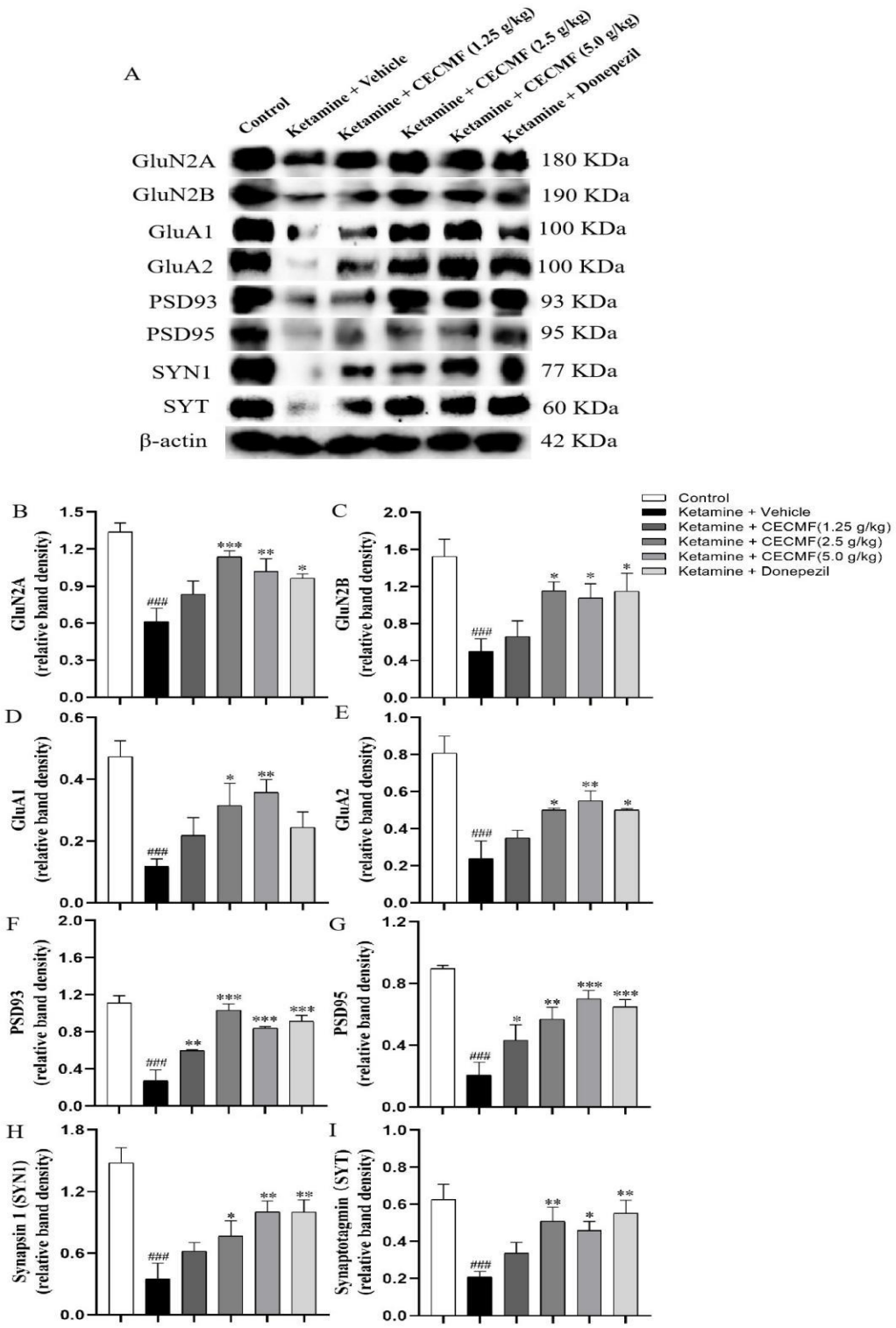


Fig. 6. Effects of CECMF on synaptotoxicity in the brains of ketamine-treated adult rats. (A) Representative western blotting images. Quantitative analysis of the protein expressions of (B) GluN2A, (C) GluN2B, (D) GluA1, (E) GluA2, (F) PSD93, (G) PSD95,

(H) SYN1 and (I) SYT. Data were expressed as the mean ± SEM (n = 3). $^{###}p < 0.001$ compared with the control group; $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared with the ketamine + vehicle group.

4.2.5. Effects of CECMF on the CaMKIIβ-ERK1/2-CREB/NF-κB pathway in hippocampus of ketamine-treated adult rats.

Our western blot results showed that (**Fig. 7**), the protein expression of p-CaMKIIβ (T287) and p-CREB (S133) were significantly reduced while p-ERK (T202/Y204) and p-p65 were markedly elevated in the brains of ketamine + vehicle rats, as compared with the control group ($p < 0.001$ for all). However, CECMF treatment at the three doses (1.25, 2.5 and 5.0 g/kg) could effectively promote the protein expressions of p-CaMKIIβ (T287) ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively) and p-CREB (S133) ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively), and decrease the expressions of p-ERK (T202/Y204) ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively) and p-p65 ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively), in the brains of ketamine treated rats as compared with the ketamine + vehicle group. Comparably, donepezil (5 mg/kg) evidently increased the expressions of p-CaMKIIβ (T287) ($p < 0.001$) and p-CREB (S133) ($p < 0.01$), and decrease the expressions of p-ERK (T202/Y204) ($p < 0.05$) and p-p65 ($p < 0.001$), in the brains of ketamine treated rats, as compared with the ketamine + vehicle control.

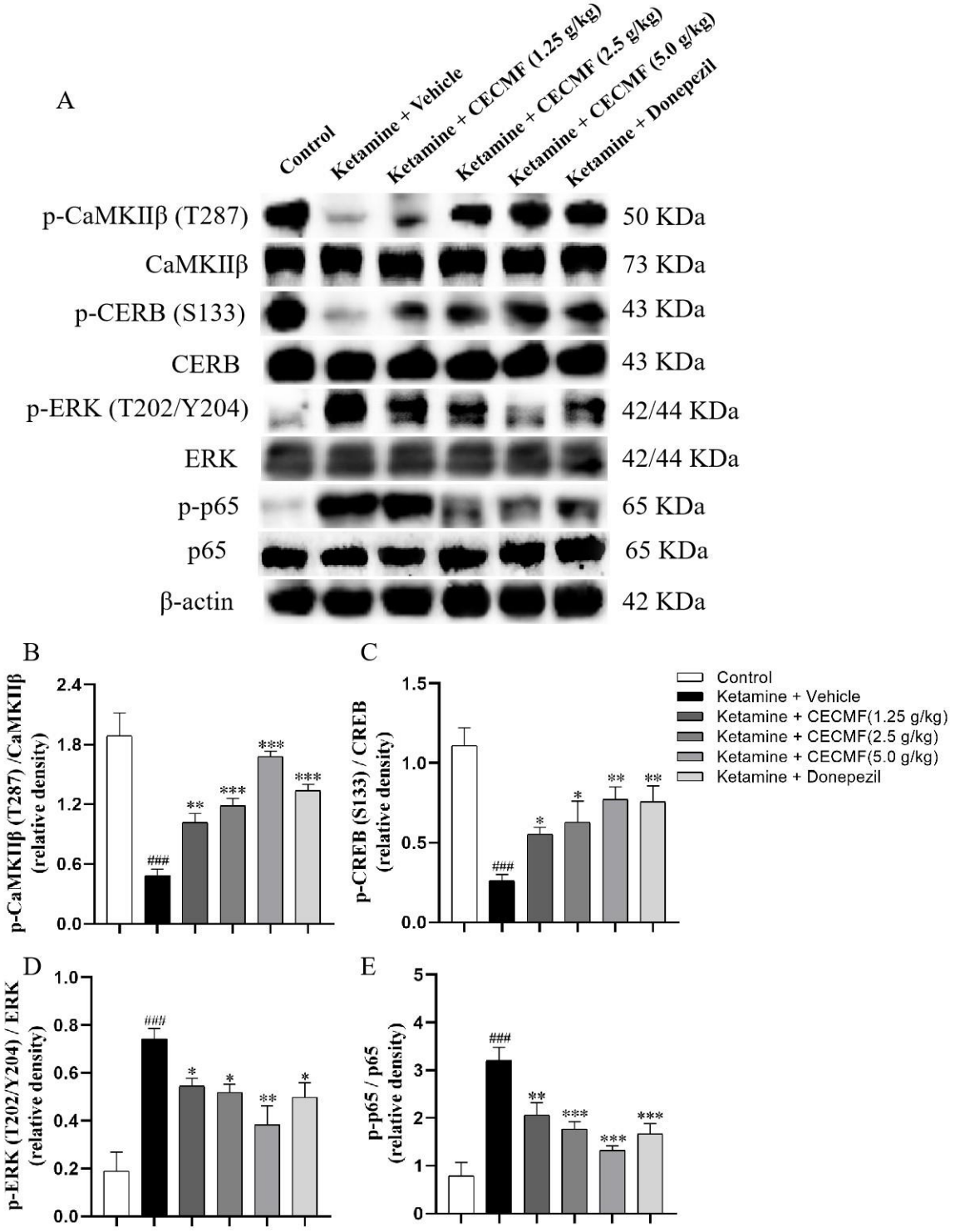


Fig. 7. Effects of CECMF on the CaMKIIβ-ERK1/2-CREB/NF-κB pathway in the brains of ketamine-treated adult rats. (A) Representative western blotting images. Quantitative analysis of the protein expressions of p-CaMKIIβ (T287)/CaMKIIβ (B), GluN2B (C), p-

CREB (S133)/CREB (D), p-ERK (T202/Y204)/ERK (E), and p-p65/p65 (F). Data were expressed as the mean ± SEM (n = 3). $^{###}p < 0.001$ compared with the control group; $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared with the ketamine + vehicle group.

4.3. To explore the effects of CECMF the cognitive deficits induced by ketamine in neonatal rats

4.3.1. Effects of CECMF on the recognition memory in ketamine-treated neonatal rats

Eight rats from each group were exploited to conduct the NORT. As shown in **Fig. 8**, the ketamine + vehicle group had a markedly lower recognition index than the control group ($p < 0.001$), suggesting that rats received ketamine had an impaired ability to discriminate between familiar and novel objects as compared with the normal control rats. The administration of CECMF (0.75, 1.5 and 3.0 g/kg) effectively increased the recognition index in the rats ($p < 0.01$, $p < 0.001$ and $p < 0.001$ respectively), when compared with the ketamine vehicle group (**Fig. 8**). In addition, the ketamine + donepezil group also had a higher recognition index than the ketamine + vehicle group ($p < 0.001$). All these experimental findings demonstrated that CECMF could improve the recognition memory impairments in neonatal rats induced by ketamine.

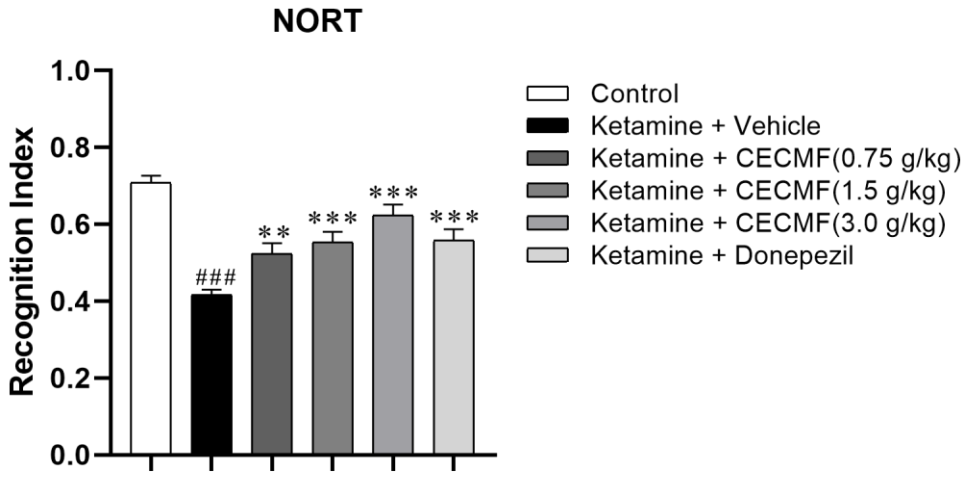


Fig. 8. Recognition Index in NORT. Data were expressed as the mean ± SEM (n = 8). $^{###}p < 0.001$ compared with the control group; $**p < 0.01$ and $***p < 0.001$ compared with the ketamine + vehicle group.

4.3.2. Effects of CECMF on cognitive and learning functions in ketamine-treated neonatal rats

As shown in **Fig. 9B**, during the training days, the rats in the ketamine + vehicle group spent significantly longer time to find the hidden platform than the normal control group (from day 2 to day 3, $p < 0.01$ for 2 days). As compared with the ketamine vehicle group, the rats in the three ketamine + CECMF (0.75, 1.5, 3.0 g/kg) groups had a shorter escape latency finding the hidden platform during the training days (CECMF at the dose of 0.75 g/kg: $p < 0.05$ for day 2 and day 3; CECMF at the dose of 1.5 g/kg: $p < 0.05$ for day 2 and $p < 0.01$ for day 3; CECMF at the dose of 3.0 g/kg: $p < 0.05$ for day 2 and day 3). In the probe trial, when compared with the control group, there were conspicuous reductions in the number of the target quadrant crossing and the time spent in the target quadrant in the ketamine + vehicle group ($p < 0.001$ for both) (**Fig. 9C & D**), demonstrating that ketamine treated rats had cognitive and memory impairments. The administration of all treatments, including CECMF (0.75, 1.5 and 3.0 g/kg) and donepezil, effectively increased the number of the target quadrant crossing in the ketamine treated-rats ($p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.05$, respectively), when compared with the ketamine vehicle group (**Fig. 9C**). The ketamine treated-rats with CECMF (0.75, 1.5 and 3.0 g/kg) and donepezil administration spent significantly more time in the target quadrant than the rats in the ketamine + vehicle group ($p < 0.01$, $p < 0.001$, $p < 0.001$ and $p < 0.05$, respectively) (**Fig. 9D**). There were no statistical differences in the swimming speed among all groups (**Fig. 9E**). These experimental results indicated that CECMF could ameliorate learning and memory impairments in ketamine treated neonatal rats.

