## Abstract

**Background:** Crush syndrome (CS) is characterized by ischemia/reperfusion (I/R)-induced rhabdomyolysis and the subsequent onset of systemic inflammation. CS is associated with a high mortality, even when patients are treated with conventional therapy. We hypothesized treatment of lethal CS rat model with dexamethasone (DEX) have therapeutic effects on the laboratory findings and clinical course and outcome.

**Methods:** To create a CS model, anesthetized rats were subjected to bilateral hind limb compression with rubber tourniquets for 5 hours, and randomly divided into 3 groups: saline-treated CS, and low (0.1 mg/kg) and high doses (5.0 mg/kg) of DEX-treated CS groups. Saline for the CS group or DEX for the DEX-treated CS groups was intravenously administered immediately before reperfusion. Under continuous monitoring and recording of arterial blood pressures, blood and tissue samples were collected for histological and biochemical analysis at designated time period before and after reperfusion.

**Results:** Under continuous monitoring and recording of arterial blood pressures, blood and tissue samples were collected for histological and biochemical analysis at designated time period before and after reperfusion. Ischemic compression of rat hind limbs reduced the nitrite content in the crushed muscle, and the subsequent reperfusion induced reactive oxygen species-mediated circulatory collapse and systemic inflammation, finally resulting in a mortality rate of 76% by 48 hours after reperfusion. A single injection of high-dose DEX

immediately before reperfusion activated endothelial nitric oxide synthase (eNOS) by sequential phosphorylation through the non-genomic phosphoinositide 3-kinase (PI3K)-Akt-eNOS signaling pathway. DEX also exhibited anti-inflammatory effects by modulating pro- and anti-inflammatory mediators, consequently suppressing myeloperoxidase activities and subsequent systemic inflammation, showing a complete recovery of the rats from lethal CS.

**Conclusion:** These results indicate that high-dose DEX reduces systemic inflammation and contributes to the improved survival rate in a rat CS model.

**Level of Evidence: Level Ⅰ**

**Key words:** crush syndrome, ischemia/reperfusion, nitric oxide, Dexamethasone, nitrite

# Acute lethal crush-injured rats can be successfully rescued by a single injection of high-dose dexamethasone through a pathway involving PI3K-Akt-eNOS signaling

**Running title: The Effects of Dexamethasone on Crush Syndrome**

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## Introduction

Crush syndrome (CS) is a serious medical condition characterized by hypovolemic circulatory shock, hyperkalemic metabolic acidosis, and acute myoglobinuric renal failure and is frequently observed after traumatic events such as earthquakes, landslides, and vehicle accidents[1].

Currently recommended treatment for CS involves early aggressive volume repletion using normal saline and alkaline diuresis with bicarbonate and mannitol, combined with the correction of hyperkalemia[1]. When such patients demonstrate acute myoglobinuric renal failure that is severe enough to produce refractory hyperkalemia, acidosis, and uremia, transfer to a hospital and hemodialysis are urgently indicated. Although the use of antioxidants and free-radical scavengers has emerged as a promising new approach for the treatment of acute kidney injury[2] and systemic inflammation, controlled studies evaluating their efficacy are currently lacking. At present, no satisfactory treatment has been established for CS because these conventional managements provide only symptomatic management, not treatment of the underlying mechanism.

The most important mechanism causing CS-related mortality is ischemia/reperfusion (I/R)-induced rhabdomyolysis and the subsequent onset of reactive oxygen species (ROS)-mediated systemic inflammation[3,4], we therefore directed our therapeutic attention to an old drug, glucocorticoid, which has recently been attracting new research attention due to

the recent recognition of its anti-inflammatory action in a receptor-dependent, but transcription-independent, manner (non-genomic pathway).Recent studies have reported beneficial effects of glucocorticoids in the treatment of experimental I/R injury due to myocardial infarction[5], cerebral stroke[6], and kidney damage[7] through the non-genomic pathway, in which glucocorticoid receptor-mediated signaling phosphorylates phosphoinositide 3-kinase (PI3K) and subsequently activates Akt and endothelial nitric oxide synthase (eNOS)[8]. However, there has so far been no experimental evidence of the potential therapeutic benefits of glucocorticoid administration for CS.

