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Glucocorticoids attenuate septic acute kidney injury

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ABSTRACT

Background: The incidence and mortality of septic acute kidney injury (AKI) remains high, whereas our understanding of pathogenesis for septic AKI is still limited. Glucocorticoids (GCs) have been clinically recommended for treatment of septic shock and also have showed favorable effect on septic AKI in several animal experiments. The aim of this study is to investigate the pathophysiology of septic AKI and the effect of GCs on septic AKI.

Methods: We induced septic AKI using cecal ligation and puncture (CLP) model in 8–10 wk-old male C57BL/6 mice. Saline or dexamethasone (2.5 mg/kg) dissolved in saline was administered after surgery. Hemodynamic, biochemical and histological changes were examined in a time-course manner.

Results: CLP resulted in hyperdynamic warm shock with multiple organ dysfunction including AKI. Despite renal dysfunction, light microscopy showed scanty acute tubular necrosis and inflammation. Instead, CLP induced significant increase in apoptosis of the kidney and spleen cells. In addition, septic kidneys showed mitochondrial injury and alterations in Bcl2 family proteins in the renal tubular cells. Dexamethasone treatment attenuated renal dysfunction, but it was not associated with improvement of hemodynamic parameters. Dexamethasone-induced organ protective effect was associated with reduced mitochondrial injury with preserved cytochrome c oxidase and suppression of proapoptotic proteins as well as reduced cytokine release.

Conclusions: Mitochondrial damage and subsequent apoptosis are thought to play important role in the development of septic AKI. GCs might be a useful therapeutic strategy for septic AKI by reducing mitochondrial damage and apoptosis.

1. Introduction

Mortality from acute kidney injury (AKI) still exceeds 50% in critically ill patients and the most common cause of AKI in those patients are severe sepsis or septic shock [1,2]. Nevertheless, our understanding of pathogenesis for septic AKI is limited and treatment has remained largely supportive. Traditionally, septic AKI has been thought to be prerenal azotemia mediated by intense renal vasoconstriction [3]. However, several recent studies demonstrated that the development of AKI is accompanied by preserved or even increased renal blood flow in large animal model of sepsis [4]. This raises a question about the long-held belief that septic AKI could be understood in the context of "ischemic paradigm" and stimulates new approaches for better understanding the pathogenesis of septic AKI, such as immunologic/cytopathic mechanisms [5].

Low-dose glucocorticoids (GCs) have been clinically recommended in sepsis for the purpose of treating septic shock [6]. Although beneficial effect of GCs in sepsis-associated kidney injury has also been shown [7], precise mechanisms underlying the renoprotective effects are still unclear.

The aim of this study is to investigate the pathophysiology of septic AKI and the effect of GCs on septic AKI using mouse cecal ligation and puncture (CLP) model, which has been known to be most relevant to human sepsis.

2. Materials and methods

2.1. Animal model

All experiments were conducted with the approval of the Korea University Institutional Animal Care and Use Committee. To induce

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septic AKI, we used CLP model [8]. Male C57BL/6 mice (8–10 week of age, weighing 22–25 g; Charles River, Seoul, Korea) were used. Briefly, after a midline laparotomy, the cecum was ligated 1 cm from its distal end, followed by double puncture and manipulated to ensure extrusion of feces into the abdominal cavity. Cecum was relocated into the abdominal cavity, and then the abdomen was closed. Animals were resuscitated by injecting saline (0.05 ml/g) with or without dexamethasone (2.5 mg/kg) immediately after surgery (CLP+V and CLP+Dex, respectively). In sham operated animals, the cecum was isolated, but neither ligated nor punctured. Dexamethasone was obtained from Sigma–Aldrich (Sigma–Aldrich, Missouri, USA).

2.2. Hemodynamic measurements

Blood pressure and heart rate (HR) were measured every hour for a duration of 6 h, and then every 3 h for 24 h, using tail-cuff plethysmography (LE 5001-Pressure Meter, Letica SA, Barcelona, Spain). Echocardiograms were performed in conscious mice using a 13-MHz linear transducer (Vivid 7 ultrasound system, GE Healthcare Co., USA) at 0, 6, 12 and 24 h. Left ventricular (LV) function, ventricular size and wall thickness were measured from M-mode frames.

2.3. Histopathology

Routine histological examination was performed in periodic acid-Schiff (PAS) stained tissues.

