Calcitonin-stimulated renal Ca\(^{2+}\) reabsorption occurs independently of TRPV5

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Abstract

Background. Calcitonin (CT) is known to affect renal Ca\(^{2+}\) handling. However, it remains unclear how CT affects Ca\(^{2+}\) transport in the distal convolutions. The aim of this study was to investigate the contribution of the renal epithelial Ca\(^{2+}\) channel, transient receptor potential vanilloid 5 (TRPV5), to renal Ca\(^{2+}\) handling in response to CT.

Methods. C57BL/6 mice received a single overnight (16 hr) injection of CT. In addition, TRPV5 knockout (TRPV5\(^{-/-}\)) mice and their wild type (TRPV5\(^{+/+}\)) controls, received three bolus injections of CT over a 40-hr study period. All experimental groups were placed in metabolic cages.

Results. C57BL/6 mice received a single bolus injection of CT, which significantly reduced the urinary Ca\(^{2+}\) excretion. In addition, urinary Na\(^{+}\) and K\(^{+}\) excretion also decreased after CT administration. No apparent changes in renal expression of TRPV5, calbindin-D\(_{28K}\) (CaBP28K) or TRPV6 could be detected between CT- and vehicle-treated mice. To evaluate whether TRPV5 activity is needed for the CT-induced increase in Ca\(^{2+}\) reabsorption, mice with genetic ablation of TRPV5 (TRPV5\(^{-/-}\)) were employed. TRPV5\(^{-/-}\) mice as well as their wild-type (TRPV5\(^{+/+}\)) controls received three bolus injections of CT over a 40-hr study period. Overnight (16 hrs) as well as the subsequent 24-hr urine was collected. Overnight urinary Ca\(^{2+}\) excretion was reduced in both TRPV5\(^{-/-}\) and TRPV5\(^{+/+}\) mice after a bolus injection of CT. The subsequent 24-hr urinary excretion of Ca\(^{2+}\) was collected after the third bolus injection showed no effect of CT on renal Ca\(^{2+}\) handling in either mice group. Accordingly, CT did not alter the intrarenal protein abundance of TRPV5 and CaBP28K after three bolus injections of CT.

Conclusion. CT augments the renal reabsorptive capacity for Ca\(^{2+}\). This increase is likely to occur independently of TRPV5.

Keywords: calbindin; calcitonin; calcium; distal convoluted tubule; TRPV5


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Introduction

Disturbances in the systemic Ca\(^{2+}\) concentration often result in instability of the neurological and cardiac systems (e.g., hypercalcemia causes fatigue, nausea and abnormal heart rhythms [1]). Thus, maintenance of serum Ca\(^{2+}\) concentration plays an important role in stabilizing these physiological processes. Several hormones have been known to affect the systemic Ca\(^{2+}\) concentration. Calcitonin (CT) is a 32 amino acid peptide synthesized by post-translational processing in the C cells of the thyroid gland in mammals [2]. CT acts primarily by inhibiting osteoclast-mediated bone resorption and is secreted in response to increases in systemic Ca\(^{2+}\) concentration [2]. Historically, CT has been extensively used in treating hypercalcemia, osteoporosis and Page's disease [2]. Throughout the kidney and elsewhere, CT acts primarily by activating the adenylate cyclase pathway [3,4].

In the kidney, vectorial NaCl transport within the thick ascending limb (TAL) drives paracellular transport of Ca\(^{2+}\). Administration of CT to rats increases the renal reabsorption of Ca\(^{2+}\), which is in part achieved by increasing the reabsorption of Ca\(^{2+}\) in the TAL. Here, application of CT also stimulates Ca\(^{2+}\) transport [14-20]. CT increases vectorial NaCl transport [5-7]. Active transcellular Ca\(^{2+}\) transport is restricted to the distal convolutions. Here, luminal Ca\(^{2+}\) enters across the apical membrane via the epithelial Ca\(^{2+}\) channel, transient receptor potential vanilloid 5 (TRPV5) [8,9]. Studies in rabbit suggest that CT exerts its effect on Ca\(^{2+}\) reabsorption only in the distal convoluted tubule (DCT), while no stimulation occurs in the connecting tubule (CNT) [10]. In addition, CT fails to stimulate cyclic adenosine monophosphate (cAMP) accumulation in the CNT of rabbits [11]. Our group has also been unable to show stimulatory effects of CT on cAMP production in rabbit primary CNT cultures [12]. In the rabbit, TRPV5 localizes primarily to the CNT where CT does not affect Ca\(^{2+}\) reabsorption [8,10]. However, in mouse, TRPV5 is highly expressed in apical membrane domains of particularly the late DCT2, with a gradual decrease along CNT and initial collecting duct (iCD) [13,14], suggesting that TRPV5-mediated Ca\(^{2+}\) uptake predominates in the DCT2 segment. Similarly, in rat, immunohistochemical data suggests that TRPV5 expression appears in the DCT2 and is further observed in the CNT [15]. The intrarenal distribution of TRPV5 in human is currently not known.