We previously reported the development of a practical animal model for crush injury using simple rubber tourniquets compressing the bilateral hind limbs of the rat for 5 hours[9]. This model has a similar clinical appearance to lethal human CS, with a high mortality due to rhabdomyolysis, serious arrhythmia with hyperkalemia, circulatory shock, and remote organ failure, including lung edema and acute renal failure. Using this model, in the present study we investigated the effects of dexamethasone (DEX) on the clinical course and outcome of CS, and showed complete recovery of the rats from lethal crush injury. We also provide evidence of the possible mechanism underlying the anti-I/R injury effects of DEX. Moreover, this study provides a simpler and more useful first-aid treatment for CS, which should be implemented as soon as possible in the field of disaster medicine.

## MATERIALS AND METHODS

### *Animal model*

Male Wistar rats weighing 250–300 g obtained from Kiwa Laboratory Animals (Wakayama, Japan) were housed in a room maintained at a temperature of 23 ± 3 °C and a relative humidity of 55 ± 15% with a 12-h light-dark cycle, with free access to food and water. All animal experiments were conducted according to the guidelines for animal use approved by the Life Science Research Center of Josai University (approval reference number: H23015). Anesthesia was induced by the intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). The body temperature was maintained by a heating lamp during the experiment. The CS model was generated as reported previously[9]. In brief, a rubber tourniquet that applied by wrapping five turns around a metal cylinder under a 2 kg load, and the end of the band was glued. After a given period of time, the compression was released by cutting and removing the tourniquet.

### *Experimental design*

The animals were randomly assigned to four groups. Three CS groups were subjected to hind limb compression for 5 hours[9] (SDC Method 1), followed by the infusion of 100 μL of normal saline (CS control group: n=44) or the infusion of 100 μL of different doses of dexamethasone 21-phosphate disodium salt (DEX, SIGMA-ALDRICH) (0.1 or 5.0 mg/kg of

DEX, CS+DEX-0.1: n=29 and CS+DEX-5.0: n=25, respectively) immediately before reperfusion. A sham group (n=25) serving as a control underwent the same procedures as all the three CS groups except for the compression and decompression with rubber tourniquets. All rats were placed under anesthesia (intraperitoneal injection of pentobarbital sodium, 50 mg/kg body weight) during the 5 h compression period, then were allowed to recover and were kept without intravenous fluid solution but with oral free access to food and water until they were killed for the sampling of blood and tissues at the designated time periods.

In a separate experiment, four groups of rats (CS control group: n=29; sham and CS+DEX-5.0 groups: n=10; and CS+DEX-0.1 group: n=14) were prepared and observed for 48 h after reperfusion to determine the survival rates.

To compare the therapeutic effects of DEX with the conventional therapies such us early fluid resuscitation and/or correction of acidosis, another set of experiment was also performed using the CS rats treated with fluid resuscitation (30mL/kg/hr, starting immediately after reperfusion for first 3 hours) of saline or bicarbonated Ringer's solution (BICARBON®, Ajinomoto Pharmaceutical Co. Ltd. Tokyo).

***Recording and analysis of the mean arterial pressure***

At the designated time period after reperfusion, each group was re-anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) to record the mean

arterial blood pressure (MAP). The femoral artery was cannulated with a polyethylene catheter (INTERMEDIC™ PE-50 tubing; Becton Dickinson and Company, Sparks, MD) connected to a pressure transducer. The blood pressures were amplified, displayed, and recorded via a PowerLab™ data acquisition system (AD Instruments, UK).