For immunohistochemical detection of neutrophils or macrophages, formalin-fixed and paraffin-embedded kidney sections were stained with Ly6G Ab (Sigma–Aldrich) or F4/80 antibody (Serotec, Oxford, UK). Cytochrome c oxidase expression was also examined (Cell Signaling Technology, MA, USA).

For detecting apoptosis, kidney and spleen sections were stained with TUNEL kit (Milipore, MA, USA) and anti-activated caspase-3 antibody (Cell Signaling Technology). The number of positively stained cells was counted in 8 randomly chosen $\times$200 fields of kidney cortex and outer medulla, or in 8, $\times$200 fields of the spleen.

Tissues for electron microscopy (EM) were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed with 1% osmium tetroxide, dehydrated in graded ethanols, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with H-600 electron microscope (Hitachi, Tokyo, Japan).

2.4. Caspase-3 activity

We measured the activity of caspase-3 in the kidney and spleen, using BD ApoAlert Caspase Colorimetric Assay Kit (BD Bioscience, CA, USA), according to the manufacturer's instructions.

2.5. Western blot

Total kidney lysates were prepared by homogenization in ice-cold RIPA buffer (1% TritonX-100, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl) supplemented with protease inhibitor tablet (Roche Diagnostics, Basel, Switzerland), 1 mM Na_3VO_4 and 50 mM NaF. Western blot analyses were performed as previously described [9]. Anti–mouse Bax and Bcl-xL antibodies were purchased from Cell Signaling Technology.

2.6. Quantitation of cytokine production by using cytometric bead array (CBA)

Quantitation of various cytokines and chemokine in the plasma and kidney tissues was done by CBA. A mouse inflammation kit (BD Bioscience) was used according to the manufacturer's instructions to simultaneously detect mouse IL-12p70, TNF-α, IL-6, MCP-1, IFN-γ and IL-10, as previously described [10].

2.7. Cell culture

The HK-2 cells (American Tissue Culture Collection, Rockville, MD, USA), an immortalized proximal tubular epithelial cell line from normal adult human kidney, were grown in DMEM Ham's F12 media (1:1) supplemented with 10% FCS and antibiotics. The cells were seeded in a 6-well tissue culture plate and allowed to adhere for 24 h in an incubator at 37 °C with 5% CO_2 in 95% air, and were subsequently serum starved overnight. Then, 100 ng/ml of TNF-α was added to induce apoptosis, and dexamethasone was also added in the indicated cells. After 24 h, the cells were harvested and examined for immunoblotting with anti-cytochrome c oxidase antibody.

2.8. Statistical analysis

Statistical analyses were performed using SPSS 14.0 for Windows. Comparisons between the groups were examined by Mann–Whitney test or Kruscal–Wallis test. Statistical significance was considered at a $p < 0.05$.

3. Results

3.1. Effect of dexamethasone on kidney dysfunction induced by CLP

Plasma BUN and creatitnine (Cr) started to increase at 12 h after CLP, and the mean plasma Cr at 24 h was significantly higher in CLP+V mice (0.18 ± 0.05 vs 0.81 ± 0.21 mg/dl), confirming the development of septic AKI (Fig. 1A and B). Table 1 shows biochemical data at 24 h after CLP, and demonstrates that our mice model of CLP induced polymicrobial peritonitis with multiple organ dysfunction including AKI and liver dysfunction.

Next, we compared kidney dysfunction between CLP+V and CLP+Dex mice. As shown in Fig. 1(C and D), dexamethasone significantly reduced plasma BUN (78.6 ± 19.7 vs 43.2 ± 21.9) and Cr (0.72 ± 0.21 vs 0.42 ± 0.20) level compared to CLP+V mice.

3.2. Effect of dexamethasone on the hemodynamic parameters

We examined hemodynamics in a time course manner in CLP mice that are awake. As shown in S.1, the mean arterial pressure (MAP) significantly decreased starting at 3 h after CLP. HR significantly increased at 1 h, but decreased to the baseline level at 18 h. Fractional shortening (FS), that is known to represent cardiac systolic function, significantly increased at 6 h, and remained high until 24 h in CLP mice, suggesting that CLP induced the hyperdynamic "warm shock" state. Although GCs has been shown to attenuate circulatory failure in a previous rat endotoxemia model [7], hemodynamic parameters showed comparable values for MAP, HR, FS and LVMI between CLP+V and CLP+Dex group during the experimental period (S.1 and 2).

