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Original Article

Calcitriol ameliorated rhabdomyolysis induced acute renal failure in rats

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Abstract: Rhabdomyolysis could cause acute renal failure through several mechanisms. It was known that 1,25-dihydroxyvitamin D3 (calcitriol) had pleiotropic effects besides its calcitropic effects. In this study, calcitriol was used to investigate the effects on glycerol-induced rhabdomyolysis with acute renal failure in rats. Rhabdomyolysis was induced after intramuscular injection of 10 mL/kg 50% glycerol in rats. Then, the rats received intravenous injection of calcitriol (10 ng/kg in 0.5 mL normal saline). Blood urea nitrogen (BUN), creatinine (Cre), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and creatine phosphokinase (CPK) were measured at 0, 1, 3, 6, 12, 18, 24, and 48 hrs. Renal tissue injury score was estimated and renal tubular cells positive of E-cadherin, nuclear factor-κB (NF-κB) and inducible nitric oxide synthase (iNOS) were semi-quantitated calculated by immunohistochemical (IHC) stain. It showed that glycerol-induced rhabdomyolysis significantly increased serum levels of BUN, Cre, GOT, GPT, and CPK. In addition, there was increased renal tissue injury score, elevated expression of NF-κB and iNOS and decreased expression of E-cadherin of renal tubular cells histopathologically. After treating with calcitriol, serum levels of BUN, Cre improved, renal tissue injury scores decreased, the renal tubular expression of NF-κB and iNOS decreased, and E-cadherin preserved. Through anti-oxidant and anti-inflammatory effects, there were beneficial effects of calcitriol against acute kidney injury caused by glycerol-induced rhabdomyolysis in rats.

Keywords: Acute renal failure, calcitriol, rhabdomyolysis

Introduction

It was well known that rhabdomyolysis was caused by breakdown and necrosis of striated muscles with the results of leakage of intramuscular constituents including electrolytes, myoglobin and other sarcoplasmic proteins into the circulation and extracellular fluid [1-3]. Severity of injuries caused by rhabdomyolysis ranged from asymptomatic elevation of serum creatine kinase (CK) levels to life-threatening conditions such as disseminated intravascular coagulation, and acute renal failure (AKI) [1]. The possible mechanisms of rhabdomyolysis-induced renal injuries were vasoconstriction, formation of intra-tubular casts and direct toxicity of myoglobin to renal tubular cells [4, 5]. Studies had revealed that released heme proteins from myoglobin produced reactive oxygen species, scavenged nitric oxide, and activated endothelin receptors which together induced a synergistic effect on renal vasoconstriction and intra-tubular cast formation resulting in myoglobinuric acute renal failure [6, 7]. The traditional strategies to treat rhabdomyolysis included aggressive hydration, mannitol usage, urine alkalization and forced dieresis [6, 8]. Additionally, recent strategies against rhabdomyolysis-induced acute kidney injury including medications lessening vasoconstriction, anti-oxidant and anti-inflammation were developed with promising results [9-13]. This highlighted the possibility of treating rhabdomyolysis-induced acute kidney injury by approaches towards the underlying mechanisms.

It was well accepted that 1,25-dihydroxyvitamin D3 (calcitriol) had an important role of regulating calcium and phosphorus homeostasis and bone mineralization [14]. Apart from this calci-

tropic effect, calcitriol was reported to have pleiotropic effects towards multiple organs and had potent anti-proliferative, stimulating cell differentiation, immune-modulatory, and anti-inflammation activities [15]. Studies had showed calcitriol could delay the progression of diabetic nephropathy through suppressing TNF-α and IL-6 [14, 16], and protected renal injury through modulating inflammation [17-19].

Since we had recently shown that through the pleiotropic effects, erythropoietin and phenobarbital could protect renal function after rhabdomyolysis [20, 21] and calcitriol was reported to have effects on anti-inflammation and anti-oxidant, it is our hypothesis that calcitriol could ameliorate the acute kidney injury after glycerol-induced rhabdomyolysis.

Materials and methods

Preparation of animals

Twenty-four male Sprague-Dawley rats weighting 280-300 g were purchased from the National Animal Center and were housed in our animal center under a controlled environment at a temperature of 22 ± 1°C with a 12-hour light/dark cycle. Food and water were provided ad libitum. Before inserting polyethylene catheters (PE-50) into the femoral artery for collecting blood samples and femoral vein for intravenous administration of drugs or fluids, these rats were anesthetized with ether inhalation for about 10 minutes and during the period of performing these procedures. These procedures were completed within 15 minutes, and the wound was kept less than 0.5 cm^2. Afterwards, the rats were placed in a conscious rat metabolic cage (Shingshieying Instruments, Hualien, Taiwan). The rats awakened soon after the procedures and rhabdomyolysis was induced 24 hours later with these rats in a conscious state [19-21]. The experimental protocol was approved by the Animal Usage Regulation Committee of Tzu Chi Hospital.