***Blood and tissue sampling***

Blood samples from each group were obtained at one, three, six, and 24 h after reperfusion (n=3). The analyses of the pH, $PO_2$, $PCO_2$, $HCO_3^-$, and base excess (BE) of the arterial blood obtained from the femoral artery were performed using a blood gas analyzer (i-STAT300F®, FUSO Pharmaceutical Industries, Ltd., Osaka, Japan). Venous blood from the postcaval vein was sampled and centrifuged to measure the plasma levels of potassium ion ($K^+$), blood urea nitrogen (BUN), creatinine, creatine phosphokinase (CPK), and lactate dehydrogenase (LDH) (measurements were performed by SRL Inc., Tokyo, Japan). Tissue samples from the gastrocnemius muscles, kidneys, and lungs were subjected to a histological analysis, measurement of the MPO activity, nitrite or nitrate level, and a Western blot analysis.

***Nitrite and nitrate concentrations in the muscle and plasma***

The nitrite concentrations in muscle were measured using a dedicated HPLC system (ENO-20; Eicom, Kyoto, Japan) according to the method previously reported[3].

***Plasma levels of interleukin 6 and 10***

The plasma levels of interleukin (IL)-6 and 10 (Rat IL-6 and 10 ELISA kit; Endogen, Inc) were measured by ELISA according to the manufacturer's instructions.

***Western blot analysis***

According to the method previously reported [6], the muscle tissue samples obtained from the rats were homogenized and centrifuged Muscle lysates were applied to SDS-PAGE using antibodies against eNOS, P-eNOS (Santa Cruz Biotechnology), Akt, P-Akt, p85, P-p85, iNOS (Cell Signaling), HO-1 (Thermo), and α-tubulin (Cell Signaling). The bound antibodies were visualized using the ECL chemiluminescence detection system (SuperSignal West Dura Extended Duration Substrate, Pierce) with HRP-conjugated secondary antibodies (Pierce). The band intensity was quantified using a Bio Imaging System, with the Gene Snap and Gene Tools software programs (Syngene Bio Imaging, Cambridge, USA). Control of the protein loading and transfer was conducted by determining the α-tubulin levels.

***Determination of ROS***

ROS production in the injured gastrocnemius muscle was determined by measuring the thiobarbituric acid reactive substances (TBARS) according to the method previously reported by Uchiyama *et al.*[10]

***Histology and MPO activity***

For the histological evaluations, tissue samples were collected 3 hours after reperfusion, then fixed in 10% formalin and embedded in paraffin wax. The sections were then cut and stained with hematoxylin and eosin, and were carefully examined microscopically (SDC Method 2). MPO activities in the muscle tissues were measured according to the technique described by Murata et al[9].

***Statistical analysis***

The results are expressed as the means ± the standard error of the mean (SEM). ANOVA was used for each of the variables to assess the differences between each group, and Tukey's honestly significant difference test or the Tukey-Kramer test was used to compare the results between groups. Survival curves were calculated by the Kaplan-Meier method, with the level of significance set at $p < 0.05$, and survival was compared by a log-rank test. Differences were considered to be significant for values of $p < 0.05$.

## RESULTS

### Survival rates

In the present study, crush injury gradually decreased the survival rate in the CS control group, with the survival rate decreasing to 76%, 45%, and 28% at 3, 6, and 18 hours after reperfusion, respectively, then finally to 24% by 48 hours after reperfusion (SDC Figure 1). On the other hand, the treatment of CS rats with CS+DEX-0.1 group significantly improved the survival rate until 18 hours after reperfusion, although it ultimately ended in failure 48 hours after reperfusion, with a final mortality rate similar to the CS control group. However, to our surprise, CS+DEX-5.0 group saved all CS rats, and maintained a 100% survival rate over the 48-hour experimental period (SDC Figure 1). On the other hand, the conventional therapies of fluid resuscitation with saline and bicarbonated Ringer's solution for this CS models (30% and 60% of survival rates by 48 hours after reperfusion, respectively) appear to be inferior to DEX treatment in survival rate (SDC Figure 2).