3.3. Effect of dexamethasone on cytokine profiles

We next examined the effect of dexamethasone on cytokine levels. As expected, CLP induced a profound increase of pro-inflammatory cytokines, such as TNF-α, MCP-1 and IL-6, as well as anti-inflammatory cytokine, IL-10 in plasma (S.3A). In mice treated with dexamethasone, all these cytokine levels were significantly reduced compared with those in the CLP mice. Similar findings were observed in cytokine levels of the septic kidneys (S.3B).

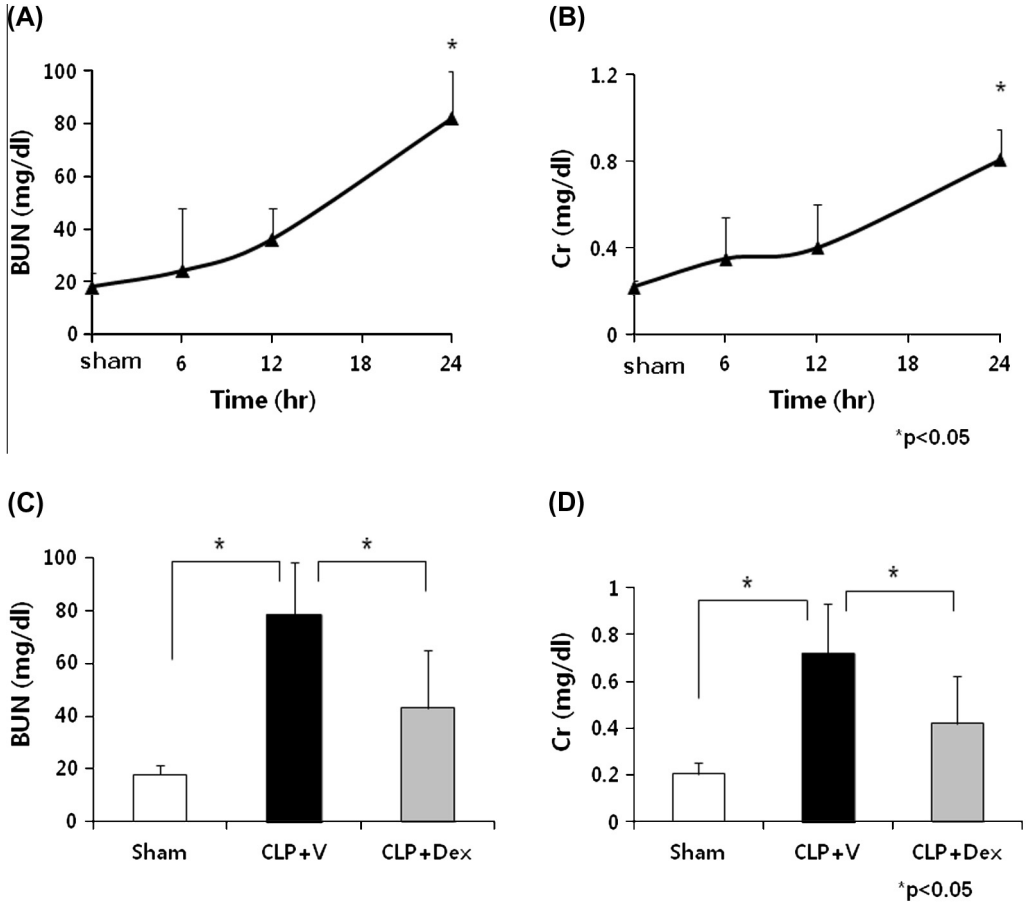

Fig. 1. Dexamethasone attenuates renal dysfunction induced by CLP. (A–B): Time course renal dysfunction in CLP mice. *$p < 0.05$ vs sham at the indicated time point. ($n = 4$ in sham; $n = 4$ at 6 h, $n = 5$ at 12 h, $n = 5$ at 24 h). (C–D): Plasma BUN and Cr concentrations were compared at 24 h after surgery in sham ($n = 4$), CLP + V (V, vehicle) ($n = 14$) and CLP + Dex mice ($n = 12$). Values are expressed as the mean (SD).

Table 1
CLP induces polymicrobial peritonitis including AKI.

	Sham	CLP
BUN (mg/dl)	18.7 ± 5.5	82.1 ± 15.1*
Cr (mg/dl)	0.18 ± 0.05	0.81 ± 0.21*
LDH (IU/L)	721 ± 126.3	5238.5 ± 788.1*
ALT (IU/L)	25.6 ± 3.2	172 ± 19.6*
Peritoneal fluid culture	none	*E. coli*, *Enterococcus fecalis*

Values are mean ± sd.
* $p < 0.05$ vs sham. Sham ($n = 5$); CLP ($n = 11$).