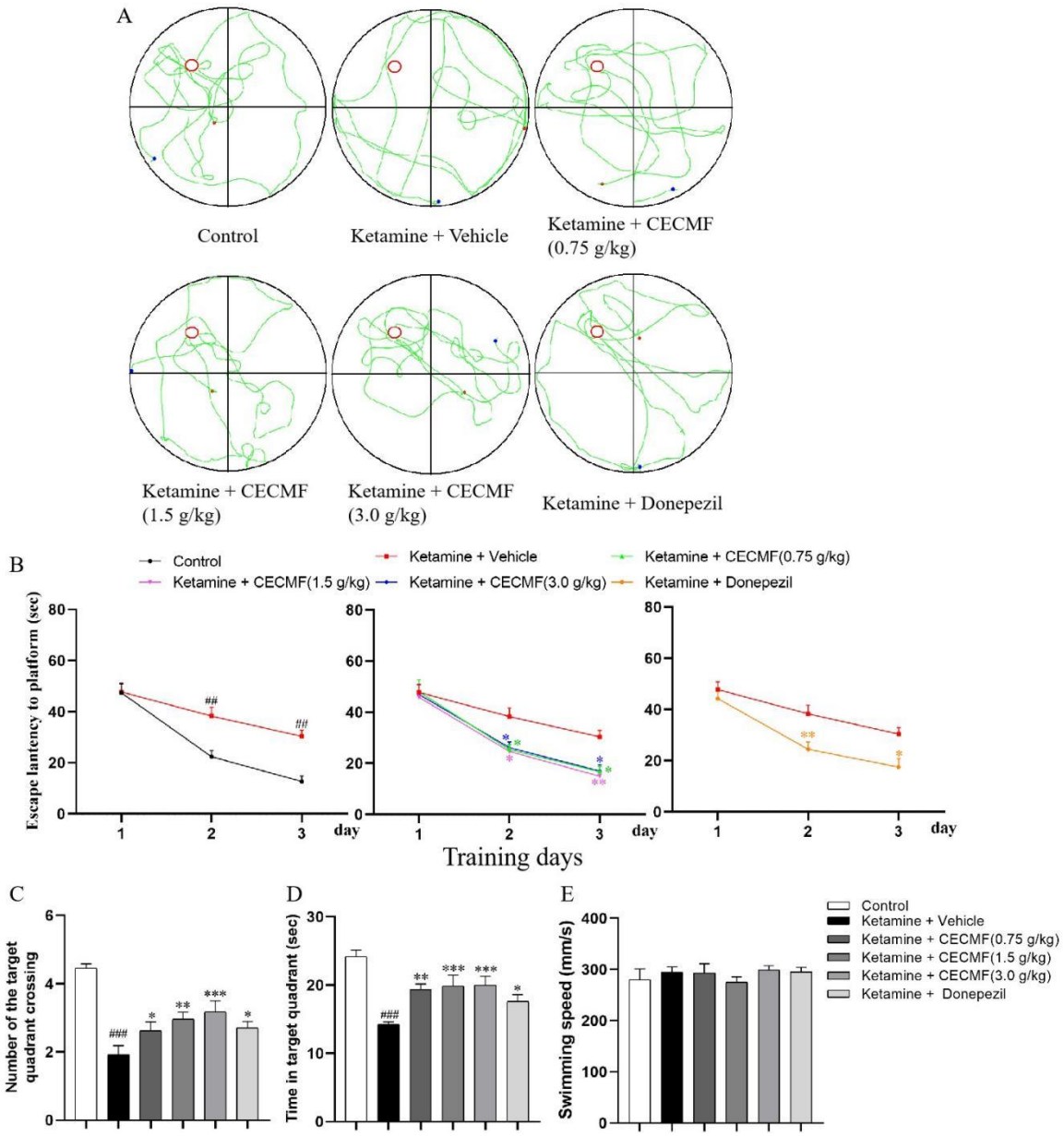


Fig. 9. Effects of CECMF on cognitive and learning functions in ketamine-treated neonatal rats. (A) Representative images of the swimming paths of rats in the MWMT probe test. (B) escape latency to platform during training days in MWMT. (C) numbers of the target crossing in the MWMT probe test. (D) the time spent in the target quadrant in the MWMT probe test. (E) swimming speed in the target quadrant in the MWMT probe test. Data were expressed as the mean ± SEM (n = 8). $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared with the control group; $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared with the ketamine + vehicle group.

4.3.3. Effects of CECMF on neuronal apoptosis in hippocampus of ketamine-treated neonatal rats.

As illustrated in **Fig. 10**, Bcl-2, Bax and cleaved caspase 3 protein expression levels were quantified in hippocampus of ketamine-treated neonatal rats by western blotting. Our results showed that the protein expression of Bcl-2 was significantly reduced while Bax and cleaved caspase 3 were markedly elevated in the hippocampus of ketamine + vehicle rats, as compared with the control group ($p < 0.001$ for all). Nevertheless, in comparison with the ketamine + vehicle group, CECMF treatment at the three doses (0.75, 1.5 and 3.0 g/kg) could effectively promote the protein expressions of Bcl-2 ($p < 0.01$, $p < 0.01$ and $p < 0.001$, respectively), and reduce the expressions of Bax ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) and cleaved caspase 3 ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively), in the hippocampus of ketamine treated rats. Furthermore, donepezil (5 mg/kg) evidently enhanced the expressions of Bcl-2 ($p < 0.01$), and lessened the expressions of Bax ($p < 0.01$) and cleaved caspase 3 ($p < 0.001$), in the hippocampus of ketamine treated adult rats, when compared with the ketamine + vehicle control.

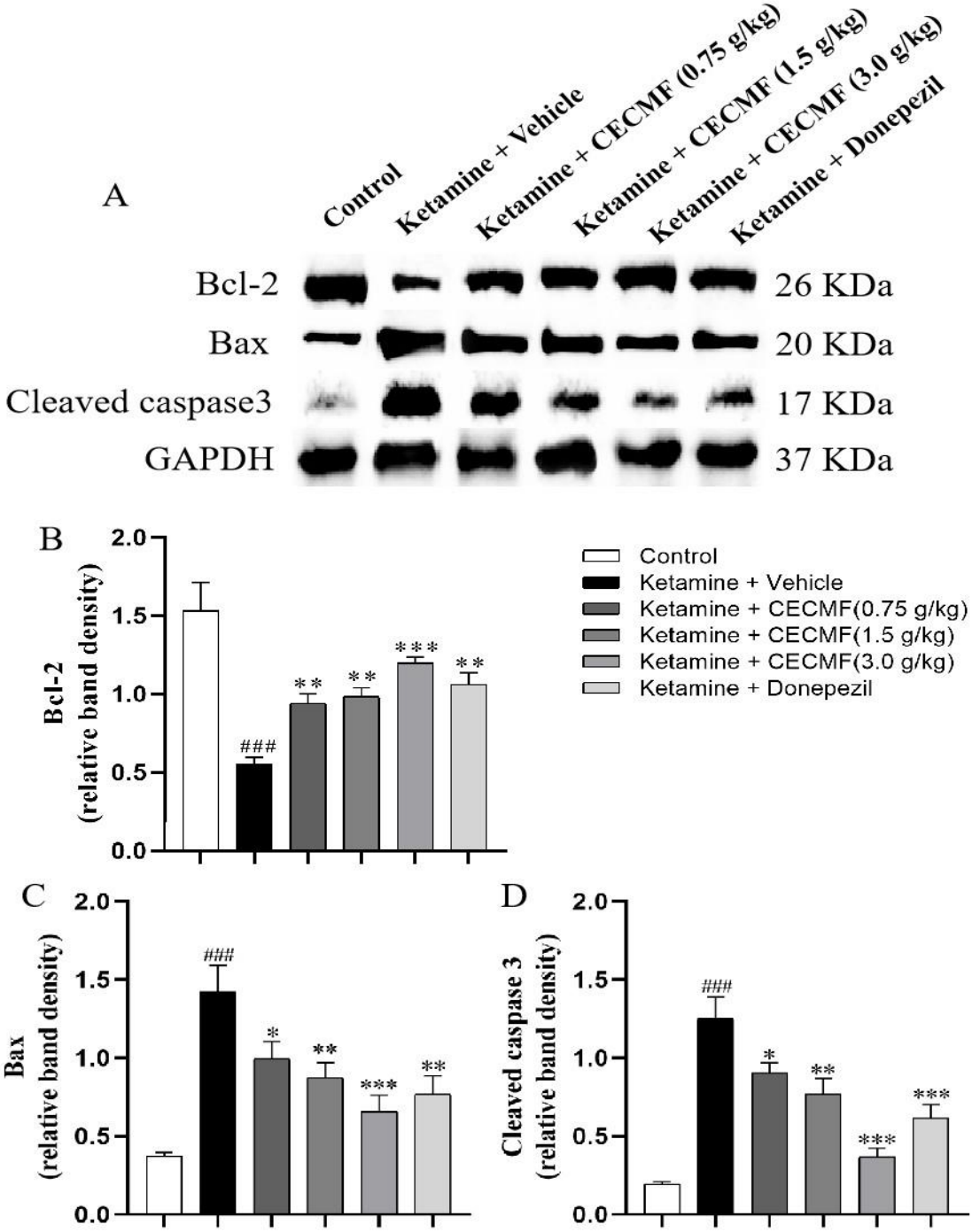


Fig. 10. Effects of CECMF on neuronal apoptosis in the brains of ketamine-treated neonatal rats. (A) Representative western blotting images. Quantitative analysis of the protein expressions of Bcl-2 (B), Bax (C), and cleaved caspase 3 (D). Data were expressed as the mean ± SEM (n = 3). $^{###}p < 0.001$ compared with the control group; $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared with the ketamine + vehicle group.

4.3.4. Effects of CECMF on synaptotoxicity in hippocampus of ketamine-treated neonatal rats.

To evaluate the effects of CECMF (0.75, 1.5 and 3.0 g/kg) on synaptic pathology, we examined the levels of GluN2A, GluN2B, GluA1, GluA2, PSD93, PSD95, SYN1 and SYT, in the hippocampus of ketamine-treated neonatal rats (**Fig. 11**). The results demonstrated that the protein levels of GluN2A, GluN2B, GluA1, GluA2, PSD93, PSD95, SYN1 and SYT were significantly decreased in the hippocampus of ketamine-treated neonatal rats as compared with the control group ($p < 0.001$ for all). Treatment with higher doses of CECMF (1.5 g/kg and 3.0 g/kg) markedly elevated the protein expressions of GluN2B ($p < 0.05$ and $p < 0.001$, respectively) and SYN1 ($p < 0.05$ and $p < 0.01$, respectively) in the hippocampus of ketamine treated rats. Moreover, CECMF treatments at all the three doses (0.75 g/kg, 1.5 and 3.0 g/kg) significantly enhanced the protein expressions of GluN2A ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively), GluA1 ($p < 0.05$, $p < 0.05$ and $p < 0.001$, respectively), GluA2 ($p < 0.01$, $p < 0.01$ and $p < 0.001$, respectively), PSD93 ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively), PSD95 ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively), and SYT ($p < 0.01$, $p < 0.01$ and $p < 0.001$, respectively), in the hippocampus of ketamine treated neonatal rats as compared with the ketamine + vehicle group. Homoplastically, donepezil (5 mg/kg) obviously increased the protein expressions of GluN2A ($p < 0.001$), GluN2B ($p < 0.01$), GluA1 ($p < 0.01$), GluA2 ($p < 0.01$), PSD93 ($p < 0.001$), PSD95 ($p < 0.001$), SYN1 ($p < 0.05$) and SYT ($p < 0.001$), in the brains of ketamine treated rats, as compared with the ketamine + vehicle control.

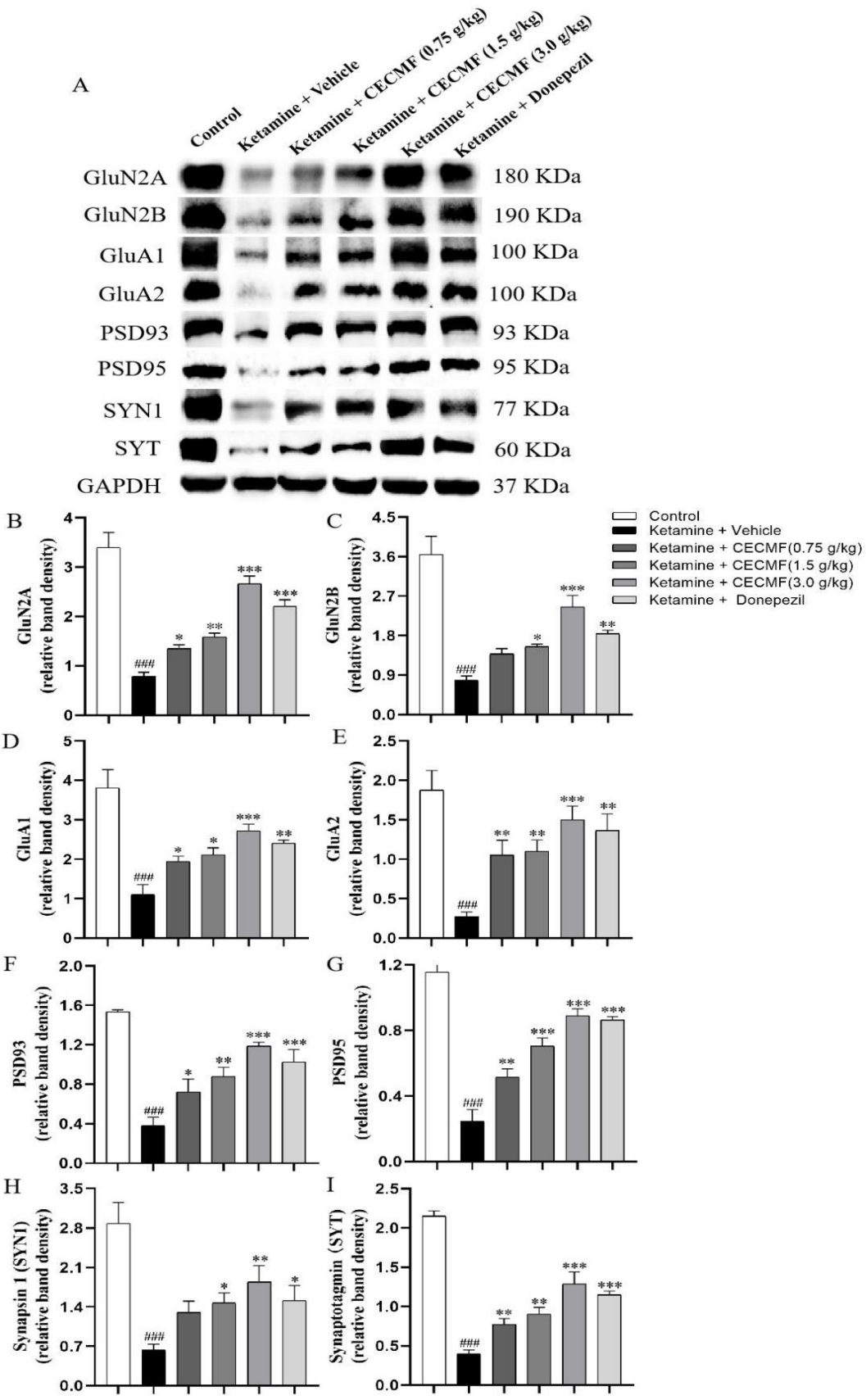

A
Control
Ketamine + Vehicle
Ketamine + CECMF (0.75 g/kg)
Ketamine + CECMF (1.5 g/kg)
Ketamine + CECMF (3.0 g/kg)
Ketamine + Donepezil
GluN2A 180 KDa
GluN2B 190 KDa
GluA1 100 KDa
GluA2 100 KDa
PSD93 93 KDa
PSD95 95 KDa
SYN1 77 KDa
SYT 60 KDa
GAPDH 37 KDa
B
GluN2A (relative band density)
C
GluN2B (relative band density)
D
GluA1 (relative band density)
E
GluA2 (relative band density)
F
PSD93 (relative band density)
G
PSD95 (relative band density)
H
Synapsin 1 (SYN1) (relative band density)
I
Synaptotagmin (SYT) (relative band density)
Control
Ketamine + Vehicle
Ketamine + CECMF(0.75 g/kg)
Ketamine + CECMF(1.5 g/kg)
Ketamine + CECMF(3.0 g/kg)
Ketamine + Donepezil

Fig. 11. Effects of CECMF on synaptotoxicity in the brains of ketamine-treated neonatal rats. (A) Representative western blotting images. Quantitative analysis of the protein expressions of GluN2A (B), GluN2B (C), GluA1 (D), GluA2 (E), PSD93 (F), PSD95 (G), SYN1 (H), and SYT (I). Data were expressed as the mean ± SEM (n = 3). $^{###}p < 0.001$ compared with the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the ketamine + vehicle group.