### Effects of DEX on plasma biochemical markers, mean arterial pressure and blood gas

As shown in Table 1, the CS control rats showed significant increases in the markers of rhabdomyolysis, such as CPK, $K^+$, and LDH in the plasma as early as one hour after reperfusion, with each peak value observed at 3, 6, and 24 hours after reperfusion, respectively. In Table 2, the CS control rats showed a significantly decreased MAP

throughout the 24-hour experimental period, which consequently induced metabolic acidosis shown in the arterial blood gas analysis (pH, base excess, $HCO_3^-$ and lactate). However, DEX remarkably suppressed not only rhabdomyolysis, but also the subsequent hypovolemic hypotension in a dose-dependent manner (Table 2). In addition, the circulatory improvement associated with DEX also decreased the plasma levels of renal damage markers, such as creatinine and BUN, which began to increase at three hours after reperfusion in the CS control rats (Table 1). More beneficial effects of DEX were also shown than the conventional therapies of fluid resuscitation on plasma biochemical markers, MAP and blood gas (SDC Table 1 and 2).

**DEX-induced NO production through the non-genomic signaling pathway and the subsequent reduction of ROS production**

We examined the non-genomic activation of the PI3K-Akt-eNOS signaling pathway in injured muscle by DEX. DEX exhibited a tendency to increase the phosphorylation of the regulatory p85 subunit of PI3K compared with untreated CS rats in the early phase (0.5-3 h) after reperfusion (Figure 1A), and CS+DEX-5.0 group significantly activated and amplified the Akt phosphorylation and subsequent eNOS phosphorylation at 1 and 3 hours after reperfusion (Figures 1B and C). However, it has been difficult to evaluate the NO production in injured muscle. Because of the difficulty associated with evaluating the biological NO

production directly using *in vivo* studies, we instead measured the muscle levels of nitrite, a stable NO oxidation product indicative of the level of NO production. Activated eNOS produced NO and resulted in increased levels of nitrite in the muscle compared with that in the CS control rats at one hour after reperfusion (Figure 2A). Despite the increased eNOS phosphorylation (P-eNOS Western blots are shown in Figure 1C) by DEX, the total eNOS protein expression was not quantitatively different among the groups (normalized by α-tubulin, SDC Figure 3). The reduced NO production represented by the decreased nitrite contents in the injured muscle (Figure 2A) led to the induction of ROS production and subsequent rhabdomyolysis, but this was blocked only by CS+DEX-5.0 group as early as 3 hours after reperfusion (Figure 2B).

**Genomic effects of DEX on the expression levels of anti- and pro-inflammatory cytokines**

We observed significant increases in the plasma levels of IL-6 and IL-10 in the CS control rats (Figures 3A and B), which started at three hours after reperfusion following a transient increase in ROS production in the injured muscle (Figure 2B). The increase lasted throughout the 24-hour experimental period. DEX dose-dependently reduced the CS-induced plasma levels of these cytokines, particularly in the late phase of the 24-hour experimental period (Figures 3A and B).

**Effects of DEX on the myeloperoxidase activities in muscle, lung, and kidney**

We determined the MPO activities in injured muscle, lung, and kidney tissue. The MPO activities in these organs were found to remarkably increase, especially at three hours after reperfusion, and they lasted for the entire 24-hour experimental period. However, DEX dose-dependently inhibited the MPO activities in the muscle, lungs, and kidneys (Figures 4A-C).

**Effects of DEX on the histological findings of the gastrocnemius muscle, lungs, and kidneys**

We next examined the histology of CS-induced damages in the muscle, lung and kidney. In comparison with sham rats, the histological changes appeared to be marked in CS rats, and dose-dependently improved by DEX treatment in these tissues (SDC Figures 4-6).