3.4. Effect of dexamethasone on apoptosis of kidney tubule cells

In contrast to ischemic AKI, PAS-stained kidney tissue section showed only minor changes, such as sparse vacuolar degeneration of the tubular cells and minimal focal necrosis (data not shown). However, substantial number of TUNEL positive apoptotic tubular cells were found in the cortex and outer medulla of the kidney in a time-dependent manner. In addition, spleen cell apoptosis was also observed and they were mainly located in white pulp.

Dexamethasone-treated mice showed significantly reduced apoptosis of the kidney tubular cells, as well as spleen cells compared with CLP mice at 24 h (Fig. 2A and B). Caspase-3 activity also increased in the septic kidneys and spleens, which was reduced by dexamethasone treated mice (Fig. 2C).

3.5. Effects of dexamethasone on mitochondrial change in sepsis

Growing body of evidence suggests that mitochondrial injury and subsequent bioenergic abnormalities underlie the development of multiorgan dysfunction in severe sepsis. In our study, ultrastructural study of the septic kidneys, using transmission EM, showed chromatin aggregation with cytoplasmic blebbing compatible with apoptosis (data not shown). In addition, we observed swollen mitochondria with rarefied cristae, as well as a number of vacuoles in the tubular cells, which was reduced by dexamethasone treatment (Fig. 3A).

Next, to assess the functional change of mitochondria in the septic kidneys, we measured the amount of cytochrome c oxidase (COX). COX is the last enzyme in the respiratory transport chain located in the mitochondrial membrane, and regarded as the major rate-limiting step for oxidative phosphorylation [11]. As shown in Fig. 3B and C, the expression of COX significantly reduced at 24 h after CLP with the most prominent reduction, affecting the cortex and outer stripe of the outer medulla. Dexamethasone treatment markedly attenuated CLP-induced COX suppression.

3.6. Direct effect of dexamethasone on renal tubular cells

We next examined the direct effect of dexamethasone on the cultured renal tubular epithelial cells. Functions of mitochondria in the tubular cells were assessed by Western blotting of COX after TNF-α exposure in the presence or absence of dexamethasone. Incubation of the HK-2 cells with TNF-α markedly decreased COX