Experimental design

These rats were randomly divided into three groups. In the Vehicle group (n = 8), rats were injected intramuscularly with 10 mL/kg of normal saline in each hind leg and then immediately received intravenous 10 ng/kg calcitriol (Sigma Chemical, St. Louis, MO, USA) in 0.5 mL normal saline [19]. In the Glycerol group (n = 8), rats were intramuscularly injected with 50% glycerol (10 mL/kg; Sigma Chemical, St. Louis, MO, USA) in each hind leg and then immediately received intravenous 0.5 mL normal saline [20, 21]. In the Glycerol + Calcitriol group (n = 8), rats were intramuscularly injected with 50% glycerol (10 mL/kg) in each hind leg and then received intravenous 10 ng/kg calcitriol (Sigma Chemical, St. Louis, MO, USA) in 0.5 mL normal saline. Rats were sacrificed by decapitation 48 hours after glycerol administration.

Biochemical analyses

Before and 1, 3, 6, 9, 12, 24, 48 hours after glycerol administration, blood urea nitrogen (BUN), creatinine (Cre), Glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and creatine phosphokinase (CPK) were measured by taking 0.5 ml blood samples. Blood samples were immediately centrifuged at 3,000 g for 10 minutes, and the serum was decanted and stored at 4°C. These biochemical analyses were performed within 1 hour after collecting blood samples. Serum levels of BUN, Cre, GOT, GPT, and CPK were measured using an auto-analyzer (Arkray spotchem EZ-SP-4430, Arkray Inc, Kyoto, Japan) [19-21].

Histopathological examination

Kidneys were removed immediately after sacrifice. Tissue specimens were fixed overnight in 4% buffered formaldehyde, processed by standard methods, and stained with hematoxylin and eosin (H&E). The observer who analyzed the tissues was blinded to the specimen groups. Renal tissue injury score was estimated by the percentage of epithelial necrosis or luminal necrotic debris or tubular dilation, and the development of heme casts of renal tubules in the cortex or the outer medulla, as follows: 0, none; 1, < 5%; 2, 5% to < 25%; 3, 25% to 75%; and 4, > 75% [20, 21]. All evaluations were made on five fields per section and five sections per kidney.

Immunohistochemical (IHC) stain

For IHC, serial 4-μm sections were deparaffinized, rehydrated, and incubated with different mouse monoclonal antibodies at 4°C overnight according to the manufacturer's direc-