In comparison to rabbit, the boundaries defining the DCT/CNT/iCD regions in human, rat and mouse are morphologically less well defined, with cell types from different segments intermingled. In the kidney of rats, the ‘bright’ portion of the DCT retains responsiveness to CT, resulting in increased cAMP production [4]. CT-binding sites have been identified in the rat kidney, including the TAL and DCT [16]. Detailed micropuncture studies performed by Elalouf et al. investigated the effects of CT on electrolyte transport in the distal tubules of rats deprived of vasopressin, parathyroid hormone (PTH) and glucagon. Here, they demonstrated that NaCl, as well as Ca\(^{2+}\) transport, was increased by CT infusion [17]. Other cAMP-elevating hormones such as PTH affect TRPV5-mediated Ca\(^{2+}\) transport. PTH supplementation to parathyroidectomized rats increases the expression of TRPV5, independent of serum 1,25-dihydroxyvitamin D\(_2\) levels, suggesting that perhaps intracellular elevations in cAMP affect the abundance of TRPV5 [18]. In addition, cAMP generation has been shown to increase TRPV5 single-channel activity via a protein kinase alpha-dependent mechanism [19]. However, it is unclear whether there is a functional overlap between PTH and CT in the DCT2 regions of rats and mice, and whether the CT-induced increase in cAMP could lead to a similar activation in the DCT2 segment as PTH.

In order to better understand the molecular actions of CT on overall renal Ca\(^{2+}\) balance and to delineate the potential effects of CT on TRPV5-dependent Ca\(^{2+}\) reabsorption, several experimental series were performed in wild-type and TRPV5-deficient mice. Due to the rapid action of CT, we investigated the short-term effects by of CT on the renal Ca\(^{2+}\) excretion.

Materials and methods

Experimental protocol 1

Male C57BL/6 mice (12 weeks of age) were housed in a light and temperature-controlled room with ad libitum access to deionized drinking water and standard chow (0.28% (wt/wt) NaCl, 1.00% (wt/wt) Ca, 0.22% (wt/wt) Mg; LabDiet, USA). Mice were injected subcutaneously with a single dose of salmon CT (20 U/100 g bodyweight, Novartis Pharmaceuticals, Taiwan, n = 6) or vehicle (n = 6) and placed in metabolic cages. After 16 hrs, overnight urine was collected, and the animals were sacrificed under halothane anaesthesia. Blood was obtained via orbital puncture before cervical dislocation. The kidneys were dissected out and snap frozen for RNA extraction. The animal ethics boards of the National Defense Medical Center (Taipei, Taiwan) approved all animal experimental procedures.

Experimental protocol 2

TRPV5(wt/wt) mice were generated by targeted ablation of the TRPV5 gene as described previously [20]. After acclimatization, TRPV5(wt/wt) and TRPV5−/− mice received salmon CT (20 U/100 g bodyweight, n = 6) or vehicle (n = 6) in three subcutaneous bolus injections spaced over 40 hrs. During this period, the mice were housed in metabolic cages. Overnight (16 hrs) urine was collected during the period between the first and second dose of CT. Subsequently, 24-h urine was collected during the remainder of the study, where the mice received the last two injections. At the end of the experiment, animals were killed under halothane anaesthesia, blood was removed, and tissue samples were harvested for immunohistochemistry and western blotting. The animal ethics board of the Radboud University Nijmegen approved the animal experimental procedures.