4.3.5. Effects of CECMF on the CaMKIIβ-ERK1/2-CREB/NF-κB pathway in hippocampus of ketamine-treated neonatal rats.

Our western blot results showed that (**Fig. 12**), the protein expression of p-CaMKIIβ (T287) and p-CREB (S133) were significantly lowered ($p < 0.01$ and $p < 0.001$, respectively) while p-ERK (T202/Y204) and p-p65 were markedly elevated ($p < 0.001$for both) in the brains of ketamine + vehicle neonatal rats, when compared with the control group. However, CECMF treatment at the two doses (1.5 and 3.0 g/kg) could effectively promote the protein expressions of p-CaMKIIβ (T287) ($p < 0.05$ and $p < 0.01$, respectively), and treatment with CECMF at all the three doses (0.75, 1.5 and 3.0 g/kg) could significantly lift the protein expressions of p-CREB (S133) ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively), and decrease the expressions of p-ERK (T202/Y204) ($p < 0.05$, $p < 0.05$ and $p < 0.001$, respectively) and p-p65 ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively), in the hippocampus of ketamine treated rats as compared with the ketamine + vehicle group. Analogously, donepezil (5 mg/kg) evidently increased the expressions of p-CaMKIIβ (T287) ($p < 0.05$) and p-CREB (S133) ($p < 0.001$), and decreased the expressions of p-ERK (T202/Y204) ($p < 0.01$) and p-p65 ($p < 0.01$), in the brains of ketamine treated rats, when compared with the ketamine + vehicle control.

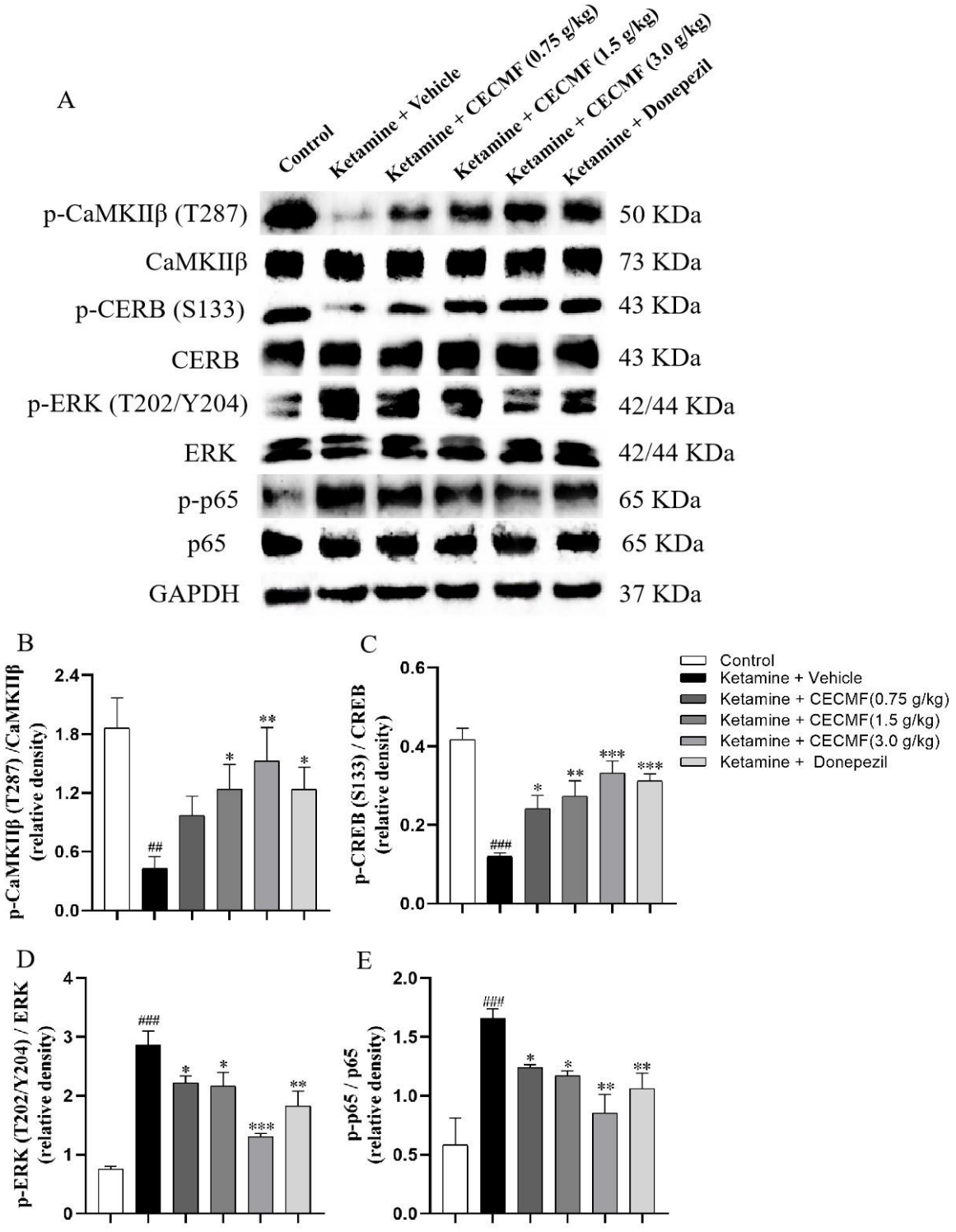


Fig. 12. Effects of CECMF on the CaMKIIβ-ERK1/2-CREB/NF-κB pathway in the brains of ketamine-treated neonatal rats. (A) Representative western blotting images. Quantitative analysis of the protein expressions of p-CaMKIIβ (T287)/CaMKIIβ (B), GluN2B (C), p-

CREB (S133)/CREB (D), p-ERK (T202/Y204)/ERK (E), and p-p65/p65 (F). Data were expressed as the mean ± SEM (n = 3). $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared with the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the ketamine + vehicle group.

4.4. To investigate the effects of CECMF on cystitis induced by ketamine in rats.

4.4.1. Effects of CECMF on urinary frequency of ketamine-treated female rats.

Fig.13A shows a typical example of 6 h urine spotting stains on the impregnated papers in each group at week 8. As shown in **Fig. 13B**, there was no significant difference between all six groups in the value of baseline urinary frequency before ketamine treatment (week 0, $p > 0.05$). At week 4 after ketamine treatment and before CECMF administration, the rats in all the five ketamine groups had significantly increased micturition frequency compared to the normal control group ($p < 0.001$ for all). Moreover, at week 8, the administration of all treatments, including CECMF (1.25, 2.5 and 5.0 g/kg) and parecoxib sodium (COX-2 inhibitor, positive control, 10 mg/kg), effectively relieved frequent micturition ($p < 0.001$ for all), when compared with the ketamine + vehicle group.

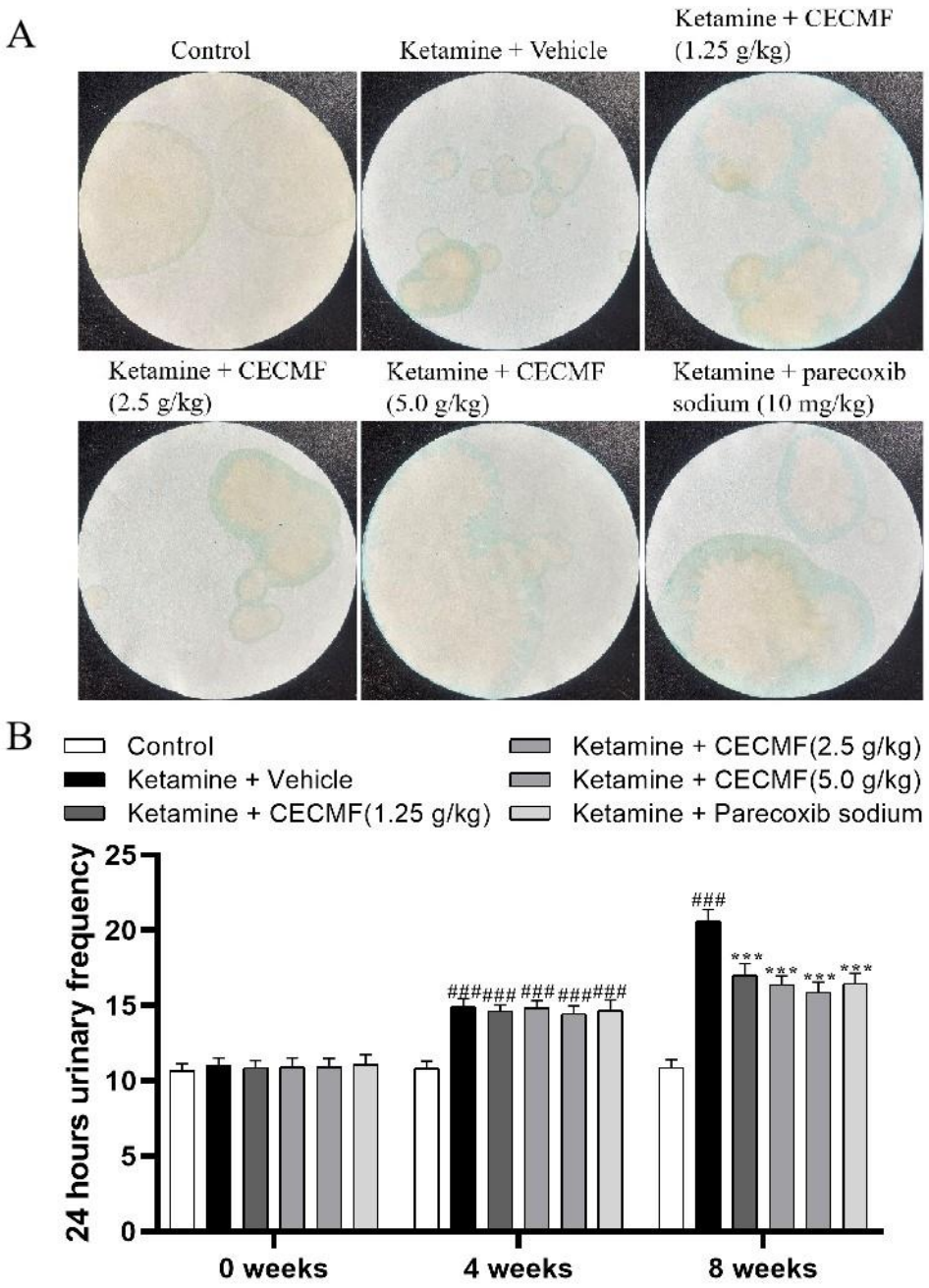


Fig. 13. Effects of CECMF on urinary frequency of ketamine-treated female rats. (A) CuSO4 treated paper sheets were used to show the presence of urine. (B) 24 hours urinary frequency at three consecutive day periods after 0 (baseline), 4, and 8 weeks of treatment. Data were expressed as the mean ± SEM (n = 8). $^{###}p < 0.001$ compared with the control group; $***p < 0.001$ compared with the ketamine + vehicle group.

4.4.2. Effects of CECMF on urine markers (NO, APF, GP51) of ketamine-treated female rats.

As shown in **Fig. 14A-C**, the ketamine + vehicle group had significantly higher levels of urine markers, including NO ($p < 0.001$) and APF ($p < 0.001$) and lower levels of GP51 ($p < 0.001$), than the control group. Nevertheless, administration of CECMF at the doses of 1.25 g/kg, 2.5 g/kg and 5.0 g/kg significantly decreased the elevated levels of urinary NO ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively), APF ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively) and promoted the levels of GP51 ($p < 0.001$ for all) in the rats. On the other hand, parecoxib sodium (10 mg/kg) treatment markedly reduced the levels of NO ($p < 0.01$), APF ($p < 0.001$) and enhanced the levels of GP51 ($p < 0.001$), when compared with the ketamine + vehicle group.

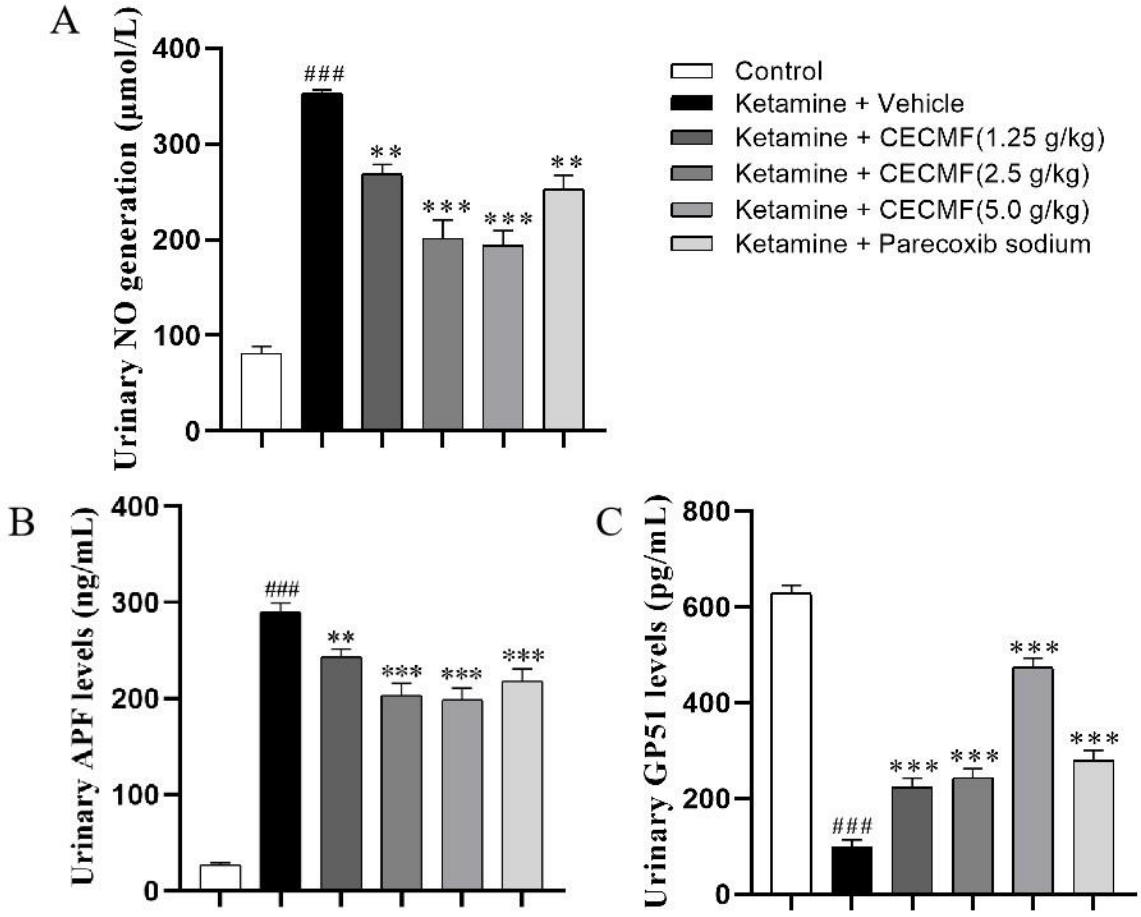
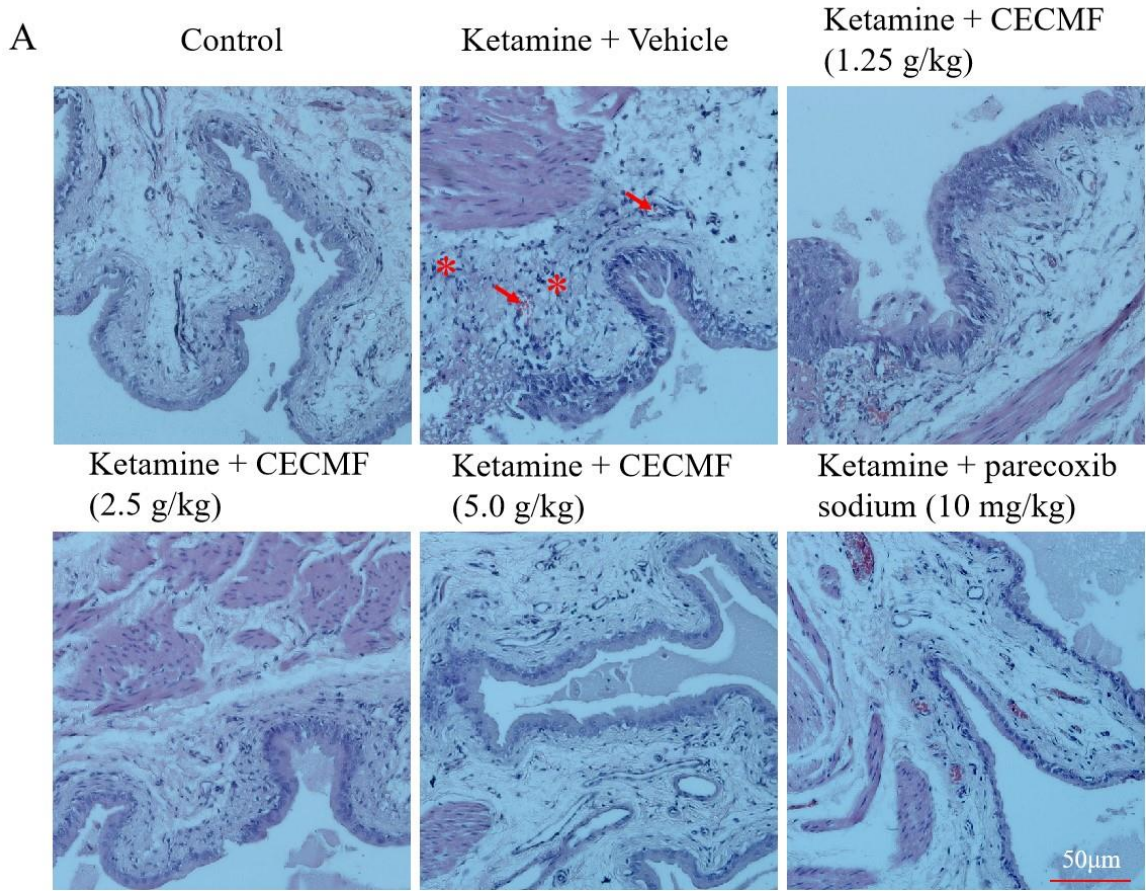