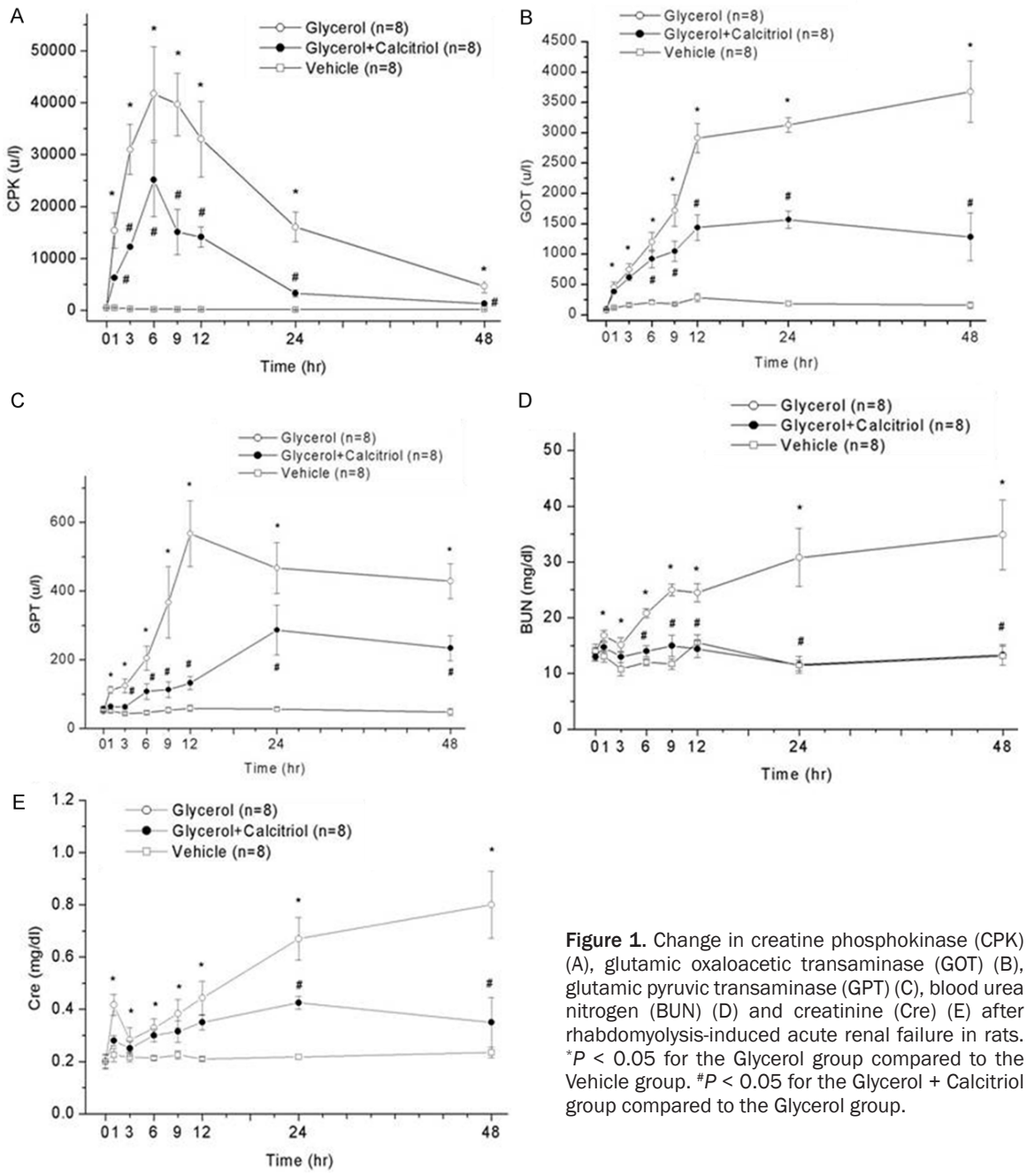

Figure 1. Change in creatine phosphokinase (CPK) (A), glutamic oxaloacetic transaminase (GOT) (B), glutamic pyruvic transaminase (GPT) (C), blood urea nitrogen (BUN) (D) and creatinine (Cre) (E) after rhabdomyolysis-induced acute renal failure in rats. $^{*}P < 0.05$ for the Glycerol group compared to the Vehicle group. $^{\#}P < 0.05$ for the Glycerol + Calcitriol group compared to the Glycerol group.

tions. Antigen retrieval was used for E-cadherin, nuclear factor-κB/P65 (NF-κB p65) and iNOS. Dilutions were 1 in 100 for E-cadherin, NF-κB/p65, and iNOS (Neomarkers, Lab Vision Corporation, Fremont, California, USA). After incubation, tissue sections were covered with biotinylated goat antimouse polyvalent secondary antibody and incubated at room temperature for 10 minutes. After washing, the slides were incubated in peroxidase conjugated streptavidin-biotin complex (Dako, Copenhagen, Denmark) for 10 minutes. Cells positive for E-cadherin, NF-κB/p65, and iNOS were semi-quantitated on paraffin-embedded tissue sections, counting 10 high-power fields (200 ×) per section; data were expressed as the percentage of positive stained renal tubular cells of the total area examined [20, 21]. All scoring was performed in a blinded manner on coded slides.

Statistical analysis

Data were expressed as means ± SEMs. Statistical comparisons between different groups