Urine and serum analyses

Urine and serum concentrations of Ca\(^{2+}\) were analysed using a colorimetric assay kit (Roche, Mannheim, Germany). Urine and serum concentrations of Na\(^{+}\) and K\(^{+}\) were determined using an automated analyser (AU 5000 chemistry analyser, Olympus, Tokyo, Japan).

RNA extraction and quantitative real-time PCR

Total RNA was extracted from kidney using Trizol Total RNA Isolation Reagent (Sigma, St Louis, MO, USA) as described previously [21]. The obtained total RNA was subjected to DNase treatment to prevent genomic DNA contamination. Thereafter, 1.5 µg of total RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). The cDNA was used to determine intrarenal mRNA expression levels of TRPV5, calbindin-D\(_{28K}\) (CaBP28K) and TRPV6 by quantitative real-time PCR, using the ABI Prism 7700 Sequence Detection System (PE Biosystems, Rotkreuz, Switzerland). The expression level of the housekeeping gene hypoxanthine–guanine phosphoribosyl transferase (HPRT) was used as an internal control to normal-
Data is presented as mean ± SEM.

Fig. 1. Renal Ca$^{2+}$ excretion (A) and serum Ca$^{2+}$ concentration (B) after a bolus injection CT (data is presented as mean ± SEM; *$P < 0.05$, statistically significant.

Immunohistochemistry
Kidney tissue was immersion fixed in 1% (wt/vol) periodate–lysine–paraformaldehyde for 2 hrs at room temperature, and subsequently incubated overnight at 4°C in phosphate-buffered saline (PBS) containing 15% (wt/vol) sucrose. The kidneys were snap frozen in liquid nitrogen and 7-μm sections cut on a cryostat microtome (Microm HM 550, MICROM International GmbH, Germany). For immunohistochemical detection of TRPV5, kidney sections were stained with a guinea pig anti-TRPV5 antibody (1:50) [8] or mouse anti-CaBP28K antibody (1:1000; Sigma). To visualize TRPV5 and CaBP28K, sections were incubated with a goat anti-guinea pig Alexa 488-conjugated anti-IgG (1:1000; Sigma) and goat anti-mouse Alexa 488-conjugated anti-IgG (1:1000; Sigma), respectively. TRPV5 protein expression was semi-quantified by taking five digital images of each kidney section on with a Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY, USA) and calculating the integrated optical density using Image-Pro Plus version 3.0 software (Media Cybernetics, Silver Spring, MD).

Immunoblotting
Total mouse kidney lysates were prepared as described previously [23]. The protein concentration of the homogenates was determined with the Bio-Rad protein assay (Bio-Rad, München, Germany). Samples were submitted to 12% (wt/vol) SDS–PAGE and blotted to polyvinylidifluoride-nitrocellulose membranes (Immobilon- P, Millipore Corp., Bedford, MA). Blots were incubated overnight with a rabbit anti-CaBP28K polyclonal antibody (1:5000; Sigma) at 4°C. Subsequently, blots were incubated with a goat anti-rabbit peroxidase-labelled secondary antibody (1 hr; 1:10000; Sigma, St. Louis, MO). Immunoreactive protein was detected by the chemiluminescence method (Pierce, Rockford, IL). Immunopositive bands were scanned using an imaging densitometer (Bio-Rad Rs-690) to determine pixel density (Molecular Analyst Software; BioRad Laboratories, Hercules, CA).

Table 1. Serum concentrations and urinary excretion of Na$^+$ and K$^+$ after a bolus injection CT

<table>
<thead>
<tr>
<th></th>
<th>Urine</th>
<th>Serum</th>
</tr>
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<tbody>
<tr>
<td>Volume (ml)</td>
<td>$Na^+$ (μmol)</td>
<td>$K^+$ (μmol)</td>
</tr>
<tr>
<td>Control</td>
<td>1.21 ± 0.11</td>
<td>268 ± 32</td>
</tr>
<tr>
<td>CT</td>
<td>0.98 ± 0.18</td>
<td>132 ± 16*</td>
</tr>
</tbody>
</table>

*$P < 0.05$ versus controls; CT, calcitonin.