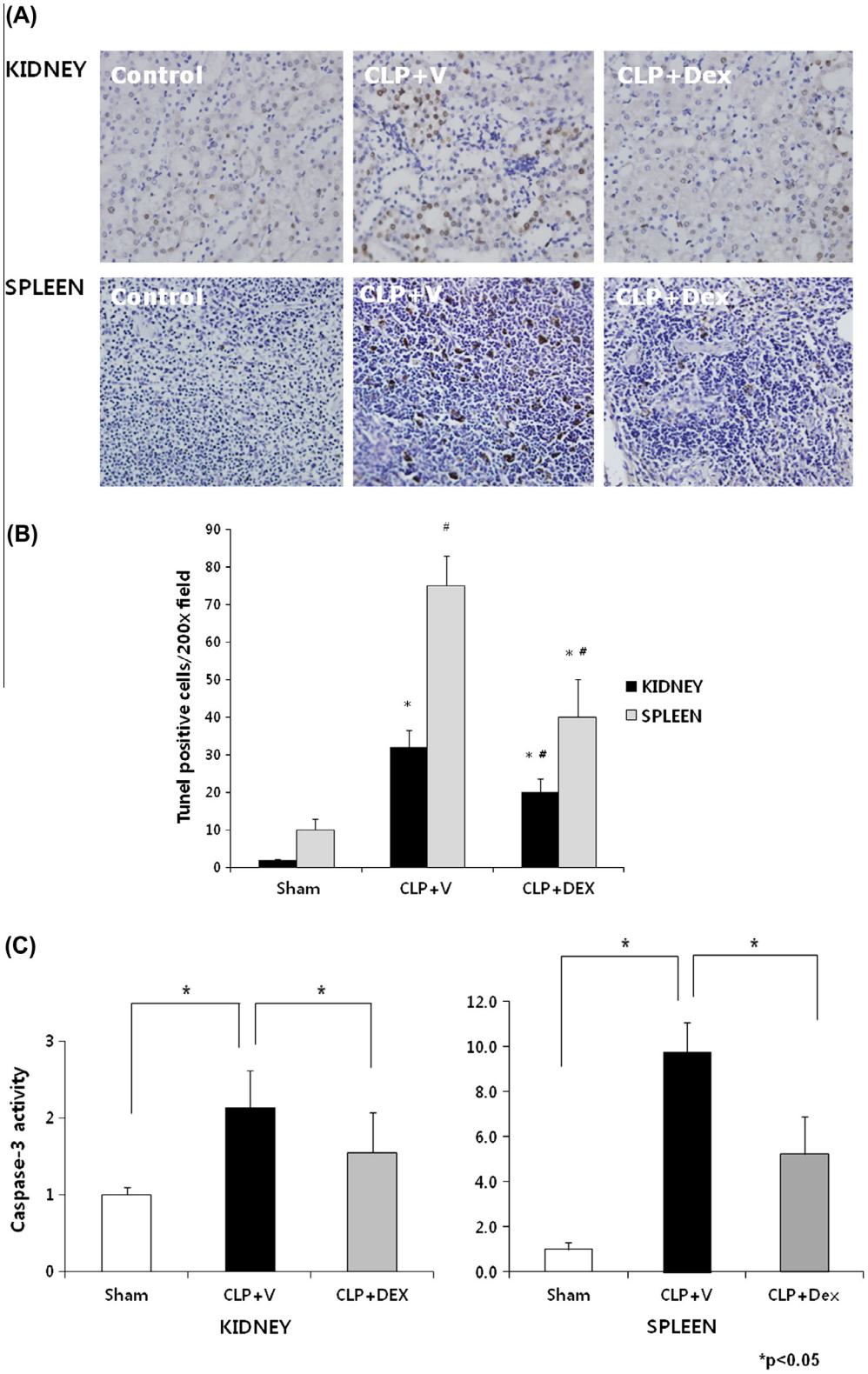

Fig. 2. Dexamethasone significantly reduces apoptosis of renal and spleen cells. (A) TUNEL assay (×200). The experiments were conducted using tissues harvested at 24 h after surgery in sham, CLP + V and CLP + Dex mice. (B) The number of TUNEL-positive cells was quantified. $^{*}p < 0.05$ vs sham; $^{\#}p < 0.05$ vs CLP + V. (C) Caspase-3 activity. Values are expressed as the mean (SD). (Sham, $n = 4$; CLP + V, $n = 7$; CLP + Dex, $n = 5$).

protein levels at 24 h (Fig. 3D). However, addition of dexamethasone significantly prevented the reduction of COX expression induced by TNF-α. There was no difference in the expression of COX between the cells incubated with dexamethasone alone and control cells.

3.7. Inhibition of proapoptotic protein expression by dexamethasone

According to the previous observations that Bcl-2 family proteins play a key role in regulating mitochondrial permeability, release of cytochrome c, COX activity and subsequent apoptosis