## DISCUSSION

An important finding of the present study is the significantly reduced nitrite content observed in muscle after ischemia (0 h after reperfusion, Figure 2A). During ischemia, the basal nitrite content in muscle is reduced because of the decreased NOS activity and increased nitrite consumption by enzymatic and non-enzymatic anaerobic bioconversion to NO[11-14], and more prolonged ischemia results in the loss of NO bioavailability and also impairs the integrity of the muscle cell membranes, thus rendering them more labile to oxidative stress upon reperfusion. The most important initial event in I/R injury is the excessive production of ROS by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, mitochondrial respiratory chain complexes, and even uncoupled eNOS following reperfusion[4,15]. The ROS thus produced destroy cell membranes as a result of lipid peroxidation and impair the sarcoplasmic reticulum with massive intracellular calcium overload, thus leading to the opening of the mitochondrial permeability transition pore, and subsequently inducing cell apoptosis and/or necrosis. The mechanism of cytoprotection against I/R injury by NO has been well demonstrated[16-20] to be through a reduction of the ROS-mediated cell damage upon reperfusion[21,22]. However, it was unclear precisely how DEX induced endogenous NO in the present study. In this study, only CS+DEX-5.0 group enhanced the muscle nitrite content as rapidly as one hour after reperfusion (Figure 2A) by activating eNOS through a dose-dependent sequential

phosphorylation of the PI3K-Akt-eNOS signaling pathway (Figure 1A-C)[22]. However, the tissue levels of nitrite might be an indirect marker for endogenous NO production, because the reaction of NO with oxygen to form nitrite is kinetically unfavorable compared with the reaction of NO with heme proteins, such as hemoglobin and myoglobin, which favorably yield nitrate instead under physiological conditions. On the other hand, NOS-derived NO has been reported to be predominantly oxidized to nitrite by ceruloplasmin, which is upregulated under ischemic conditions[23]. It is therefore possible that the increase in muscle nitrite observed in the present study might be due to either enhanced NO production by DEX and/or a tendency of NO to be oxidized to nitrite in the early phase of the I/R injury (Figure 2A). Either way, these results suggest that the early temporary recovery of the NO stores to increase the NO bioavailability may contribute to the DEX-mediated reduction of I/R injury. In fact, these DEX-activated NO signaling pathways have been well established to have a protective effect against the early phase of I/R injury in other organs, including the myocardium[5] and cerebrum[6]. In line with these observations, we recently reported that the treatment of CS rats with intravenous nitrite before reperfusion enhanced the muscle levels of nitrite, which was associated with reduced rhabdomyolysis and systemic inflammation, with a remarkable improvement in the survival rates[3]. These results clearly indicate that the tissue NO-releasing capacity in the early phase of reperfusion plays an important role in modulating the process of serious I/R injury.

In addition to the local non-genomic signaling mediated by DEX (as discussed above), DEX also exerts its anti-inflammatory actions at the transcriptional level via the genomic pathway by either direct or indirect interactions with transcription factors, such as NF-kB and activator protein-1[24]. While ROS are well known to activate these redox-sensitive transcription factors, DEX can inhibit them and the subsequent induction of pro-inflammatory mediators, such as IL-6. The plasma level of IL-6 is a clinically relevant and feasible parameter to estimate the severity of injury and make a prognosis after trauma[25,26]. Furthermore, IL-6 induces the immediate-early gene encoding intercellular adhesion molecule-1 after I/R injury[27,28]. Therefore, an enhanced level of IL-6 reflects abnormal interactions between the vascular endothelium and circulating leukocytes, which was observed in this study as enhanced MPO activities indicative of systemic inflammation and multiple organ failure. The present study clearly showed DEX to have dose-dependent inhibitory effects on the pro-inflammatory mediators and MPO activities, possibly through a transrepression-mediated pathway, which attenuated the systemic inflammation and prevented the occurrence of lethal remote organ failure (Figure 4).

On the other hand, IL-10 is an anti-inflammatory cytokine known to be induced by glucocorticoids via the genomic pathway (transactivation), and it is capable of inhibiting the production of pro-inflammatory mediators, such as TNF-α and IL-6(Figure 3A). However, the effect of glucocorticoid on the plasma levels of IL-10 still remains controversial, as it has

been reported to have the opposite effect or to be biphasic, depending on the type of stimulus, the dose of glucocorticoid, and the study setting (*in vivo* or *in vitro*)[29]. Although further studies will be required to delineate this complicated issue, the present study showed the increased plasma levels of IL-10 following I/R injury, possibly reflecting a response to systemic inflammation and the suppressive effect of DEX on local and systemic inflammation could result in decreased plasma levels of IL-10 (Figure 3B).