Data is presented as mean ± SEM. $n = 6$ animals per group. $*P < 0.05$, statistically significant.

Statistical analyses
Values are expressed as mean ± SEM. Statistical significance ($P < 0.05$) in experimental protocol 1 was determined using the student’s t-test. In experimental group 2, significance was determined by a one-way ANOVA. In case of significance, the Tukey–Kramer multiple comparisons test was applied. The analyses were performed using the Statview Statistical Package Software (Power PC, version 4.5.1, Berkeley, CA).

Results

CT increases renal Ca$^{2+}$ reabsorption in C57BL/6 mice
To assess the acute effects of CT, 16-hr urine from overnight collections was obtained following a bolus injection of the hormone (20 U/100 g body weight) or vehicle, respectively. The effect of overnight CT exposure on renal and systemic Ca$^{2+}$ handling is depicted in Figure 1. Administration of CT resulted in a significant decrease in the urinary excretion of Ca$^{2+}$ ($P < 0.05$). Serum Ca$^{2+}$ concentrations were slightly reduced after overnight CT administration ($P < 0.05$). Urinary excretion of Na$^+$ and K$^+$ showed a significant decrease after CT injection (Table 1). In addition, the urinary Na$^+$/K$^+$ ratio remained unchanged excluding an effect on the collecting duct. These data are consistent with a primary effect of CT on electrolyte transport in the TAL and DCT [5–7,17].

CT does not change the renal mRNA expression of TRPV5, CaBP28K and TRPV6
To evaluate whether the increased Ca$^{2+}$ reabsorption could in part be due to transcriptional changes in Ca$^{2+}$ transport proteins located in the distal convolutions, quantitative real-time PCR was used to estimate TRPV5, CaBP28K and TRPV6 abundance. As can be seen in Figure 2, no changes were observed in the renal mRNA expression of these transcripts. Although CT did not modulate the changes of TRPV5, it does not exclude the potential involvement of TRPV5. Therefore, we investigated the effect of CT in TRPV5$^{+/−}$ and TRPV5$^{−/−}$ mice after one and three injections of CT.

CT stimulates Ca$^{2+}$ reabsorption in TRPV5$^{+/−}$ mice
Administration of CT to TRPV5$^{+/−}$ and TRPV5$^{−/−}$ mice resulted in a decreased overnight urinary Ca$^{2+}$ excretion in both groups. However, after repeated administration of CT, the urinary Ca$^{2+}$ excretion returned to normal in both TRPV5$^{+/−}$ and TRPV5$^{−/−}$ mice, as compared to their corresponding ve-
hicle-injected controls (Figure 3). Serum Ca$^{2+}$ concentrations were measured after 40 hrs, but no difference was observed after CT administration in either strain (Figure 3). This is in line with the observed rapid effect of CT.

**Renal protein expression of Ca$^{2+}$ transporters remains unaltered after three CT injections**

Kidneys retrieved from TRPV5$^{+/+}$ and TRPV5$^{-/-}$ mice by the end of the experiment were used to assess the effects of CT on renal Ca$^{2+}$ transporter expression. TRPV5 and CaBP28K protein abundance was semi-quantified by immunohistochemistry (Figure 4) and immunoblotting (Figure 5), respectively. Computerized analysis of immunohistochemical images did not reveal any changes in TRPV5 protein expression after CT administration in TRPV5$^{+/+}$ mice compared to their vehicle-injected control TRPV5$^{+/+}$ mice (Figure 4). This is in line with the observation that CT affects renal Ca$^{2+}$ transport independent of TRPV5. In addition, the renal CaBP28K protein expression remained unchanged after CT administration in TRPV5$^{-/-}$ mice (Figure 4 and 5). The CaBP28K protein expression of TRPV5$^{-/-}$ mice was significantly reduced compared to TRPV5$^{+/+}$ mice [20] (Figure 4 and 5).