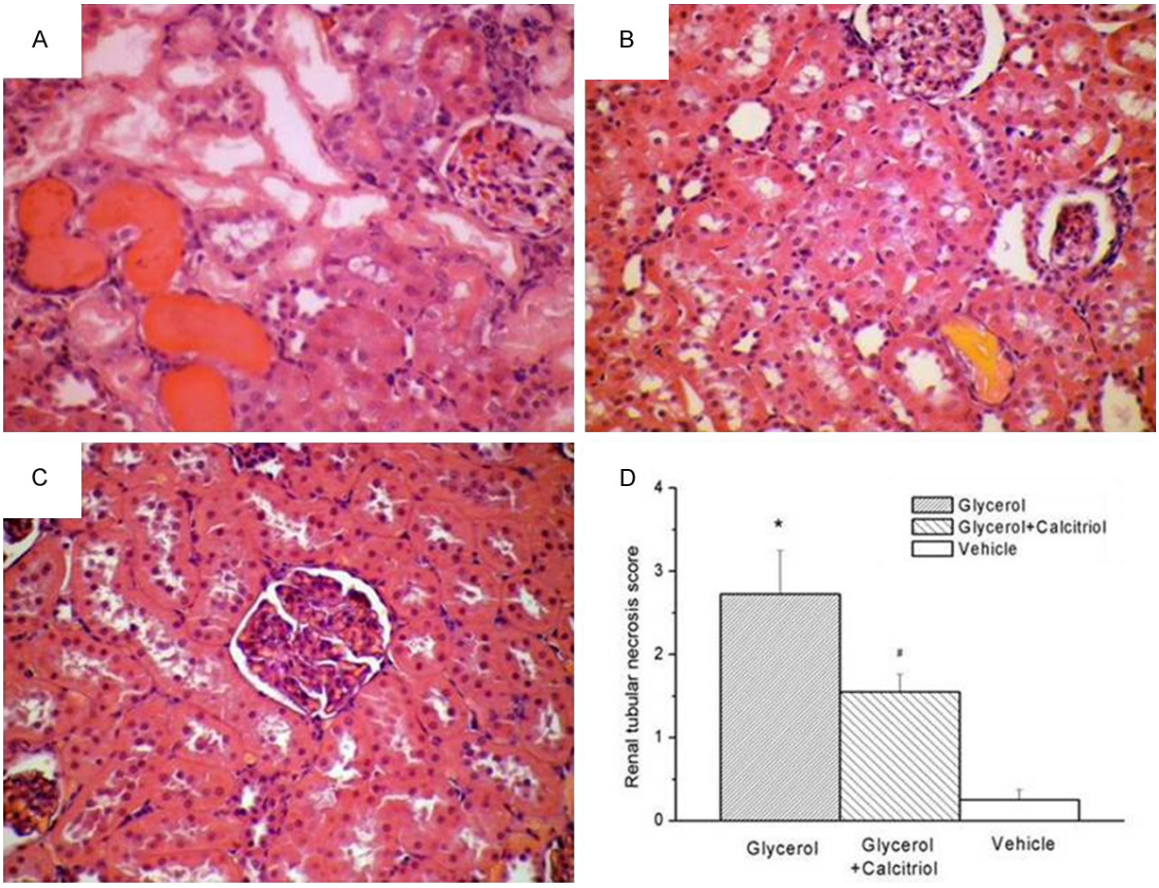

Figure 2. Post-treatment with calcitriol affected the histopathologic changes in the kidneys. Histological findings from the Glycerol group (A), Glycerol + Calcitriol group (B), and Vehicle group (C), stained with hematoxylin and eosin (magnification × 200). Renal tissue injury score after rhabdomyolysis-induced acute renal failure in rats (D). $^{*}P < 0.05$ for the Glycerol group compared to the Vehicle group. $^{\#}P < 0.05$ for the Glycerol + Calcitriol group compared to the Glycerol group.

at corresponding time points were made by repeated measures two-way analysis of variance, followed by a post hoc test (Bonferroni's method). Histopathological scores were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney U test. A *P* value of less than 0.05 was considered statistically significant.

Results

Serum levels of hemoglobin and CPK

After glycerol injection, serum CPK peaked at 6 hour and increased significantly at 1, 3, 6, 9, 12, 24, and 48 hours compared to the corresponding values in the Vehicle group ($^{*}P < 0.05$; **Figure 1A**). Serum CPK values in the Glycerol + Calcitriol group were significantly lower at 1, 3, 6, 9, 12, 24, and 48 hours compared to the corresponding values in the Glycerol group ($^{\#}P < 0.05$; **Figure 1A**).

Serum levels of GOT and GPT

After glycerol injection, serum GOT peaked at 48 hours and increased significantly at 1, 3, 6, 9, 12, 18, 24, and 48 hours compared to the Vehicle group ($^{*}P < 0.05$; **Figure 1B**). Serum GOT values in the Glycerol + Calcitriol group were significantly lower at 6, 9, 12, 24, and 48 hours compared to the Glycerol group ($^{\#}P < 0.05$; **Figure 1B**). After glycerol injection, serum GPT peaked at 12 hours and increased significantly at 1, 3, 6, 9, 12, 24, and 48 hours compared to the Vehicle group ($^{*}P < 0.05$; **Figure 1C**). Serum GPT values in the Glycerol + Calcitriol group were significantly lower at 3, 6, 9, 12, 24, and 48 hours compared to the Glycerol group ($^{\#}P < 0.05$; **Figure 1C**).