Fig. 14. Effects of CECMF on urine markers of ketamine-treated female rats. (A) NO generation. (B) APF levels. (C) GP51 levels. Data were expressed as the mean ± SEM (n

= 8). $^{###}p < 0.001$ compared with the control group; $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the ketamine + vehicle group.

4.4.3. Effects of CECMF on histological damage in the bladders of ketamine-treated female rats.

H & E staining (**Fig. 15A**) and Masson's trichrome staining (**Fig. 15B**) demonstrated the pathology of ketamine-induced cystitis in rats and the effects of CECMF treatment. In comparison with the control group, markedly higher erythrocyte debris (red arrow), increased mononuclear cells infiltration (asterisk) and increased collagen deposition in the bladder (yellow arrow) were found in ketamine-treated rats, indicating severe inflammatory infiltration (H & E staining) and fibrosis (Masson's trichrome staining) of the bladder. However, rats treated with CECMF (1.25, 2.5 and 5.0 g/kg) and parecoxib sodium (10 mg/kg) showed less monocyte/macrophage infiltration, and less collagen deposition (fibrosis) under ketamine insults.



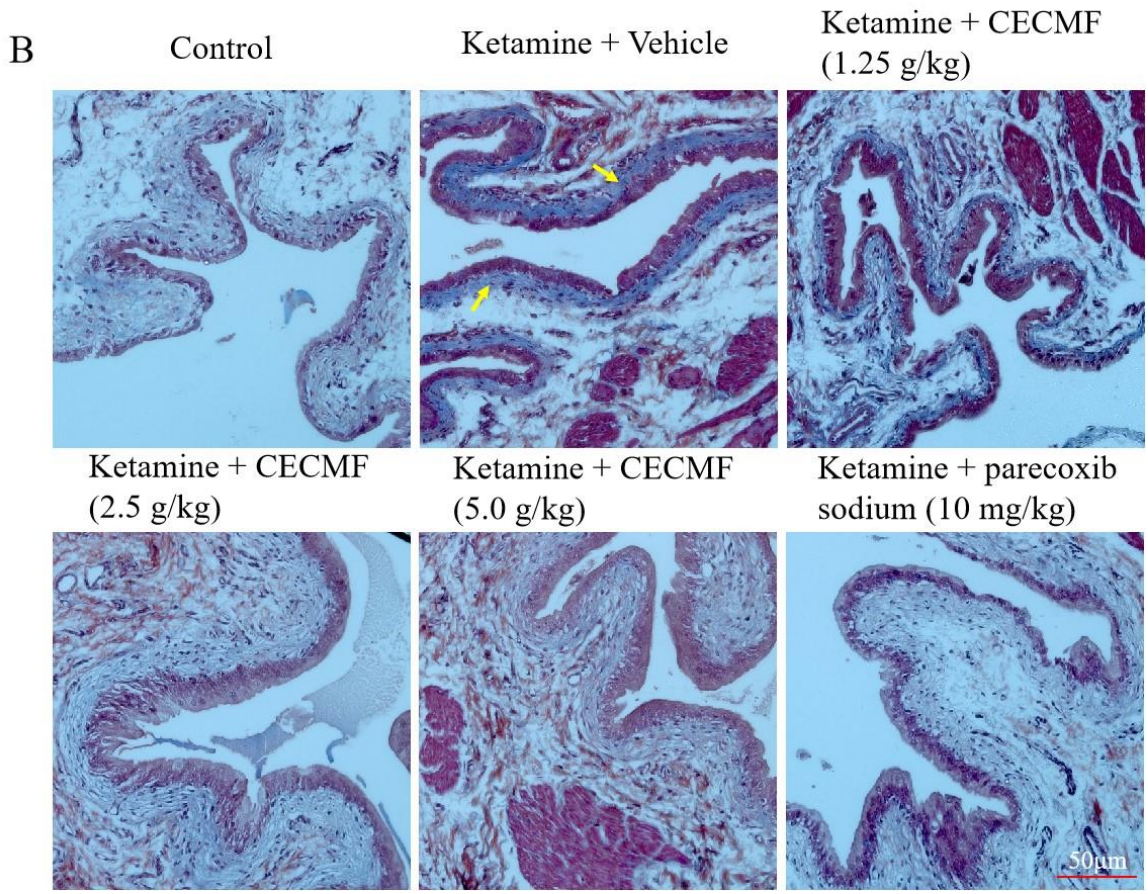
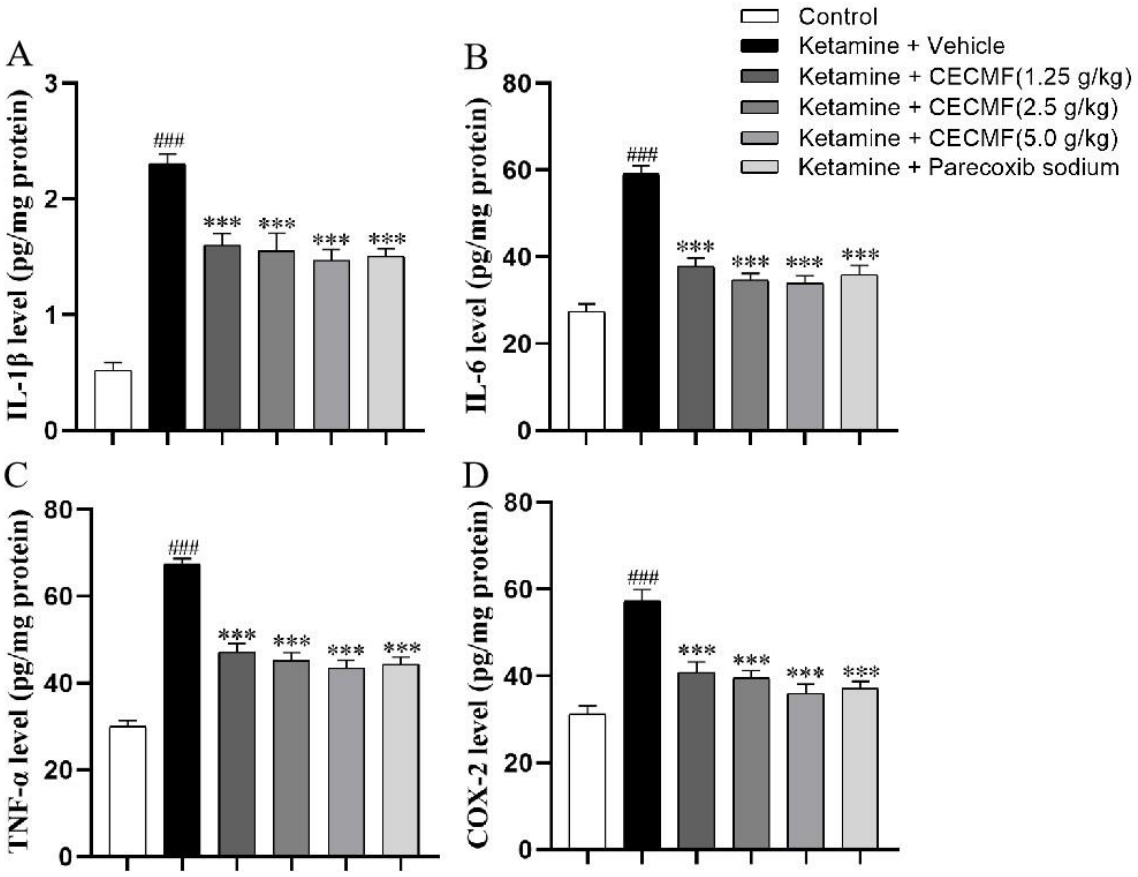


Fig. 15. Effects of CECMF on histological damage in the bladders of ketamine-treated female rats. Histological images of bladders with (A) H&E staining and (B) Masson's trichrome staining.

4.4.4. Effects of CECMF on inflammation and fibrogenesis in the bladders of ketamine-treated female rats.

As shown in **Fig. 16A-D**, the bladder tissues of rats in ketamine + vehicle group had significantly higher levels of inflammatory cytokines, including IL-1β ($p < 0.001$), IL-6 ($p < 0.001$), TNF-α ($p < 0.001$) and COX-2 ($p < 0.001$), than the control group. However, administration of CECMF at the doses of 1.25 g/kg, 2.5 g/kg and 5.0 g/kg significantly decreased the elevated levels of cystic IL-1β ($p < 0.001$ for all), IL-6 ($p < 0.001$ for all), TNF-α ($p < 0.001$ for all) and COX-2 ($p < 0.001$ for all) in the rats. Treatment with parecoxib sodium (10 mg/kg) also markedly reduced the levels of IL-1β ($p < 0.001$), IL-6 ($p < 0.001$), TNF-α ($p < 0.001$) and COX-2 ($p < 0.001$), when compared with the ketamine + vehicle group.

As depicted in **Fig. 16E-J**, the protein expression of ICAM-1, TGF-β1, Collagen I, Collagen III and Fibronectin were significantly elevated ($p < 0.001$) in the bladders of ketamine + vehicle female rats, as compared with the control group. However, treatment with CECMF at the low dose (1.25 g/kg) could significantly lessen the protein expressions of ICAM-1 ($p < 0.05$) and Collagen III ($p < 0.001$) in the bladders of ketamine treated rats as compared with the ketamine + vehicle group. Treatment with CECMF at the higher two doses (2.5 and 5.0 g/kg) could significantly reduce the protein expressions of ICAM-1 ($p < 0.01$ and $p < 0.001$, respectively), TGF-β1 ($p < 0.01$ and $p < 0.001$, respectively) , Collagen I ($p < 0.05$ and $p < 0.001$, respectively), Collagen III ($p < 0.001$ for both), and Fibronectin ($p < 0.01$ and $p < 0.001$, respectively) in the bladders of ketamine treated rats as compared with the ketamine + vehicle group. Coordinately, parecoxib sodium (10 mg/kg) treatment evidently decreased the cystic expressions of ICAM-1 ($p < 0.05$), TGF-β1 ($p < 0.01$), Collagen I ($p < 0.01$), Collagen III ($p < 0.001$) and Fibronectin ($p < 0.01$), in the bladders of ketamine treated rats, as compared with the ketamine + vehicle control group.