![Fig. 2. mRNA expression of TRPV5, CaBP28K and TRPV6 after overnight bolus injection of CT [representative histograms of mRNA expression of TRPV5 (A), CaBP28K (B) and TRPV6 (C) in kidney; data are presented as mean ± SEM].](image)

![Fig. 3. Renal Ca$^{2+}$ excretion after one and three bolus injections spaced over 40 hrs in TRPV5$^{+/+}$ and TRPV5$^{-/-}$ mice [16 hrs urinary Ca$^{2+}$ excretion after a single bolus injection of CT (A) and the consecutive 24 hrs of urinary Ca$^{2+}$ excretion after three injections (B); serum Ca$^{2+}$ concentrations after three CT bolus injections spaced over 40 hrs (C); data is presented as mean ± SEM. *P < 0.05, statistically significant].](image)
Discussion

A bolus injection of CT significantly reduced the urinary excretion of Ca\(^{2+}\) in wild-type mice. This decrease was associated with a reduction in the urinary Na\(^+\) and K\(^+\) excretion, leaving the urinary Na\(^+\)/K\(^+\) ratio unchanged. These data are consistent with the previously reported effects of CT on stimulating TAL and DCT transport of NaCl and K\(^+\) [6,7,17,24]. Earlier studies in the TAL of mice and rats have demonstrated that CT activates NaCl transport only in the cortical TAL [6,24]. This segment of the TAL is also thought to drive paracellular Ca\(^{2+}\) reabsorption in these species. Al-
though never delineated in detail, CT is likely to activate a cAMP cascade leading to increased NKCC2 transport, perhaps via increased membrane trafficking and phosphorylation [5,6,25]. Currently, it remains unknown which transporter CT stimulates in the DCT [17]. One option is the thiazide-sensitive NaCl cotransporter that resides there. A significant, albeit small, change in serum Ca\(^{2+}\) concentrations was observed after overnight CT administration. Earlier reports show that infusion of CT, depending on dose, acutely reduces serum Ca\(^{2+}\) concentrations that frequently revert to normal range within 1 day [26]. In line with this, no change was observed in systemic Ca\(^{2+}\) concentrations after three injections of CT spaced over 40 hrs. One may suggest that the reduced excretion of Ca\(^{2+}\) in the presence of CT occurs to compensate for the acute surge in systemic Ca\(^{2+}\) concentrations normally associated with CT administration. However, in thyroparathyroidectomized (TPTX) rats, Ca\(^{2+}\) infusion prior to CT treatment was given in order to avoid hypocalcaemia in the animals. In these TPTX rats, CT was still able to reduce the fractional excretion of Ca\(^{2+}\) despite normocalcaemia [27]. This is consistent with a direct effect of CT on the kidney. The effect of CT on urinary Ca\(^{2+}\) excretion is also absent after repeated administration of CT to either TRPV5\(^{+/+}\) or TRPV5\(^{-/-}\) mice. Reduced surface expression of CT receptors and decreased mRNA expression has been shown to explain such escape phenomena in other cell types [28]. This may also explain why patients with a CT-secreting tumour frequently have normal systemic concentrations of Ca\(^{2+}\) [29].

The present study was initiated to investigate the potential effect of CT on TRPV5-mediated Ca\(^{2+}\) reabsorption. Injections of CT for 16 or 40 hrs did not change the renal abundance of TRPV5. In addition, TRPV5\(^{+/+}\) and
TRPV5−/− mice responded in a similar manner to CT administration. Given the data obtained in these experimental models, it is likely that the effect of CT on renal Ca2+ transport occurs independently of TRPV5. Although CT does not affect TRPV5-mediated Ca2+ reabsorption, it is clear from this study that CT strongly stimulates renal Ca2+ reabsorption. The stimulatory effect of CT is likely to occur primarily through changes in Ca2+ transport, as previously described [6,7,24]. It should be stated that although the effects of CT on renal Ca2+ transport are present in TRPV5+/− as well as in TRPV5−/− mice, and that TRPV5 expression is unaltered after CT administration, this does not fully exclude an effect upon TRPV5. Although unlikely, a lack of stimulation of CT on TRPV5 in TRPV5−/− mice may be masked due to the pronounced effect of CT in the TAL. TRPV6 is a highly selective Ca2+ channel which has been localized to apical membrane domains in kidney [30]. As TRPV6 may play a role in apical Ca2+ entry of tubules in the distal part of the nephron, mRNA abundance of the channel was determined after an overnight bolus injection of CT. Administration of CT did not change the expression level of renal TRPV6. These data indicate that the effect of CT on renal Ca2+ transport occurs independently of TRPV6.