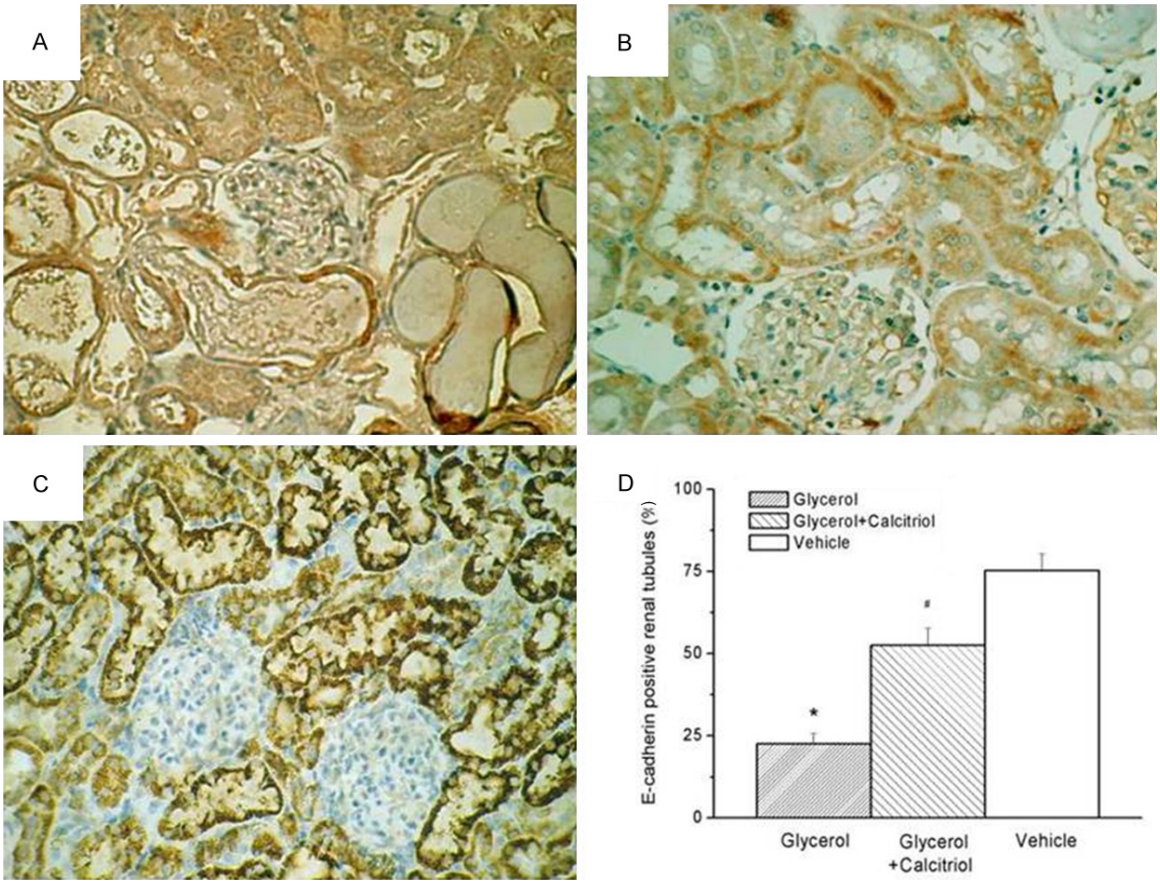

Figure 3. Immunohistochemical staining for E-cadherin in the kidneys. Histological findings from the Glycerol group (A), Glycerol + Calcitriol group (B), and Vehicle group (C) (magnification × 200). E-cadherin-positive tubule score after rhabdomyolysis-induced acute renal failure in rats (D). $^{*}P < 0.05$ for the Glycerol group compared to the Vehicle group. $^{\#}P < 0.05$ for the Glycerol + Calcitriol group compared to the Glycerol group.

Serum levels of BUN and Cre

After glycerol injection, serum BUN increased significantly at 1, 3, 6, 9, 12, 24, and 48 hours compared to the Vehicle group ($^{*}P < 0.05$; **Figure 1D**). Serum BUN values in the Glycerol + Calcitriol group were significantly lower at 6, 9, 12, 24, and 48 hours compared to the Glycerol group ($^{\#}P < 0.05$; **Figure 1D**). After glycerol injection, serum Cre increased significantly at 1, 3, 6, 9, 12, 24, and 48 hours compared to the Vehicle group ($^{*}P < 0.05$; **Figure 1E**). Serum Cre values were significantly lower in the Glycerol + Calcitriol group at 24 and 48 hours compared to the Glycerol group ($^{\#}P < 0.05$; **Figure 1E**).

Renal tissue injury score

In the kidneys of the Vehicle group, we found no epithelial necrosis, and no heme casts (**Figure 2C**). The kidneys obtained from Glycerol group showed moderate epithelial necrosis, tubular dilation, and several heme casts after rhabdomyolysis (**Figure 2A**). The renal tubules of rats in the Glycerol + Calcitriol group showed mild epithelial necrosis, and mild tubular dilation (**Figure 2B**). The renal tissue injury scores significantly increased after glycerol injection compared to the corresponding scores in the Vehicle group at 48 hrs ($^{*}P < 0.05$; **Figure 2D**). Compared with the Glycerol group, the renal tissue injury scores in the Glycerol + Calcitriol group were significantly lower ($^{\#}P < 0.05$; **Figure 2D**).

Expression of E-cadherin

In the Vehicle group, renal tubular expression of E-cadherin showed normal quantity and distribution by IHC staining (**Figure 3C**). After glycerol-induced rhabdomyolysis, there was signifi-