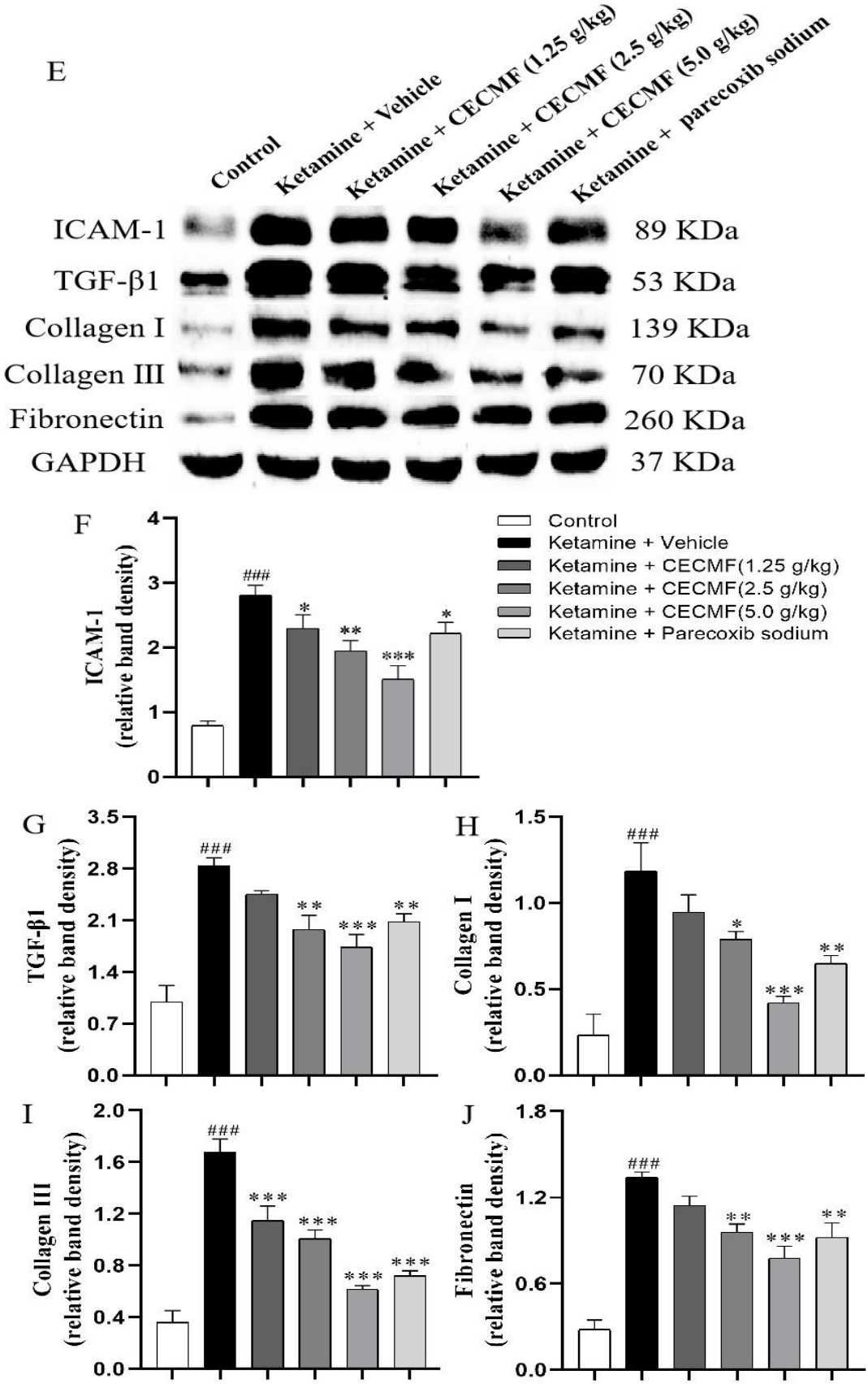

E
Control
Ketamine + Vehicle
Ketamine + CECMF (1.25 g/kg)
Ketamine + CECMF (2.5 g/kg)
Ketamine + CECMF (5.0 g/kg)
Ketamine + parecoxib sodium
ICAM-1
TGF-β1
Collagen I
Collagen III
Fibronectin
GAPDH
89 KDa
53 KDa
139 KDa
70 KDa
260 KDa
37 KDa
F
ICAM-1 (relative band density)
Control
Ketamine + Vehicle
Ketamine + CECMF(1.25 g/kg)
Ketamine + CECMF(2.5 g/kg)
Ketamine + CECMF(5.0 g/kg)
Ketamine + Parecoxib sodium
G
TGF-β1 (relative band density)
H
Collagen I (relative band density)
I
Collagen III (relative band density)
J
Fibronectin (relative band density)

Fig. 16. Effects of CECMF on inflammatory cytokines and fibrogenesis of ketamine-treated female rats. (A) IL-1β, (B) IL-6, (C) TNF-α and (D) COX-2 levels were examined by ELISA (n = 8). (E) Representative western blotting images. Quantitative analysis of the protein expressions of (F) ICAM-1, (G) TGF-β1, (H) Collagen I, (I) Collagen III and (J) Fibronectin (n = 3). Data were expressed as the mean ± SEM. $^{###}p < 0.001$ compared with the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the ketamine + vehicle group.

4.4.5. Effects of CECMF on neuroreceptors in the bladders of ketamine-treated female rats.

To evaluate the effects of CECMF (1.25, 2.5 and 5.0 g/kg) on urothelial neuroreceptors alteration, we examined the levels of TRPV1, M2-mAChR, M3-mAChR, purinergic receptor P2X1, P2X2, and P2X3, in the bladders of ketamine-treated rats (**Fig. 17**). The results demonstrated that the vesical protein levels of TRPV1, M2-mAChR, M3-mAChR, P2X1, P2X2, and P2X3 were significantly higher than that in the control group ($p < 0.001$ for all). Treatment with CECMF (1.25, 2.5 and 5.0 g/kg) significantly attenuated the protein expressions of TRPV1 ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively), M2-mAChR ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively), M3-mAChR ($p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively), P2X1 ($p < 0.001$ for three), P2X2 ($p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively), and P2X3 ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively), in the bladders of ketamine treated rats as compared with the ketamine + vehicle group. Moreover, parecoxib sodium (10mg/kg) treatment also notably decreased the protein expressions of TRPV1 ($p < 0.001$), M2-mAChR ($p < 0.01$), M3-mAChR ($p < 0.001$), P2X1 ($p < 0.001$), P2X2 ($p < 0.01$) and P2X3 ($p < 0.001$), in the bladders of ketamine treated rats, when compared with the ketamine + vehicle control group.

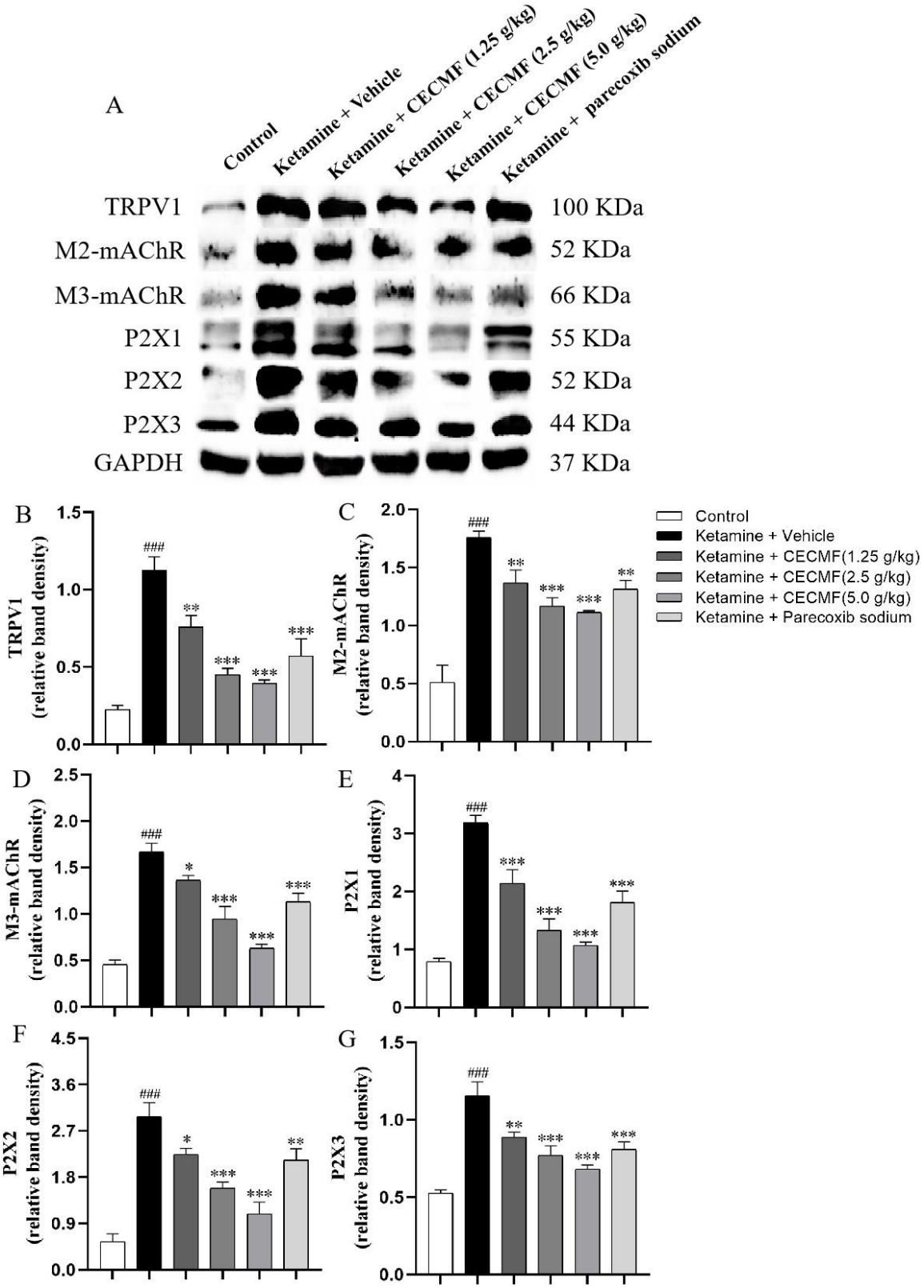


Fig. 17. Effects of CECMF on neuroreceptors in the bladders of ketamine-treated female

rats. (A) Representative western blotting images. Quantitative analysis of the protein expressions of TRPV1 (B), M2-mAChR (C), M3-mAChR (D), P2X1 (E), P2X2 (F) and P2X3 (G). Data were expressed as the mean ± SEM (n = 3). $^{###}p < 0.001$ compared with the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the ketamine + vehicle group.

4.4.6. Effects of CECMF on Nrf2/HO-1 and NF-κB pathways in the bladders of ketamine-treated female rats.

Our western blot results revealed that (**Fig. 18**), the protein expression of HO-1 and Nrf2 were significantly lowered ($p < 0.001$ for both) while p-p65 and p-IκBα were vividly boosted ($p < 0.001$for both) in the bladder of ketamine + vehicle rats, when compared with the control group. However, treatment with CECMF at all the three doses (1.25, 2.5 and 5.0 g/kg) could signally enhance the protein expressions of HO-1 ($p < 0.01$, $p < 0.01$ and $p < 0.001$, respectively) and Nrf2 ($p < 0.001$ for three), and deaden the expressions of p-p65 ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively) and p-IκBα ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively), in the bladders of ketamine treated rats as compared with the ketamine + vehicle group. Simultaneously, parecoxib sodium (10 mg/kg) clearly lifted the expressions of HO-1 ($p < 0.01$) and Nrf2 ($p < 0.001$), and weaken the expressions of p-p65 ($p < 0.001$) and p-IκBα ($p < 0.001$), in the bladders of ketamine treated rats, when compared with the ketamine + vehicle control group.

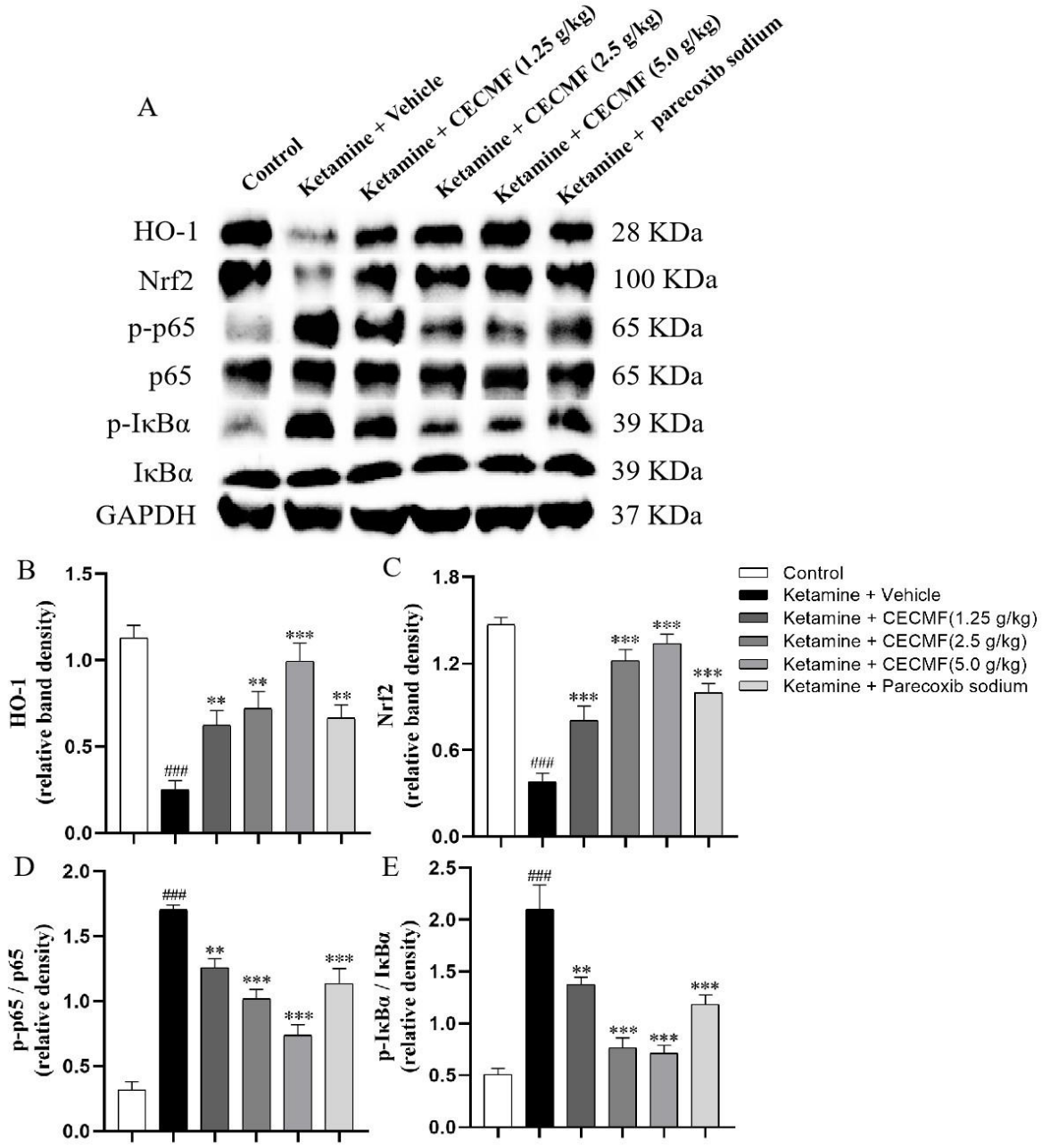


Fig. 18. Effects of CECMF on the Nrf2/HO-1 and NF-κB pathways in the bladders of ketamine-treated female rats. (A) Representative western blotting images. Quantitative analysis of the protein expressions of HO-1 (B), Nrf2 (C), p-p65/p65 (D), and p-IκBα/IκB (E). Data were expressed as the mean ± SEM (n = 3). $^{###}p < 0.001$ compared with the control group; $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the ketamine + vehicle group.

4.5. Acute toxicity of CECMF in mice

The results on its acute toxicity demonstrated that CECMF at up to the dose of 128 g/kg, which was the maximum tolerable dose of CECMF in mice, did not exert any overt toxicity.

4.6. Sub-chronic toxicity assessment of CECMF extract

No mortality or morbidity was observed in this study. Clinical and neurobehavioral observations, ophthalmological examination of the skin, fur, mucous membranes, occurrence of secretions and excretions, and autonomic activity did not reveal any abnormal effects in the animals during the 90-day and 30-day post-90-day study period.

4.6.1. Influence of CECMF on body weight of rats

There was no statistical difference in weekly body weight between the control and experimental groups for either sex ($p > 0.05$ for all) (**Fig. 19**). The weight gain of male and female rats displayed similar trends.