In summary, the data presented herein provide evidence that high-dose DEX inhibited the I/R-induced local and systemic inflammation at multiple levels of the inflammatory processes, and consequently led to a complete recovery from lethal experimental CS. We therefore recommend that this treatment should be implemented in clinical situations, particularly in combination with the current conventional therapies, including early fluid resuscitation, the correction of acidosis, and hemodialysis[1,33-32].

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## AUTHOR CONTRIBUTIONS

I. M. and J. K. led the project and designed and performed most of the experiments, with the excellent help of S.S. , M. K. and Y. M. K. O. and Y. M. developed the crush syndrome rat model. K. S. and K.O. provided technical expertise. S. K., H. U., G. N. and Y. I. provided intellectual input. I. K., H. U. and Y. M. supervised the entire project. J. K. and I. M. wrote the manuscript.

## REFERENCES


1. Sever MS, Vanholder R, Lameire N. Management of crush-related injuries after disasters. *N Engl J Med.* 2006; **354**:1052-1063.

2. Huerta-Alardin AL, Varon J, Marik PE. Bench-to-bedside review: rhabdomyolysis, an overview for clinicians. *Crit Care.* 2005; **9**:158-169.

3. Murata I, Nozaki R, Ooi K, Ohtake K, Kimura S, Ueda H, Nakano G, Sonoda K, Inoue Y, Uchida H, et al. Nitrite reduces ischemia/reperfusion-induced muscle damage and improves survival rates in rat crush injury model. *J Trauma.* 2012; **72**:1548-1554.

4. Tran TP, Tu H, Pipinos II, Muelleman RL, Albadawi H, Li YL. Tourniquet-induced acute ischemia-reperfusion injury in mouse skeletal muscles: involvement of superoxide. *Eur J Pharmacol.* 2011; **650**:328-334.

5. Hafezi-Moghadam A, Simoncini T, Yang Z, Limbourg FP, Plumier JC, Rebsamen MC, Hsieh CM, Chui DS,Thomas KL, Prorock AJ, et al. Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. *Nat Med.* 2002; **8**:473-479.

6. Limbourg FP, Huang Z, Plumier JC, Simoncini T, Fujioka M, Tuckermann J, Schütz G, Moskowitz MA, Liao JK. Rapid non-transcriptional activation of endothelial nitric oxide synthase mediates increase in cerebral blood flow and stroke protection by corticosteroids. *J Clin Invest.* 2002; **110**:1729-1738.

7. Kumar S, Allen DA, Kieswich JE, Patel NS, Harwood S, Mazzon E, Cuzzocrea S, Raftery MJ, Thiemermann C, Yaqoob MM. Dexamethasone ameliorates renal ischemia-reperfusion injury. *J Am Soc Nephrol.* 2009; **20**:2412-2425.

8. Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids. New mechanisms for old drug. *N Engl J Med.* 2005; **353**:1711-1723.

9. Murata I, Ooi K, Sasaki H, Kimura S, Ohtake K, Ueda H, Uchida H, Yasui N, Tsutsui Y, Yoshizawa N, et al. Characterization of systemic and histologic injury after crush syndrome and intervals of reperfusion in a small animal model. *J Trauma.* 2011; **70**:1453-1463.

10. Uchiyama M, Mihara M. Determination of Malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem.* 1978; **86**:271-278.

11. Li H, Samouilov A, Liu X. Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrate reduction: evaluation of its role in nitrite and nitric oxide generation in anoxic tissues. *Biochemistry.* 2003; **42**:1150-1159.

12. McNulty PH, Scott S, Kehoe V, Kozak M, Sinoway LI, Li J. Nitrite consumption in ischemic rat heart catalyzed by distinct blood-borne and tissue factors. *Am J Physiol Heart Circ Physiol.* 2008; **295**:H2143-H2148.