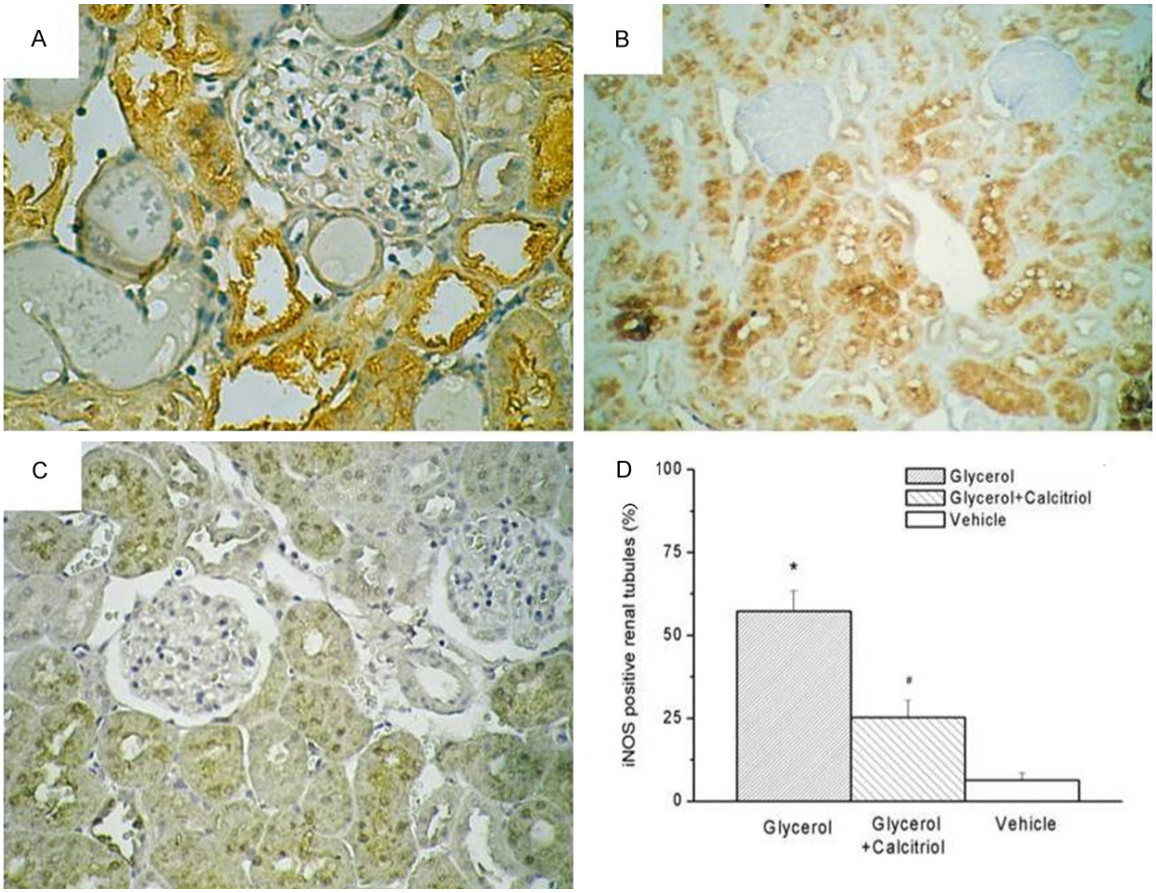

Figure 4. Immunohistochemical staining of iNOS in the kidneys. Histological sections from the Glycerol group (A), Glycerol + Calcitriol group (B), and Vehicle group (C) (magnification × 200). Inducible nitric oxide synthase (iNOS)-positive tubule score after rhabdomyolysis-induced acute renal failure in rats (D). $^{*}P < 0.05$ for the Glycerol group compared to the Vehicle group. $^{\#}P < 0.05$ for the Glycerol + Calcitriol group compared to the Glycerol group.

cantly decreased renal tubular expression of E-cadherin (**Figure 3A**). In the Glycerol + Calcitriol group, renal tubular expression of E-cadherin after glycerol injection showed much preserved (**Figure 3B**). There was significantly decreased renal tubular expression of E-cadherin in the Glycerol group compared to that of the Vehicle group ($^{*}P < 0.05$; **Figure 3D**). The Glycerol + Calcitriol group showed significantly greater renal tubular expression of E-cadherin compared to the Glycerol group ($^{\#}P < 0.05$; **Figure 3D**).

Expression of iNOS

The renal tubular expression of iNOS showed mildly increased in the Vehicle group by IHC stain (**Figure 4C**). After glycerol-induced rhabdomyolysis, there was increased renal tubular expression of iNOS in the Glycerol group (**Figure 4A**). The Glycerol + Calcitriol group showed decreased renal tubular expression of iNOS after rhabdomyolysis (**Figure 4B**). There was significantly greater renal tubular expression of iNOS in the Glycerol group compared to the Vehicle group ($^{*}P < 0.05$; **Figure 4D**). The Glycerol + Calcitriol group showed significantly less renal tubular expression of iNOS compared to the Glycerol group ($^{\#}P < 0.05$; **Figure 4D**).

Expression of NF-κB

In the Vehicle group, there were few renal tubular cells expressing of NF-κB (**Figure 5C**). After glycerol-induced rhabdomyolysis, there was increased renal tubular expression of NF-κB in the Glycerol group (**Figure 5A**). The Glycerol + Calcitriol group showed fewer renal tubular expression of NF-κB after rhabdomyolysis (**Figure 5B**). The renal tubular expression of NF-κB was significantly higher in the Glycerol group compared to the Vehicle group ($^{*}P < 0.05$;