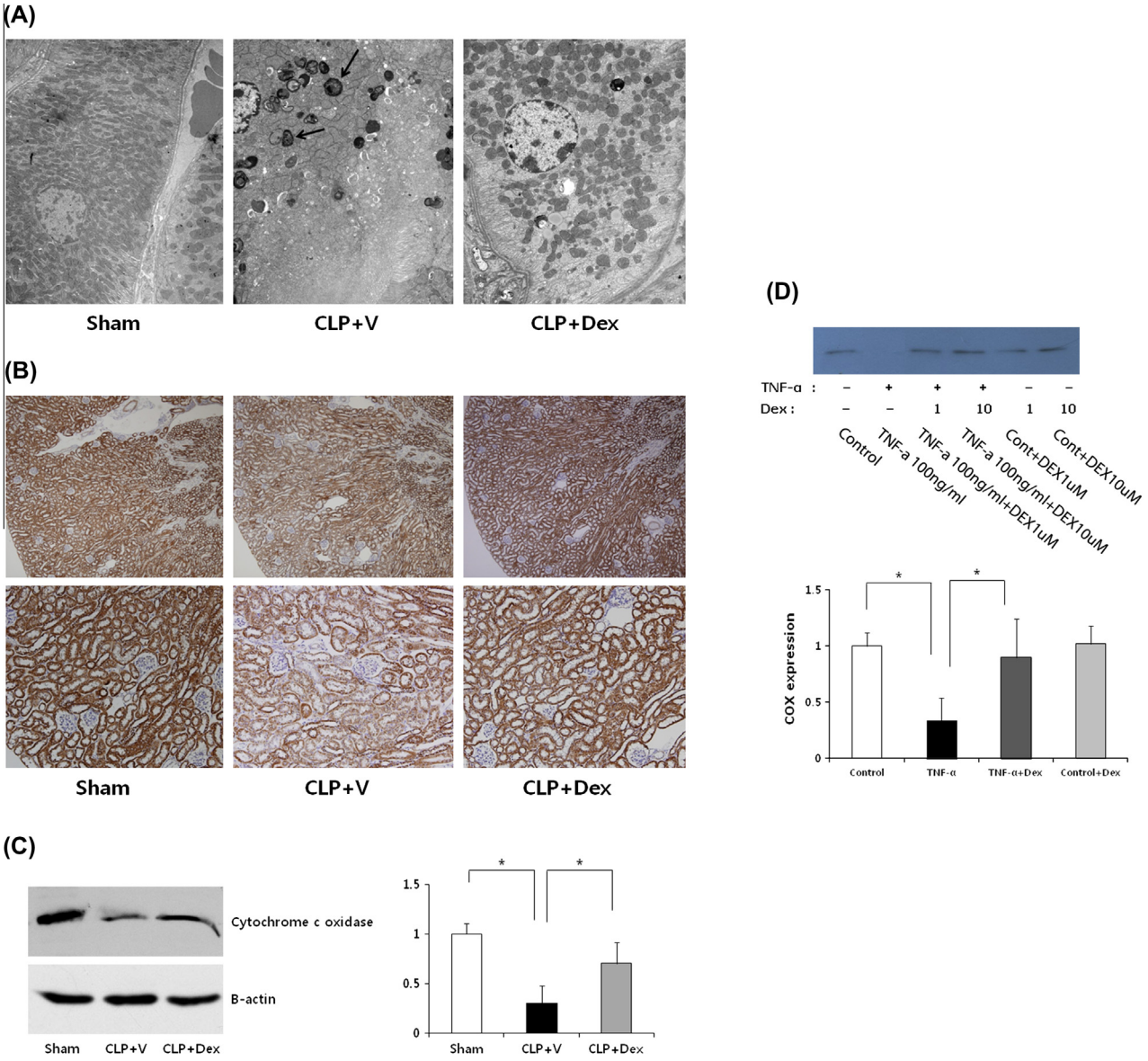

Fig. 3. Dexamethasone attenuates sepsis-induced changes in structure and function of renal tubular mitochondria. (A) Transmission electron microscopy (TEM) (3500×). Arrows indicate damaged mitochondria. (B) Staining for cytochrome *c* oxidase (COX) of kidneys.40× (upper panel),100× (lower panel) (C) Western blot analysis of whole kidney lysates for COX. $^{*}p < 0.05$. (Sham, $n = 3$; CLP + V, $n = 4$; CLP + Dex, $n = 4$). (D) HK-2 cells were incubated with TNF-α (100 ng/ml) and indicated doses of Dexamethasone (Dex) for 24 h, and Western blot analysis for COX was done. $^{*}p < 0.05$.

[11–13], we next examined the levels of Bcl-2 family proteins; Bax (proapoptotic) and Bcl-xL (antiapoptotic) to explore molecular mechanisms underlying the anti-apoptotic effect of dexamethasone in the septic kidneys. While the expression of both Bax and Bcl-xL were significantly increased at 24 h in the septic kidneys, the ratio of Bax/Bcl-xL increased significantly in the septic kidneys, and dexamethasone treatment was associated with more striking reduction in Bax expression, resulting in partial restoration of Bax/Bcl-xL ratio to normal (Fig. 4).

4. Discussion

In this study, we found that (1) CLP induced hyperdynamic warm shock with multiorgan dysfunction, including AKI, (2) despite renal dysfunction, CLP showed scanty tubular necrosis and inflammation. Instead, septic kidneys were characterized by immune cell and renal tubular cell apoptosis, (3) septic AKI was also associated with mitochondrial ultrastructural changes with reduced amount of COX and increased Bax/Bcl-xL ratio, (4) Dexamethasone treatment attenuated septic AKI irrespective of hemodynamic parameters, and its beneficial effect was associated with reduced mitochondrial damage, restoration of COX and Bax/Bcl-xL ratio, and subsequent inhibition of tubular cell apoptosis.