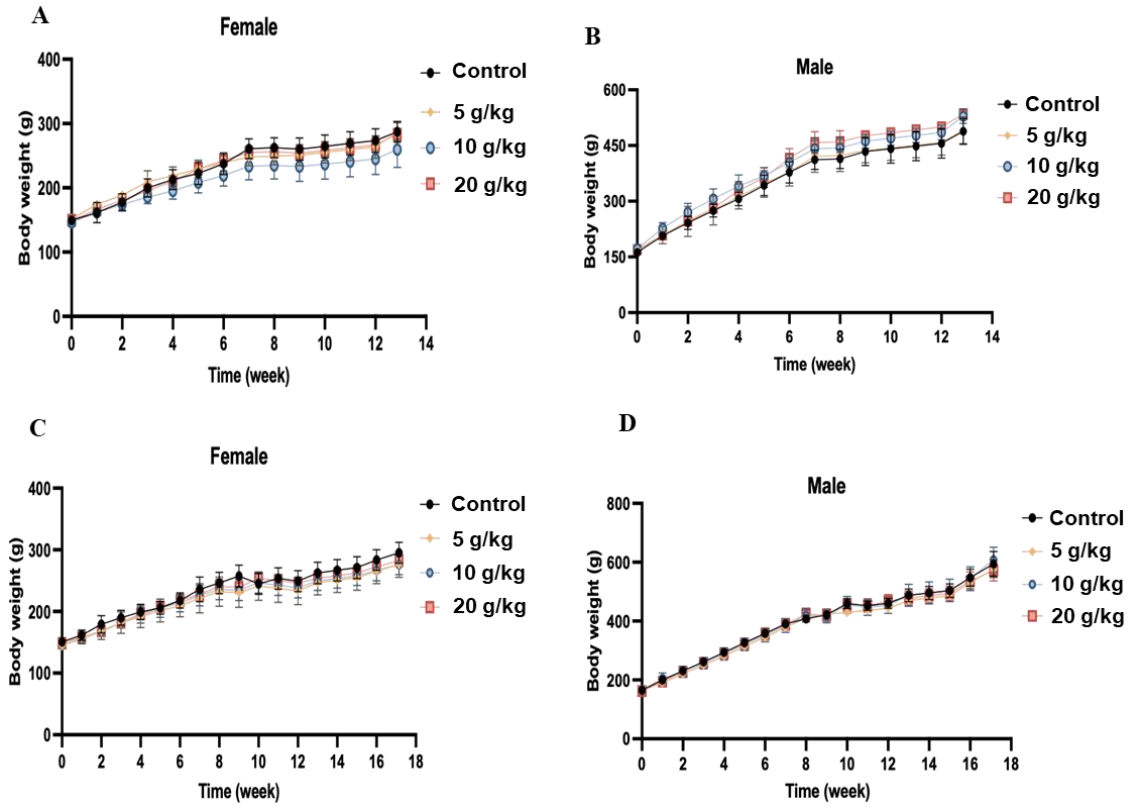


Fig. 19. Effect of repeated administration of CECMF on body weight of rats. (A and B) Body weights of 90-day-treated male and female rats (n = 5; mean ± SD). (C and D) Body weights of male and female rats 30 days after 90-day treated (n = 5; mean ± SD).

4.6.2. Influence of CECMF on the relative organ weights

The results of the gross examination did not reveal any abnormal changes or palpable lesions in any of the animals. The relative organ weights of Brain, Heart, Thymus, Spleen, Adrenals, Ovaries, Uterus, Testes, Epididymides, Kidney, Lung, Liver, and Pancreas were determined for all rats. There was no statistical difference in all relative organ weights between the control and experimental groups for either sex ($p > 0.05$ for all). (**Tables 2 and 3**).

Table 2. Effects of CECMF on the relative organ weights after treatment female and male rats for 90 days

	Organ	Control	CECMF (5 g/kg)	CECMF (10 g/kg)	CECMF (20 g/kg)
Female	**Brain**	0.67 ± 0.04	0.66 ± 0.04	0.71 ± 0.08	0.67 ± 0.03
	Heart	0.36 ± 0.02	0.33 ± 0.02	0.33 ± 0.06	0.35 ± 0.03
	Thymus	0.11 ± 0.04	0.13 ± 0.02	0.14 ± 0.02	0.13 ± 0.02
	Spleen	0.17 ± 0.05	0.14 ± 0.01	0.17 ± 0.04	0.19 ± 0.01
	Adrenals	0.07 ± 0.10	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
	Ovaries	0.05 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.00
	Uterus	0.19 ± 0.02	0.20 ± 0.06	0.25 ± 0.11	0.18 ± 0.02
	Kidney	0.75 ± 0.04	0.77 ± 0.02	0.75 ± 0.04	0.75 ± 0.04
	Lung	0.50 ± 0.10	0.45 ± 0.05	0.46 ± 0.06	0.45 ± 0.05
	Liver	3.53 ± 0.24	3.51 ± 0.23	3.65 ± 0.27	3.54 ± 0.06
	Pancreas	0.20 ± 0.05	0.21 ± 0.03	0.26 ± 0.08	0.22 ± 0.05
Male	**Brain**	0.40 ± 0.01	0.41 ± 0.03	0.38 ± 0.03	0.39 ± 0.02
	Heart	0.29 ± 0.02	0.31 ± 0.01	0.30 ± 0.02	0.30 ± 0.02
	Thymus	0.09 ± 0.02	0.07 ± 0.01	0.07 ± 0.02	0.06 ± 0.00
	Spleen	0.12 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.15 ± 0.02
	Adrenals	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.04 ± 0.06
	Testes	0.73 ± 0.04	0.70 ± 0.05	0.66 ± 0.06	0.70 ± 0.05
	Epididymides	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Kidney	0.70 ± 0.04	0.78 ± 0.13	0.71 ± 0.03	0.75 ± 0.02
	Lung	0.36 ± 0.01	0.37 ± 0.03	0.38 ± 0.04	0.35 ± 0.03
	Liver	3.86 ± 0.24	2.74 ± 1.35	3.84 ± 0.23	3.74 ± 0.10
	Pancreas	0.19 ± 0.06	0.17 ± 0.03	0.17 ± 0.06	0.17 ± 0.01

(mean ± SD, n=5)

Table 3. Effects of CECMF on the relative organ weights after treatment female and male rats for 90 days with 30 post drug withdraw

	Organ	Control	CECMF (5 g/kg)	CECMF (10 g/kg)	CECMF (20 g/kg)
Female	**Brain**	0.61 ± 0.10	0.67 ± 0.06	0.71 ± 0.03	0.66 ± 0.03
	Heart	0.33 ± 0.03	0.35 ± 0.03	0.34 ± 0.01	0.33 ± 0.01
	Thymus	0.14 ± 0.09	0.10 ± 0.04	0.09 ± 0.02	0.11 ± 0.06
	Spleen	0.14 ± 0.01	0.15 ± 0.02	0.16 ± 0.01	0.16 ± 0.02
	Adrenals	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
	Ovaries	0.06 ± 0.01	0.05 ± 0.01	0.07 ± 0.03	0.06 ± 0.01
	Uterus	0.23 ± 0.05	0.28 ± 0.09	0.33 ± 0.11	0.23 ± 0.10
	Kidney	0.72 ± 0.05	0.76 ± 0.05	0.77 ± 0.04	0.80 ± 0.04
	Lung	0.44 ± 0.03	0.46 ± 0.07	0.54 ± 0.08	0.50 ± 0.07
	Liver	3.36 ± 0.16	3.68 ± 0.22	3.60 ± 0.18	3.48 ± 0.12
	Pancreas	0.22 ± 0.05	0.25 ± 0.06	0.19 ± 0.06	0.24 ± 0.05
Male	**Brain**	0.36 ± 0.04	0.37 ± 0.02	0.35 ± 0.02	0.36 ± 0.02
	Heart	0.27 ± 0.02	0.26 ± 0.01	0.28 ± 0.04	0.27 ± 0.01
	Thymus	0.08 ± 0.03	0.07 ± 0.04	0.08 ± 0.02	0.09 ± 0.05
	Spleen	0.13 ± 0.02	0.14 ± 0.02	0.14 ± 0.03	0.14 ± 0.02
	Adrenals	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Testes	0.64 ± 0.08	0.70 ± 0.06	0.61 ± 0.03	0.66 ± 0.07
	Epididymides	0.27 ± 0.04	0.28 ± 0.03	0.26 ± 0.03	0.26 ± 0.04
	Kidney	0.70 ± 0.04	0.71 ± 0.02	0.59 ± 0.13	0.66 ± 0.06
	Lung	0.35 ± 0.04	0.35 ± 0.04	0.35 ± 0.05	0.34 ± 0.07
	Liver	3.46 ± 0.35	3.40 ± 0.23	3.33 ± 0.24	3.17 ± 0.34
	Pancreas	0.13 ± 0.03	0.13 ± 0.02	0.12 ± 0.01	0.14 ± 0.03

(mean ± SD, n=5)

4.6.3. Influence of CECMF on the biochemical indices

Hematological examination did not reveal any noticeable findings or treatment-associated effects in all experimental groups ($p > 0.05$ for all) (**Tables 4 & 5**).

Table 4. Effects of CECMF on the hematological analysis after treatment rats for 90 days.

Parameter	Control		CECMF (5 g/kg)		CECMF (10 g/kg)		CECMF (20 g/kg)	
Sex	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
RBC	8.30±0.26	8.75±0.19	8.49±0.31	8.70 ± 0.17	8.75±0.11	8.83±0.32	8.55±0.26	8.69±0.17
Haemoglobin	141.2±2.64	141.2±2.93	146.80±3.12	142.6±1.96	148.40±2.24	140.2±4.58	147.4±2.87	138.6±2.6
Haematocrit	46.08±0.96	48.56±1.14	47.60±1.79	48.84±0.98	47.52±1.34	48.26±1.87	47.64±1.53	47.58±0.35
MCV	55.54±0.68	55.54±0.88	56.06±0.63	56.20±1.04	54.30±0.94	54.62±0.56	55.76±0.87	54.74±0.74
MCH	17.04±0.47	16.16±0.15	17.28±0.31	16.40±0.23	16.96±0.08	15.86±0.14	17.26±0.26	15.98±0.15
MCHC	306.80±6.05	290.60±5.35	308.60±5.85	291.80±3.87	312.40±4.54	290.8±2.71	309.4±3.80	291.6±3.80
% Reticulocyte	3.34±0.14	3.13 ± 0.65	2.82±0.30	3.20±0.48	2.84±0.60	3.06±0.37	2.92±0.19	3.53±0.36
Reticulocyte Count	277.60±11.36	217.0±117.6	237.80±18.40	222.4±115.5	248.6±56.00	270.7±31.8	250.1±20.50	245.0±124.20
WBC	3.49±2.57	7.39±3.31	2.84 ± 1.74	8.66±2.60	3.64±1.24	8.66±2.19	2.24±1.15	9.42±1.35
% Neutrophil	18.03±3.89	14.78±4.62	15.26 ± 5.05	18.88±2.71	11.60±3.14	18.30±3.45	18.64±5.32	15.86±1.82
% Lymphocyte	71.40±10.49	82.22±5.38	79.38 ± 7.14	77.20±3.87	85.22 ± 3.64	78.32±3.29	74.66±5.83	80.54±1.62
% Monocyte	1.74 ± 0.31	1.10 ± 0.38	1.76 ± 0.65	1.26 ± 0.35	0.94 ± 0.43	1.22±0.42	1.92 ± 0.58	1.08 ± 0.17
% Eosinophil	0.84 ± 0.47	0.64 ± 0.42	1.10 ± 0.57	0.62 ± 0.21	0.96 ± 0.22	0.62±0.20	1.85 ± 0.46	0.76 ± 0.36
% Basophil	1.22 ± 0.68	0.30 ± 0.23	0.96 ± 0.77	0.32 ± 0.15	0.22 ± 0.07	0.30±0.06	1.12 ± 0.83	0.34 ± 0.14
% Large unstained cells	1.76 ± 0.48	1.00 ± 0.36	1.48 ± 0.70	1.74 ± 1.43	1.04 ± 0.24	1.16 ± 0.42	1.94 ± 0.63	1.38 ± 0.18
Neutrophil Count	1.06 ± 1.32	0.97 ± 0.33	0.38 ± 0.16	1.77 ± 0.64	0.44 ± 0.23	1.53 ± 0.35	0.39 ± 0.17	1.46 ± 0.26
Lymphocyte Count	2.45 ± 1.25	6.22 ± 2.94	2.33 ± 1.55	6.50 ± 1.71	3.08 ± 0.98	6.83 ± 1.84	1.72 ± 0.98	7.62 ± 1.11
Monocyte Count	0.07 ± 0.06	0.08 ± 0.04	0.05 ± 0.03	0.11 ± 0.07	0.04 ± 0.03	0.11 ± 0.05	0.04 ± 0.02	0.11 ± 0.01
Eosinophil Count	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.02	0.04 ± 0.02	0.06 ± 0.03	0.03 ± 0.01	0.06 ± 0.03
Basophil Count	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.01 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
Large unstained cells Count	0.06 ± 0.05	0.08 ± 0.05	0.04 ± 0.03	0.07 ± 0.02	0.04 ± 0.02	0.11 ± 0.05	0.04 ± 0.01	0.14 ± 0.03
Platelet	872±130	1053±195	925±152	1054±134	816±94	1119±77	887±121	1143±80

(mean ± SD, n=5)

Table 5. Effects of CECMF on the hematological analysis after treatment rats for 90 days with 30 drug.