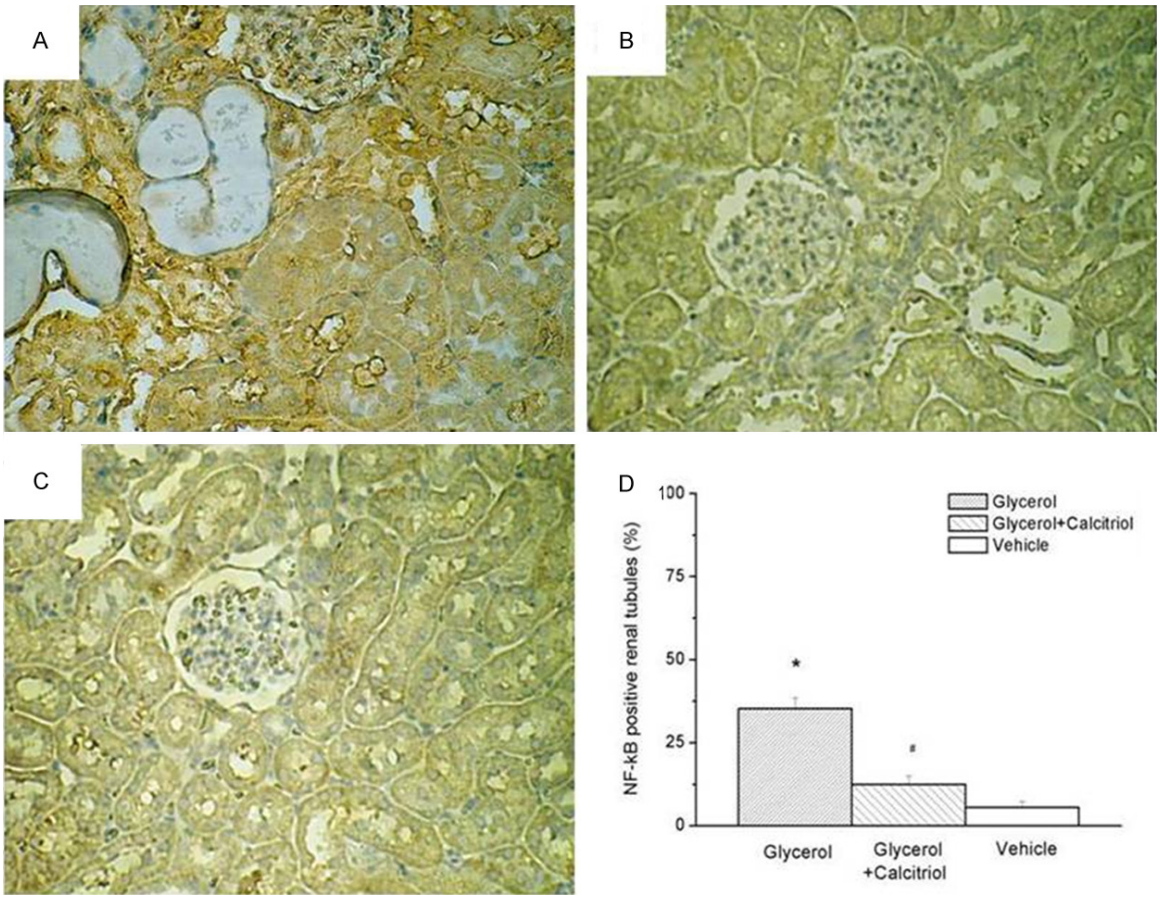

Figure 5. Immunohistochemical staining for NF-κB in the kidneys. Histological sections from the Glycerol group (A), Glycerol + Calcitriol group (B), and Vehicle group (C) (magnification × 200). NF-κB-positive tubule score after rhabdomyolysis-induced acute renal failure in rats (D). $^{*}P < 0.05$ for the Glycerol group compared to the Vehicle group. $^{\#}P < 0.05$ for the Glycerol + Calcitriol group compared to the Glycerol group.

Figure 5D). The Glycerol + Calcitriol group showed significantly less renal tubular expression of NF-κB compared to the Glycerol group ($^{\#}P < 0.05$; **Figure 5D**).

Discussion

In this study of glycerol-induced rhabdomyolysis, it was found that treatment with calcitriol could improve serum markers indicative of organ damages and ameliorates rhabdomyolysis-induced acute renal failure with decreased renal tubular expression of NF-κB, iNOS and increased expression of E-cadherin.

Rhabdomyolysis, regardless of its mechanisms such as damage to the membrane by toxins, crash injuries, or failure to provide adequate ATP following ischemia, could cause a cascade of events that lead to leakage of extracellular calcium ions into the intracellular space and induced a pathologic interaction of actin and myosin with the results of activating intra-cellular proteases to destruct muscle fibers and necrosis [22]. After muscle injuries, large quantities of potassium, phosphate, CK, GOT, urate, and heme protein myoblobin would leak into the circulation, which in the renal glomerular filtrate could precipitate and damage the renal tubules with the results of acute renal failure [1-3, 23]. Acute renal failure was reported to develop in up to 15% of patients after rhabdomyolysis and is associated with high morbidity and mortality [24]. Released heme proteins were metabolized in the kidney, generating free-radicals, scavenging nitric oxide, and activating endothelin receptors, which produced a synergistic effect on renal vasoconstriction, caused intraluminal cast formation, induced inflammation and caused direct toxicity to renal tubular cells to cause acute renal failure [4, 6,

7, 25]. Indirect activation of rennin-angiotensin-aldosteron system because of leakage of extracellular fluid into the damaged muscles or the myoglobin-induced nitric oxide scavenging with release of cytokines could cause renal vasoconstriction [25, 26], and these releasing immune-stimulatory molecules could activate infiltrated macrophages and lymphocytes by activating complement factors, Toll-like receptors and NF-κB with the results of promoting the production of pro-inflammatory cytokines [27]. Moreover, Plotnikov et al. reported increased oxidative stress and mitochondrial dysfunction with uncoupling of oxidative phosphorylation and increasing nitric oxide synthesis when adding myoglobin to renal tubules [12]. Similarly, we demonstrated that glycerol-induced rhabdomyolysis could increase renal tubular necrosis scores, increased renal tubular expression of iNOS and NF-κB, and decreased expression of E-cadherin, which together indicated a possible role of inflammation and oxidative stress to cause acute renal injuries.