Sepsis is the most common cause of AKI in critically ill patients, and the presence of AKI in these patients is known to be an independent risk factor for mortality [1]. Despite progress in supportive care, mortality from septic AKI still remains high, and this is largely due to our poor understanding of the mechanisms leading to AKI in septic patients, sepsis-induced organ dysfunction in other words. Lack of histopathologic information adds uncertainty and makes it more difficult to assess what is really happening in the septic kidneys. Although excessive innate immune activation has been thought to be critical in the development of sepsis or sepsis induced organ dysfunction, strategies targeting inflammation was found to be unsuccessful [14]. Instead, recent evidence suggests

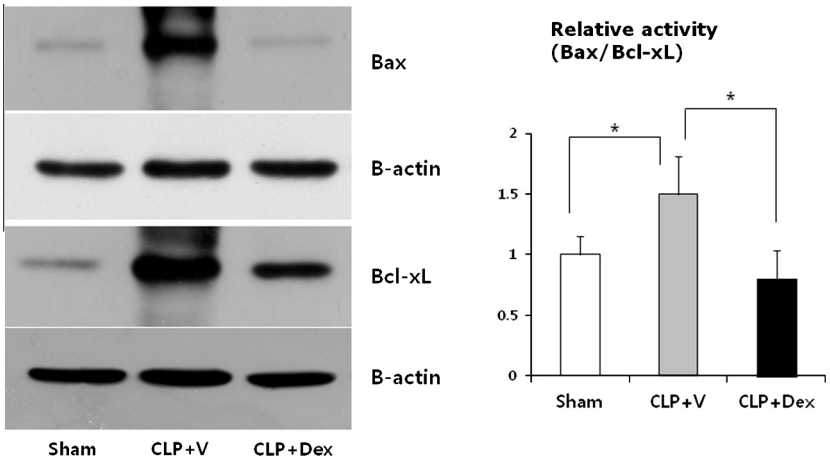

Fig. 4. Dexamethasone alters the expression of Bcl-2 family protein and attenuates the activation of pro-apoptotic proteins in septic kidney. Expression of proapoptotic protein, Bax and anti-apoptotic protein, Bcl-xL at 24 h after CLP were examined by western blot analysis. Values are expressed as the mean (SD). $^{*}p < 0.05$. (Sham, $n = 3$; CLP, $n = 4$; CLP + Dex, $n = 4$).

that mitochondrial damage and subsequent defect in mitochondrial respiratory chain might be key factors in cellular dysfunction, leading to end-organ failure in sepsis [15]. However, the presence of mitochondrial cytopathy in kidneys with sepsis has not been thoroughly investigated.

In our model of polymicrobial sepsis with AKI, light microscopic examination demonstrated that septic kidneys were only characterized by very subtle tubular damage with occasional vaculolization and lack of inflammatory cell infiltration. The only remarkable histologic abnormality was increased tubular cell apoptosis. The possible contribution of apoptosis in septic AKI has been suggested in several other studies. Lerolle et al. analyzed postmortem histopathological findings in 19 patients with septic AKI and reported that tubular cell apoptosis was major histologic abnormality, while tubular necrosis was only sparsely found, even among patients with severe septic shock. [16]. The significance of tubular cell apoptosis in septic AKI can also be supported by *in vitro* observation that the renal tubular cells undergo apoptotic cell death by inflammatory cytokines and LPS [17]. In addition, recent observation by Lee et al. showing that kidney caspase-3 activity was positively correlated with renal dysfunction in experimental septic AKI, can also support the notion that renal cell apoptosis might contribute to the development of AKI in sepsis [18]. In this study, we confirmed the occurrence of tubular cell apoptosis morphologically and also by demonstrating increased caspase-3 activity. We also observed that substantial number of the immune cells in the spleen underwent apoptosis. However, the role of immune cell apoptosis in the development of AKI cannot be determined from this study.

Mitochondria is a key cellular organelle that regulates events related to energy production. Various effectors that can induce changes in mitochondrial membrane integrity are known to provoke cellular apoptosis by releasing cytochrome c and subsequent activation of downstream caspases [12]. The recent observation that endotoxemia caused decreased oxygen delivery to the kidney without changing tissue oxygen level, strongly suggest alteration or failure of mitochondrial oxygen consumption by the kidney cells in sepsis [19].