In the rat, CT has been shown to increase NaCl as well as CaCl2 transport in the DCT [17], thus vectorial transfer of Ca2+ does occur to a greater extent in the presence of CT. The present study suggests that these effects occur largely independent of TRPV5. In addition, microperfusion experiments in rabbits show that CT stimulates Ca2+ transport in the early DCT, where TRPV5 is not expressed [10]. This raises the question, how does Ca2+ transport occurs in the early DCT and does it contribute significantly to overall renal Ca2+ handling? Further studies are needed to determine the potential effect of CT on electrolyte transporters in these segments.

In conclusion, overnight CT administration increases renal Ca2+ reabsorption in mice. This effect occurs independently of TRPV5 as no change can be detected in TRPV5 and CaBP28K expression and similar responses to CT is observed in TRPV5+/− and TRPV5−/− mice.

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Conflict of interest statement. None declared.

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N-acetylcysteine attenuates glycerol-induced acute kidney injury by regulating MAPKs and Bcl-2 family proteins

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Abstract

Background. Rhabdomyolysis-induced acute kidney injury (AKI) accounts for about 10 to 40% of all cases of AKI. It is known that N-acetylcysteine (NAC) is effective in various experimental renal injury models; however, little information is available about the rat model of glycerol-induced rhabdomyolysis. In this study, we hypothesize that NAC plays a renoprotective role via the anti-apoptotic pathway.

Methods. Male Sprague-Dawley rats were divided into four groups: (i) saline control group, (ii) NAC-treated group (N-acetylcysteine) (150 mg/kg), (iii) glycerol-treated group (50%, 8 ml/kg, IM) and (iv) NAC plus glycerol-treated group. Rats were sacrificed at 24 h after glycerol injection, and the blood and renal tissues were harvested.

Results. Glycerol administration caused severe renal dysfunction, which included marked renal oxidative stress, significantly increased blood urea nitrogen (BUN) and serum creatinine levels. Histopathological findings, such as cast formation and tubular necrosis, confirmed renal impairment. We noted a marked activation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), but not p38, in the glycerol-treated group. We also observed high expression of Bax and Bad but only weak expression of Bcl-2 and Bcl-xL in the glycerol-treated group. However, NAC pretreatment significantly improved renal function and decreased the activation of ERK, JNK, Bax and Bad, whereas it increased Bcl-2 and Bcl-xL.

Conclusion. These results demonstrate that NAC protects against renal dysfunction, morphological damage and biochemical changes via the anti-apoptotic pathway in the glycerol-induced rhabdomyolysis model in rats.

Keywords: acute kidney injury; Bcl-2 family proteins; MAPKs; N-acetylcysteine; rhabdomyolysis

Introduction

Rhabdomyolysis is a syndrome involving the breakdown of skeletal muscle, which causes myoglobin and other intracellular proteins and electrolytes to leak into the circulation [1]. It is often complicated by acute kidney injury (AKI), electrolyte imbalance and disseminated intravascular coagulation. About 10 to 50% of patients suffering from significant rhabdomyolysis develop some degree of AKI [2]. Although the treatment has been much improved, the mortality rate may still be as high as 8% [1, 3, 4]. The experimental model for rhabdomyolysis is easily acquired by injecting glycerol intramuscularly into rats or mice [5].

AKI by rhabdomyolysis has three pathogenic mechanisms: tubular obstruction, renal vasoconstriction and oxidative stress. Oxidative stress has been an important target in the prevention of myoglobin-induced renal injury [6]. The administration of antioxidants has been shown to provide partial protection against myoglobinuric-induced AKI [7–11]. N-acetylcysteine (NAC), one of these antioxidants, is a source of sulfhydryl and glutathione (GSH) groups in cells and, due to its interaction with reactive oxygen species, is a scavenger of free radicals [12]. The protective effect of NAC with respect to renal injury has been proven in various models, such as cisplatin [13], ischemia–reperfusion injury [14, 15] and chronic kidney disease [16]. However, there is little data for administering NAC in the rhabdomyolysis model, and the results are controversial [17, 18].


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