Parameter	Control		CECMF (5 g/kg)		CECMF (10 g/kg)		CECMF (20 g/kg)	
Sex	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
RBC	8.29 ± 0.20	8.46 ± 0.24	8.66 ± 0.23	8.85 ± 0.19	8.67 ± 0.34	8.47 ± 0.28	8.29 ± 0.43	8.38 ± 0.28
Haemoglobin	144.40 ± 2.94	145.20 ± 3.12	150.20 ± 4.45	146.00 ± 3.29	152.00 ± 5.48	139.80 ± 5.04	146.8±6.68	141.40 ± 4.13
Haematocrit	45.98 ± 1.03	47.90 ± 1.18	47.32 ± 1.45	49.34 ± 1.39	47.50 ± 2.04	47.38 ± 1.40	45.28 ± 2.37	46.98 ± 1.89
MCV	55.46 ± 1.00	56.68 ± 0.66	54.68 ± 0.68	55.74 ± 1.08	54.80 ± 0.66	55.96 ± 0.60	54.60 ± 0.44	56.08 ± 0.93
MCH	17.44 ± 0.23	17.14 ± 0.34	17.38 ± 0.10	16.50 ± 0.22	17.54 ± 0.20	16.52 ± 0.29	17.72 ± 0.25	16.90 ± 0.33
MCHC	314.40 ± 2.58	303.00 ± 4.34	317.60 ± 2.65	296.00 ± 3.79	320.40 ± 2.94	295.20 ± 5.31	324.2±4.26	301.60 ± 5.08
% Reticulocyte	2.28 ± 0.46	3.54 ± 0.64	2.32 ± 0.40	3.30 ± 0.17	2.52 ± 0.40	3.88 ± 0.53	2.08 ± 0.52	3.34 ± 0.36
Reticulocyte Count	187.78±39.58	297.98±45.73	199.64±32.33	291.68±13.06	216.16±29.85	326.96±41.67	172.6±38.3	278.74±28.02
WBC	2.18 ± 0.59	6.75 ± 1.80	3.31 ± 0.67	8.41 ± 0.83	3.54 ± 0.55	9.23 ± 1.44	3.53 ± 0.83	7.16 ± 2.27
% Neutrophil	19.52 ± 7.56	16.38 ± 2.43	19.80 ± 10.33	17.52 ± 1.88	22.00 ± 4.01	23.20 ± 5.15	21.38 ± 4.34	25.56 ± 9.84
% Lymphocyte	74.86 ± 9.75	79.46 ± 2.56	74.32 ± 10.86	79.12 ± 2.10	72.98 ± 4.18	73.22 ± 5.40	73.72 ± 4.44	70.38±10.44
% Monocyte	1.44 ± 0.39	1.22 ± 0.30	2.43 ± 0.39	1.36 ± 0.26	2.00 ± 0.19	1.28 ± 0.40	2.10 ± 0.30	1.76 ± 0.47
% Eosinophil	1.27 ± 0.12	0.80 ± 0.25	1.14 ± 1.01	0.48 ± 0.21	1.72 ± 0.43	0.66 ± 0.31	1.58 ± 0.29	0.68 ± 0.17
% Basophil	0.30 ± 0.14	0.40 ± 0.16	0.22 ± 0.12	0.38 ± 0.13	0.34 ± 0.12	0.28 ± 0.07	0.32 ± 0.25	0.24 ± 0.10
% Large unstained cells	0.92 ± 0.38	1.52 ± 0.40	0.64 ± 0.39	1.16 ± 0.26	0.94 ± 0.10	1.32 ± 0.21	0.94 ± 0.30	1.36 ± 0.54
Neutrophil Count	0.38 ± 0.05	1.07 ± 0.21	0.67 ± 0.39	1.48 ± 0.25	0.79 ± 0.27	2.20 ± 0.81	0.76 ± 0.25	1.85 ± 1.00
Lymphocyte Count	1.68 ± 0.64	5.40 ± 1.50	2.46 ± 0.69	6.65 ± 0.62	2.57 ± 0.29	6.70 ± 0.71	2.60 ± 0.62	5.01 ± 1.81
Monocyte Count	0.03 ± 0.01	0.08 ± 0.04	0.10 ± 0.02	0.11 ± 0.03	0.07 ± 0.01	0.12 ± 0.05	0.07 ± 0.02	0.13 ± 0.06
Eosinophil Count	0.05 ± 0.03	0.05 ± 0.03	0.04 ± 0.04	0.04 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.05 ± 0.01	0.05 ± 0.01
Basophil Count	0.01 ± 0.00	0.04 ± 0.02	0.01 ± 0.01	0.03 ± 0.01	0.01 ± 0.00	0.03 ± 0.00	0.01 ± 0.01	0.02 ± 0.01
Large unstained cells Count	0.02 ± 0.01	0.11 ± 0.05	0.02 ± 0.01	0.10 ± 0.02	0.03 ± 0.01	0.12 ± 0.02	0.03 ± 0.01	0.10 ± 0.05
Platelet	824.6±243.1	1061.0±61.8	898.6±170.9	1216.2±154.3	969.40±133.2	1114.2±138.7	850.8±125.4	1207.4±129.4

(mean ± SD, n=5)

4.6.4. Effects of CECMF on serum parameters of the rats of sub-chronic toxicity study

After 90-day oral administration of CECMF, results of the biochemical analysis showed no significant differences among the parameters in the CECMF treated groups compared to their respective control groups, both in the 90-day drug treatment experiment ($p > 0.05$ for all) (**Fig. 20A-F**) and 30-day drug withdrawal experiment ($p > 0.05$ for all) (**Fig. 21A-F**). Moreover, no significant changes were observed in male and female rats treated with CECMF (5, 10 and 20 g/kg), either in the 90-day drug treatment or 30-day drug withdrawal experiments in the serum parameters of rats such as AST, ALT, ATP, CRE, TBL and BUN, when compared with the control group ($p > 0.05$ for all).

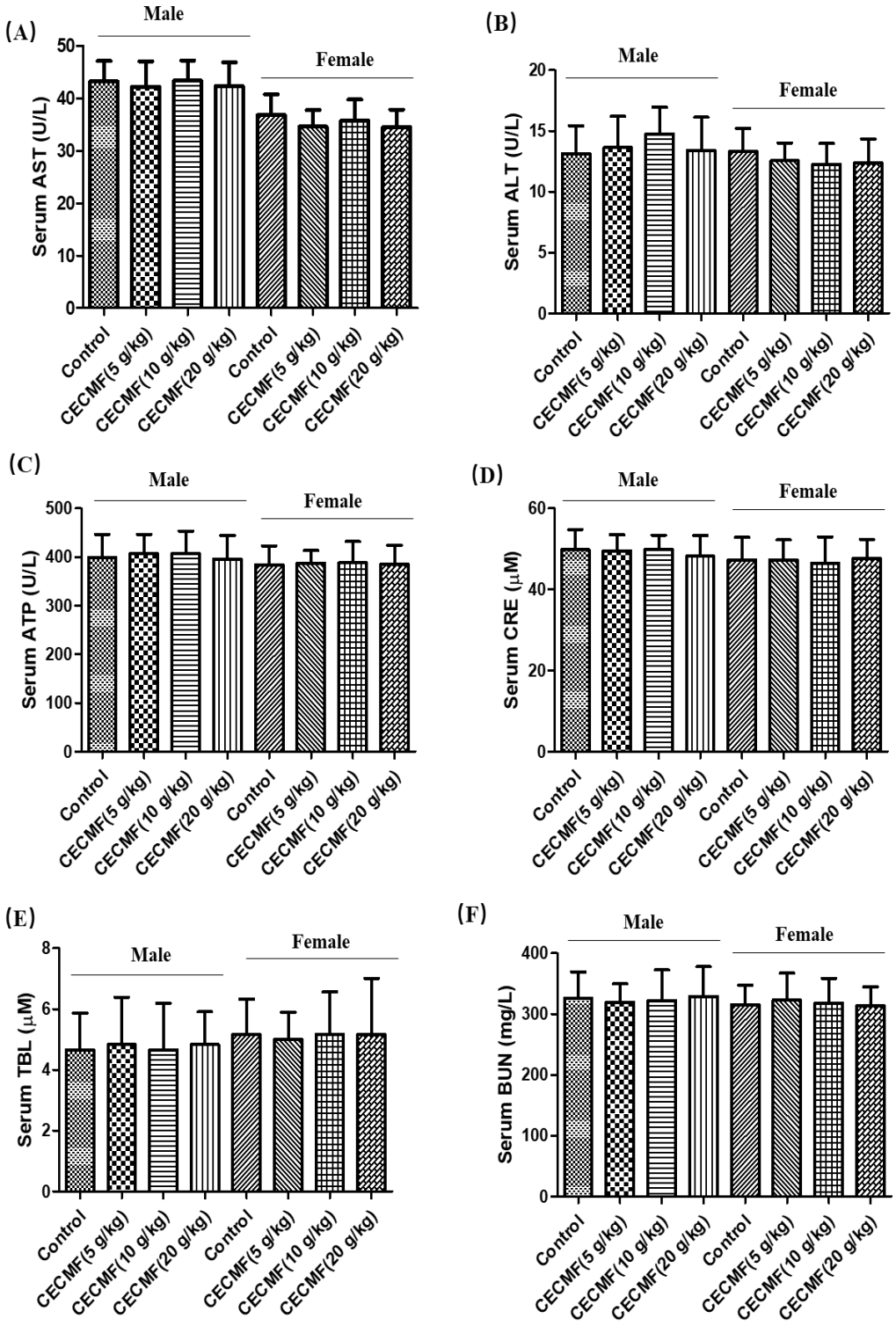


Fig. 20. Effects of CECMF on the serum parameters of the rats in the 90-day drug treatment experiment of sub-chronic toxicity study. (A) AST level, (B) ALT level, (C) ATP level, (D) CRE level, (E) TBL level and (F) BUN level. Data were presented as mean ± SEM (n = 8).

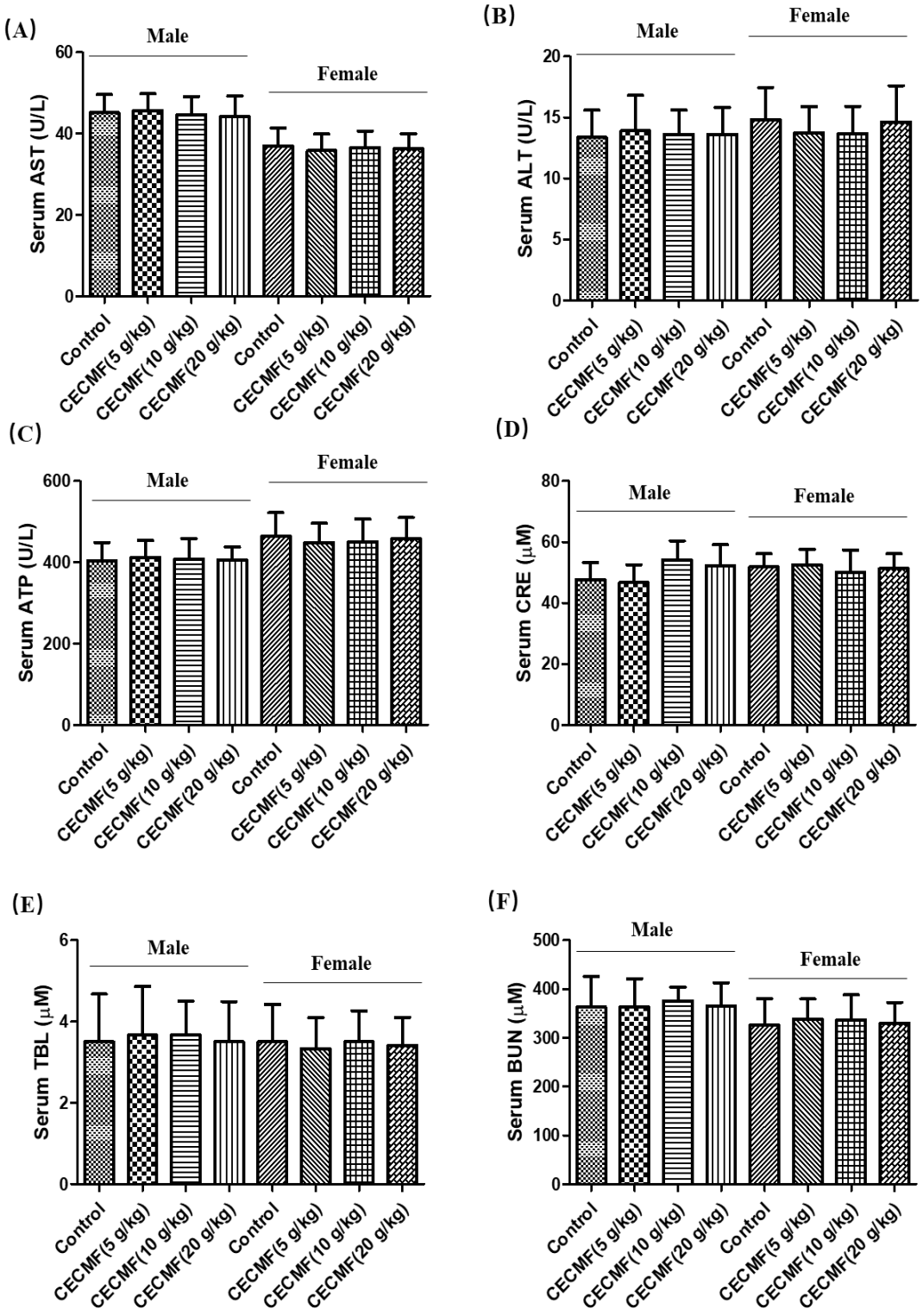


Fig. 21. Effects of CECMF on serum parameters of the rats in the 30-day drug withdrawal experiment of sub-chronic toxicity study. (A) AST level, (B) ALT level, (C) ATP level, (D) CRE level, (E) TBL level and (F) BUN level. Data were presented as mean ± SEM (n = 8).

4.6.5. Effects of CECMF on histopathologic evaluation of the rats of sub-chronic toxicity study

Results of histopathologic evaluation of lung, liver, and kidney showed that rats treated with the 90-day oral application of CECMF up to the dose of 20 g/kg had few pathological changes in both sexes when compared with the respective control rats (**Fig. 22A-B**). These pathological changes, such as lymphocytic infiltration in the liver and kidney, were incidental. The frequency and severity were comparable to the untreated rats, and there was a lack of dose relationship. The same situations were also observed in the 30-day withdrawal experiment (**Fig. 23A-B**).

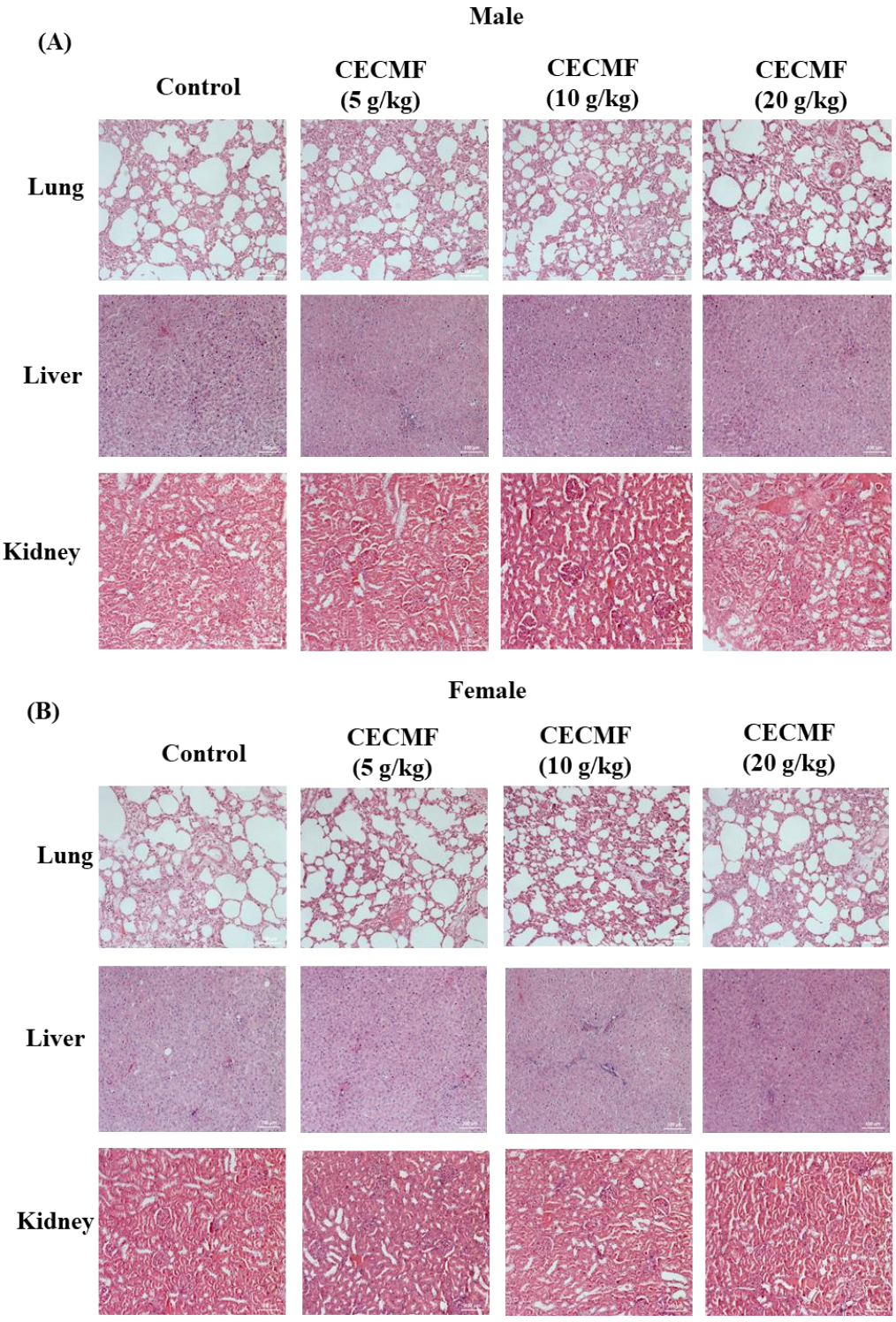


Fig. 22. Effects of CECMF on histopathologic evaluation of the lung, liver, and kidney tissues of rats of sub-chronic toxicity study. Respective photos of histopathologic evaluations (magnification 100 ×) on lung, liver, and kidney of rats treated by CECMF and the normal control rats in the 90-day drug treatment experiment. (A) male rats. (B) female rats.