13. Feelisch M, Fernandez BO, Bryan NS, Garcia-Saura MF, Bauer S, Whitlock DR, Ford PC, Janero DR, Rodriguez J, Ashrafian H. Tissue processing of nitrite in hypoxia. An intricate

interplay of nitric oxide-generating and -scavenging system. *J Biol Chem.* 2008; **283**:33927-33934.

14. Shiva S. Mitochondria as metabolizers and targets of nitrite. *Nitric Oxide.* 2010; **22**:64-74.
15. Cuzzocrea S, Riley DP, Caputi AP, Salvemini D. Antioxidant therapy: a new pharmacological approach in shock, inflammation and ischemia/reperfusion injury. *Pharmacol Rev.* 2001; **53**:135-159.
16. Sun J, Morgan M, Shen RF, Steenbergen C, Murphy E. Preconditioning results in S-nitrosylation of proteins involved in regulation of mitochondrial energetics and calcium transport. *Circ Res.* 2007; **101**:1155-1163.
17. Selemidis S, Dusting GJ, Peshavariya H, Kemp-Harper BK, Drummond GR. Nitric oxide suppresses NADPH oxidase-dependent superoxide production by S-nitrosylation in human endothelial cells. *Cardiovasc Res.* 2007; **75**:349-358.
18. Leite AC, Oliveira HC, Utino FL, Garcia R, Alberici LC, Fernandes MP, Castilho RF, Vercesi AE. Mitochondria generated nitric oxide protects against permeability transition via formation of membrane protein S-nitrosothiols. *BBA-Bioenergetics.* 2010; **1797**:1210-1216.
19. Shiva S, Huang Z, Grubina R, Sun J, Ringwood LA, MacArthur PH, Xu X, Murphy E, Darley-Usmar VM, Gladwin MT. Deoxymyoglobin is a nitrite reductase that generates

nitric oxide and regulates mitochondrial respiration. *Circ Res.* 2007; **100**:654-661.

20. Burwell LS, Brookes PS. Mitochondria as a target for the cardioprotective effects of nitric oxide in ischemia-reperfusion injury. *Antioxid Redox Signal.* 2008; **10**:579-599.

21. Murphy E, Steenbergen C. Mechanism underlying acute protection from cardiac ischemia-reperfusion injury. *Physiol Rev.* 2008; **88**:581-609.

22. Murillo D, Kamga C, Mo L, Shiva S. Nitrite as a mediator of ischemic preconditioning and cytoprotection. *Nitric Oxide.* 2011; **25**:70-80.

23. Shiva S, Wang X, Ringwood LA, Xu X, Yuditskaya S, Annavajjhala V, Miyajima H, Hogg N, Harris ZL, Gladwin MT. Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis. *Nat Chem Biol.* 2006; **29**:486-493.

24. Stahn C, Buttgereit F. Genomic and nongenomic effects of glucocorticoids. *Nat Clin Pract Rheum.* 2008; **4**:525-533.

25. Biffl WL, Moore EE, Moore FA, Peterson VM. Interleukin-6 in the injured patient. Marker of injury or mediator of inflammation? *Ann Surg.* 1996; **224**:647-664.

26. Tsukamoto T, Chanthaphavong RS, Pape HC. Current theories on the pathophysiology of multiple organ failure after trauma. *Injury.* 2010; **41**:21-26.

27. Youker K, Smith CW, Anderson DC, Miller D, Michael LH, Rossen RD, Entman ML. Neutrophil adherence to isolated adult cardiac myocyte. Induction by cardiac lymph collected during ischemia and reperfusion. *J clin Invest.* 1992; **89**:602-609.

28. Kukielka GL, Youker KA, Hawkins HK, Perrard JL, Michael LH, Ballantyne CM, Smith CW, Entman ML. Regulation of ICAM-1 and IL-6 in myocardial ischemia: effect of reperfusion. *Ann N Y Acad Sci.* 1994; **574**:258-270.

29. Franchimont D, Martens H, Hagelstein MT, Louis E, Dewe W, Chrousos GP, Belaiche J, Geenen V. Tumor necrosis factor α decreases, and interleukin-10 increases, the sensitivity of human monocytes to dexamethasone: potential regulation of the glucocorticoid receptor. *J Clin Endocrinol Metab.* 1999; **84**:2834-2839.