In addition to treat the underlying etiologies causing rhabdomyolysis, treatments to preserve renal function including aggressive hydration, using mannitol, urine alkalization and forced dieresis [6, 8]. Moreover, mannitol, as a diuretic agent, besides its effects to increase renal blood flow, glomerular filtration, prevent intratubular heme pigment trapping and cast formation, was reported to have anti-oxidant effects by hydroxyl anion scavenging but with controversial results [9, 10]. By decreasing oxidative injuries, acetaminophen was studied in vitro and found to inhibit hemoprotein-induced lipid peroxidation by reducing ferryl heme to its ferric state and subsequent free radicals [11]. Mitochondrial pore inhibitor, cyclosporine A, mitochondria-targeted antioxidant and deferoxamine could also abrogate respiratory control drop, uncoupling of oxidative phosphorylation, an increase of lipid peroxidation products and stimulated NO synthesis in myoglobin incubated mitochondrial [12]. N-acetylcysteine, vitamin E, flavonoids, L-carnitine, or pentoxifylline were also reported to have anti-oxidant or anti-inflammatory effects against rhabdomyolysis-induced acute kidney injury [13]. Moreover, we recently reported that erythropoietin and phenobarbital could have protective effects against rhabdomyolysis-induced kidney injuries with the demonstration of decreased serum markers of BUN and Cre, suppressed renal tubular expression of NF-κB and iNOS, and preserved the expression E-cadherin [20, 21]. Taken together, we considered that therapeutic strategies towards vasoconstriction, anti-inflammation and anti-oxidant effects could lessen the deteriorating effects of rhabdomyolysis-induced acute renal failure.

Calcitriol, 1,25-dihydroxyvitamin D, the physiologically active form of vitamin D, was traditionally considered as a key regulator of bone metabolism, calcium and phosphorous homeostasis [14]. Besides its traditional effects, the synthetic analogs of vitamin D were being increasingly recognized for their potent anti-proliferative, stimulating cell differentiation, immune-modulatory, and anti-inflammation activities [15]. Studies had shown that vitamin D might mediate inflammation by inhibiting prostaglandin synthesis and actions, by inhibiting stress-activated kinase signaling and resultant production of inflammatory cytokines, and by inhibiting nuclear factor κB (NF-κB) signaling [15, 28]. Serum 25(OH)D3 was reported to be negatively associated with serum and urinary pro-inflammatory markers, and proteinuria and the serum levels of TNF-α and IL-6 improved to an apparent level after calcitriol supplement, which indicated an anti-inflammatory effects of calcitriol [16, 29]. In addition, studies had shown that calcitriol or synthetic vitamin D analog could protect kidneys from lipopolysaccharide-induced or ischemia/reperfusion-induced acute renal injury by down-regulating renal inflammation via inhibiting renal Toll like receptors and NF-κB signaling through the interaction between vitamin D receptor with NF-κB p65 subunit [17, 18], and we recently proved that calcitriol had organs protective effects with decreasing serum pro-inflammatory levels of TNF-α and IL-6 in rats with hemorrhagic shock [19], which together indicated calcitriol could have an important role of renal protection as regulating renal anti-oxidant and anti-inflammatory responses. In this study, we similarly demonstrate that treatment with calcitriol after rhabdomyolysis could protect kidneys by demonstrating improved serum BUN and Cre and renal tissue injury scores. Moreover, we considered the possible mechanism of protection from acute kidney injury might through the anti-oxidant or anti-inflammatory effects of cal-

citriol by demonstrating decreased renal tubular activity of iNOS and NF-κB and preserved expression of E-cadherin in the glycerol + Calcitriol group compared to that of the glycerol group. However, the detailed mechanism of calcitriol on rhabdomyolysis-induced acute kidney injury needed to be further studied.

Conclusions

In conclusion, treating calcitriol in a rat model of glycerol-induced rhabdomyolysis could decrease the levels of BUN, Cre, GOT, GPT, and CPK, suppress the renal tubular expression of NF-κB, iNOS, and preserve the expression of E-cadherin. Taken together, it is our hypothesis that calcitriol could ameliorate the rhabdomyolysis-induced organ damages and acute kidney injury, which supposed calcitriol as a potential promising modality of treatment against rhabdomyolysis.

Acknowledgements

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Disclosure of conflict of interest

None.

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