In our study using polymicrobial sepsis model, we also confirmed the structural mitochondrial alteration as well as functional defect. In addition to structural mitochondrial damage in ultrastructural examination, suppressed COX expression was evident in the septic kidneys. Cytochrome c oxidase, or complex IV is the last enzyme constituent in the respiratory electron transport chain of mitochondria, helping to establish a transmembrane difference of proton electrochemical potential for synthesis of ATP. In addition, we also observed that the ratio of Bax to Bcl-xL markedly increased in the septic kidneys. Proteins of Bcl-2 family has been known to regulate mitochondrial respiration through COX activity in various condition, and alterations in their expression can lead to mitochondrial dysfunction and release of cytochrome c, which is a crucial event in the pro-apoptotic process [13]. As previous studies have demonstrated that COX suppression sensitize the cells to apoptosis by increasing ROS production [11,20], mitochondrial damage and COX suppression in septic kidneys are likely to contribute to renal tubular cell apoptosis and subsequent development of AKI in sepsis. Tran et al. recently observed the selective suppression of gene expression profiles that are involved in oxidative phosphorylation in sepsis-associated AKI [19]. They also demonstrated that mice deficient in PGC-1α, a major regulator of mitochondrial biogenesis and metabolism, suffered more persistent injury in an endotoxemic AKI model, suggesting the important role of mitochondrial biogenesis in the development of organ dysfunction in sepsis.

Low dose GCs are currently recommended for treatment of refractory septic shock [6]. Although presence of relative adrenal insufficiency, GCs' immune modulatory effect or inhibitory effect on iNOS with enhancing the effect of vasopressors are possible explanations for GCs usage, precise mechanisms of action have not been fully clarified yet [21,22]. In addition, GCs' pleiotrophic effects that may alter cell biology have been recently reported, and anti-apoptotic effect of GCs in several different cell types have been previously demonstrated [23,24]. More recently, Kumar et al. have first reported that dexamethasone, a synthetic GCs, ameliorated renal ischemia–reperfusion injury by its direct anti-apoptotic effect on the proximal tubule cells via nongenomic activation of a survival pathway [25]. On the basis of these findings, we tested whether GCs could reduce mitochondrial injury and renal tubular apoptosis in septic AKI.

First, we observed that dexamethasone treatment after CLP markedly attenuated kidney dysfunction. However, the observed renoprotective effect was not accompanied by improvement in hemodynamic parameters. This finding is important because it might support the notion that tubular mitochondrial damage and apoptosis are not simply caused by renal hypoperfusion from systemic hypotension or intrarenal vasoconstriction, and thus, dexamethasone-induced renoprotective effect was independent of hemodynamic effect. More importantly, we observed that tubular cell mitochondrial structural alterations with decreased expression of COX were partially restored in the septic kidneys by dexameth-

asone treatment. Increased Bax/Bcl-xL ratio observed in septic kidneys were also partially restored by dexamethasone treatment, suggesting that it has the protective effect on mitochondria by preserving the enzyme in respiratory electron transport chain, and also altering the expression of Bcl_2-family proteins. Finally, we observed that dexamethasone directly prevented TNF-α-induced COX suppression in cultured renal tubular cells. Incubation with proinflammatory cytokine, TNF-α decreased the expression of COX, indicating that TNF-α provoke mitochondrial damage. Although dexamethasone treatment alone had no effect on COX expression in the tubular cells, co-treatment with dexamethasone partially restored the suppression of COX induced by TNF-α, suggesting the direct protective effect of dexamethasone on mitochondrial respiratory chain.

However, precise downstream mechanisms of dexamethasone on mitochondria are not clear in this study, despite that mitochondria is known to have glucocorticoid receptors [26]. Direct or indirect interactions between steroids and mitochondria possibly through steroid receptor on mitochondria are relatively a new area and need future research.

In elucidating the mechanisms of GCs in septic AKI, its anti-inflammatory action that is inherent in steroid cannot be overlooked. Although several clinical trials targeting inflammation, such as TNF-α have failed to show any clinical benefit, it is clear that overwhelming activation of early innate immune activation is a prerequisite in sepsis-induced mortality or organ dysfunction. We observed that dexamethasone-induced beneficial effect in septic AKI was accompanied by generalized suppression of cytokine and chemokine surge following CLP. However, because not only pro-inflammatory but also anti-inflammatory cytokines decreased, the exact mechanism of dexamethasone-induced beneficial effect in terms of cytokine level cannot be determined in this study. In the present study, GCs treatment of mice was performed with dose of 2.5 m/kg, the human equivalent dose of which is 0.20 mg/kg, that is administered to septic patients as low dose GCs [27].

Further studies are needed to elucidate the more detailed molecular mechanisms involved in the pathogenesis of septic AKI, and also in the beneficial effect of GCs to overcome this devastating disease.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.042.

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