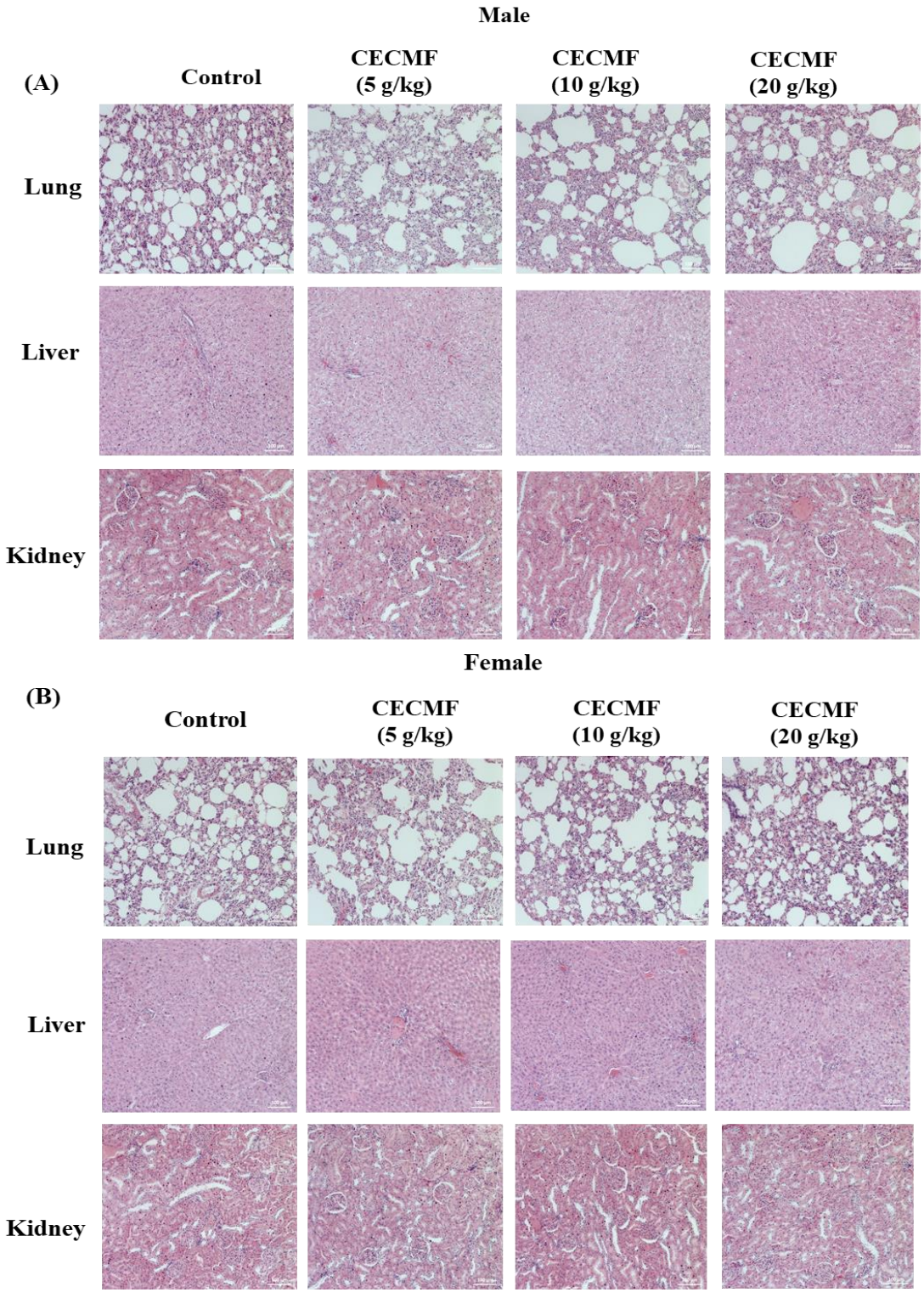


Fig. 23. Effects of CECMF on histopathologic evaluation of the lung, liver, and kidney tissues of rats of sub-chronic toxicity study. Respective photos of histopathologic evaluations (magnification 100 ×) on lung, liver, and kidney of rats treated by CECMF and the normal control rats in the 30-day drug withdrawal experiment. (A) male rats. (B) female rats.

5. Discussion

Ketamine is a noncompetitive N-Methyl-D-aspartate (NMDA) receptor antagonist known for its potent anaesthetic properties and rapid onset of action [56,57]. Several studies have confirmed that ketamine can induce apoptosis of nerve cells and cognitive dysfunction in immature or young animals depending on the timing of exposure and dosage [58,59]. The NORT was conducted to assess recognition memory in rats. MWMT is another behavioral assay of cognitive function by assessing spatial learning and memory. Our study found that ketamine treatment induced impaired function in all aspects of the NORT and MWMT, including reduced recognition memory during crossing the target quadrant, decreased learning ability during the training trials and impaired memory during the probe trial. CECMF treatment could significantly attenuate recognition memory damage and cognitive impairment in ketamine treated rats.

In an effort to search for the molecular and cellular mechanism associated with CECMF mediated restoration of cognitive function that was disrupted by ketamine, we examined neuronal loss in the hippocampus. Neuronal loss has been shown to be associated with cognitive deficits in various scenarios [60,61]. In this study, we showed that long-term exposure of ketamine in rats induced both neuronal loss in neonatal and adult rat brain, manifested as cleaved caspase 3 activation, a crucial apoptosis mediator [62], as well as disruption of two key apoptosis regulators, including upregulation of Bax and downregulation of Bcl-2, which is consistent with previous findings [59]. Importantly, our study showed that CECMF restored the levels of these apoptotic-related proteins (decreasing the expressions of cleaved caspase 3 and Bax and increasing the expression of Bcl-2) in rats treated with ketamine in a dose-dependent matter.

As a glutamate receptor that is a major excitatory neurotransmitter in the brain, the NMDA receptor plays an important role in the mechanisms of synaptic plasticity, which is central to learning and memory [63,64]. Specifically, increased glutamatergic neurotransmission results in membrane depolarization mediated by postsynaptic AMPA

receptors activation, the opening of the NMDAR following the release of the Mg^{2+} block, and Ca^{2+} influx that facilitates postsynaptic plasticity including upregulation of synaptic AMPA receptors [65]. As an NMDA receptor antagonist, ketamine hence has a negative effect on cognitive function [66]. In this study, we found the repressive productions of NMDA receptor subunits (GluN2A and GluN2B) and AMPA receptors subunits (GluA1 and GluA2) by ketamine treatment were dramatically promoted by CECMF in a dose-dependent manner in both neonatal and adult rat model. Furthermore, the postsynaptic density proteins 95 (PSD95) and 93 (PSD93) are critical synaptic proteins that bind to the NMDA receptors and AMPA receptors [67,68], and controls synaptic transmission and plasticity [69]. In addition, some presynaptic markers, such as SYN1 and SYT, forms the molecular foundation of learning and memory in the central nervous system [70,71]. Our present study demonstrated that the suppressive levels of PSD95, PSD93 SYN1 and SYT by ketamine treatment were obviously elevated by CECMF in both neonatal and adult rat model.

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a Ser/Thr protein kinase that is highly abundant in the brain [72]. CaMKIIβ is crucial for controlling synaptic plasticity direction and cognition in rodents [73–75]. Previous studies have shown that mutations in the CaMKIIβ gene that influence autophosphorylation at Thr287 affected neuronal migration and caused intellectual disability [76]. Besides, phosphorylation of ERK1/2, CREB, and NF-κB, as the downstream signaling molecules of CaMKIIβ, is important for the transcription of genes that promote spine formation, synaptic plasticity, learning, and memory [77,78]. In the present study, chronic exposure to ketamine reduced the phosphorylation of CaMKIIβ (Thr287) and CREB (Ser133) and enhanced the phosphorylation of ERK1/2 (Thr202/Tyr204) and NF-κB. CECMF treatment notably up-regulated the phosphorylation of CaMKIIβ (Thr287) and CREB (Ser133) and down-regulated the phosphorylation of ERK1/2 (Thr202/Tyr204) and NF-κB in the hippocampus of ketamine treated neonatal and adult rats, indicating that the above alterations may be related to the modulation of CaMKIIβ-ERK1/2-CREB/NF-κB signaling pathway.

Secondly, ketamine-treated rats experienced bladder hyperactivity with increases in peak micturition pressure, micturition frequency, voiding contraction, and non-voiding contraction, but decrease in bladder volume. Morphological evaluation of ketamine-induced bladder damages was shown by increasing ulcerated urothelium, erythematous mucosa, and interstitial fibrosis [79-84]. However, in our study, these changes were all ameliorated in the ketamine + CECMF groups in a dose dependent manner and the ketamine + parecoxib sodium group (COX-2 inhibitor, positive control). The results were also consistent with those observed with other rat models that were treated with ketamine by I.P. injection [81, 84,85]. In present study, three different urine markers were characterized in an attempt to detect the progression of bladder damage induced by ketamine. NO is synthesized by a family of NOS enzymes, which is a gaseous signaling molecule that regulates various physiological and pathophysiological response [86]. It has been shown that gaseous NO levels in the urinary bladder are much higher in patients with cystitis than in controls [87]. Pathological conditions activate iNOS in mitochondria to produce large amounts of NO as a defense mechanism. The iNOS dependent pathological generation of NO in the bladder can increase the permeability of urothelium and cause urothelium barrier dysfunction [88].

Measurement of urinary APF also provided evidence of the acute toxic effects of ketamine on the urothelium [89]. APF has been shown to inhibit bladder epithelial cell proliferation, decrease tight junction protein expression, and increase paracellular permeability, and it has been proposed as a sensitive and specific biomarker for cystitis [90]. GP51 is another urine marker that has been shown to be differentially expressed in patients with cystitis [91]. Urinary levels of GP51 in patients with cystitis have been shown to be significantly lower than in healthy subjects [92]. It functions as a protective mucosal barrier that protects the underlying urothelium from the access of bacteria, urea, and potassium [93]. In present study, the generation of NO and APF increased and the generation of GP51 decreased in rat urine after treatment with ketamine. CECMF treatment vividly lowed the urine levels of NO and APF and lifted the levels of GP51 in ketamine induced cystitis (KIC) rats, portending that CECMF could attenuate the

discomfort symptoms of KIC. Based on the investigation of patients with KIC, chronic bladder inflammation and overproduction of reactive oxygen may result from the direct toxic effect, neurogenic inflammation, or immunomodulatory imbalance [94-96]. The increase in the levels of inflammatory cytokines (eg, IL-1β, IL-6, TNF-α, and COX-2) and decrease in the expressions of antioxidant biomarkers in the serum or bladder tissue has been reported in patients with KIC [95, 97, 98]. Direct toxic damage caused by ketamine and its metabolites is considered to induce a cascade of bladder pathology of KIC, namely bladder barrier dysfunction, neurogenic infammation, and COX-mediated inflammation [99].

Juan et al. reported that NF-κB translocation into the nucleus could activate COX-2 mediated inflammation and fibrosis of KIC [99]. In the current study, we observed that CECMF treatment inhibited the overexpression of inflammatory mediators (i.e., IL-1β, IL-6, TNF-α, COX-2, ICAM-1, NF-κB p-p65 and p-IκBα) and boosted the antioxidant biomarkers (HO-1 and Nrf2) in KIC rats. Furthermore, during chronic persistent inflammation, TGF-β1 may activate fibrogenesis and contribute to bladder interstitial fibrosis [100,101]. In the late stage of KIC, the extensive fibrosis of a contracted bladder is the main pathology and causes severe urinary symptoms in patients [99]. Usually, repeated relapse and remission of inflammatory phases would progress the fibrogenesis of the interstitial tissue [101]. In the present study, we observed that CECMF treatment reduced TGF-β1 protein overexpression and collagen I, collagen III, and fibronectin accumulation in the bladder in ketamine-treated rats. Ketamine toxicity may elicit a broad spectrum of urothelial neuroreceptors alteration in KIC patients [102]. In animal models, researchers have disclosed that using different dosage and chronicity of ketamine exposure could induce various degrees of dysregulation of bladder muscarinic and purinergic systems [103,104]. Our study results showed that CECMF treatment could inhibit the overexpression of the TRPV1 receptor, muscarinic receptor (M2-mAChR and M3-mAChR) and purinergic receptor (P2X1, P2X2, and P2X3) in the bladder of ketamine-treated rats.

Taken together, our experimental findings have unequivocally demonstrated that

CECMF could promote neuronal survival and synaptic plasticity, thereby eventually attenuate cognitive deficit in rats treated with ketamine by regulation of the CaMKIIβ-ERK1/2-CREB/NF-κB signaling pathway. Our study also for the first time proved that CECMF was able to attenuate bladder damages following the KIC in rats, and the molecular mechanisms involve reduction of both inflammatory infiltration and oxidative stress via suppression of muscarinic and purinergic receptors. We believe that CECMF has the potential to be developed into an innovative Chinese medicine-based for treatment of cognitive deficits and cystitis induced by ketamine.

Importantly, the results of the acute toxicity demonstrated that CECMF at up to the dose of 128 g/kg, which was the maximum tolerable dose of CECMF in mice, did not exert any overt toxicity. The results of sub-chronic toxicity study demonstrated that CECMF (5, 10 and 20 g/kg) did not affect the body weight, relative organ weights, the functions of liver and kidney and hematological analysis in male and female rats after treatment with CECMF for 90 days or 30 post drug withdraw.

CECMF contains 12 Chinese herbal medicines, including *Talcum* 15g, *Lysimachiae Herba* 15g, *Salviae Milti Radix et Rhizoma* 15g, *Puerariae Lobatae Radix* 15g, *Ramulus Uncariae cum Uncis* 12g, *Corydalis Rhizoma* 12g, *Polygoni Cuspidati Rhizoma et Radix* 12g, *Plantaginis Semen* 12g, *Akebiae Caulis* 9g, *Dianthi Herba* 9g, *Polygoni Avicularis Herba* 9g, *Gardeniae Fructus* 9g, which is prescribed at a daily dose for an adult. CECMF is a Chinese medicine-based herbal formula developed from the clinical experience. Based on Chinese medicine theory, this compound herbal formula has the therapeutic functions of invigorating blood circulation to remove blood stasis, clearing heat and toxicity to quench inflammation, and promoting urination to treat gonorrhea. This study is essentially a preclinical project to unravel the efficacy and safety of CECMF using *in vivo* animal experimental models and to elucidate the underlying molecular mechanisms. Positive results from this study provide scientific rationale and justification for future clinical study this herbal formula on drug abusers. Additionally, we can disseminate the research results of our study to the Chinese medicine practitioners via academic conferences and benefit the ketamine abusers with

cognitive deficits and cystitis in clinical practice.

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