30. Gunal AI, Celiker H, Dogukan A, Ozalp G, Kirciman E, Simsekli H, Gunay I, Demircin M, Belhan O, Yildirim MA, et al. Early and vigorous fluid resuscitation prevents acute renal failure in the crush victims of catastrophic earthquakes. *J Am Soc Nephrol.* 2004; **15**:1862-1867.

31. Better OS, Abassi ZA. Early fluid resuscitation in patients with rhabdomyolysis. *Nat Rev Nephrol.* 2011; **7**:416-422.

32. Sever MS, Lameire N, Vanholder R. Renal disaster relief: from theory to practice. *Nephrol Dial Transplant.* 2009; **24:**1730-173.

## Figure Legends

**Figure 1: DEX-induced NO production through the non-genomic signaling pathway.**

The non-genomic effect of DEX on the PI3K-Akt-eNOS signaling pathway in the injured muscles. The lower panels show Western blots of each signaling protein (data are representative of three independent experiments). The band intensities were quantified by densitometry, and the ratios of the levels of phosphorylated to total protein were determined: P-p85 to p85 (A), P-Akt to Akt (B), P-eNOS to eNOS (C), White bar: sham, black bar: CS control, gray bar: CS+DEX-0.1, shaded bar: CS+DEX-5.0 groups, respectively. Values are shown as the means ± SEM. n=3 per group. *: $p<0.05$ vs. CS control group, #: $p<0.05$ vs. sham group, by a one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test.

**Figure 2: Effects of DEX on the nitrite levels and ROS production in the injured muscles.**

Effects of DEX on the nitrite levels in the injured muscles (A), the effects of DEX on the ROS production in the injured muscles, that the ROS levels were determined by measuring the TBARS (B) of sham, CS control, CS+DEX-0.1, and CS+DEX-5.0 groups.. White bar: sham, black bar: CS control, gray bar: CS+DEX-0.1, shaded bar: CS+DEX-5.0 groups,

respectively. Values are shown as the means ± SEM. n=3 per group.*: $p<0.05$ vs. CS control group, **: $p<0.01$ vs. CS control group, #: $p<0.05$ vs. sham group, ##: $p<0.01$ vs. sham group, by a one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test.

**Figure 3: Effect of DEX on the expression levels of anti- and pro-inflammatory cytokines.**

Plasma IL-6 levels (A), plasma IL-10 levels (B) of sham, CS control, CS+DEX-0.1, and CS+DEX-5.0 groups. White bar: sham, black bar: CS control, gray bar: CS+DEX-0.1, shaded bar: CS+DEX-5.0 groups, respectively. Values are shown as the means ± SEM. n=3 per group. *: $p<0.05$ vs. CS control group, **: $p<0.01$ vs. CS control group, ##: $p<0.01$ vs. sham group,†: $p<0.05$ vs. CS+DEX-0.1 group, ††: $p<0.01$ vs. CS+DEX-0.1 group by a one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test.

**Figure 4: Effects of DEX on the MPO activities in muscle, lung and kidney tissues.**

MPO activities of the muscles (A), lungs (B), and kidneys (C) of sham, CS control, CS+DEX-0.1, and CS+DEX-5.0 groups. White bar: sham, black bar: CS control, gray bar: CS+DEX-0.1, shaded bar: CS+DEX-5.0 groups, respectively. The values are shown as the means ± SEM with n=3 per group. *: $p<0.05$ vs. CS control group, **: $p<0.01$ vs. CS control group, #: $p<0.05$ vs. sham group, ##: $p<0.01$ vs. sham group,†: $p<0.05$ vs. CS+DEX-0.1

group, ††: $p<0.01$ vs. CS+DEX-0.1 group by a one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test.

**Supplemental Digital Content (SDC)**

# Acute lethal crush-injured rats can be successfully rescued by a single injection of high-dose dexamethasone through a pathway involving PI3K-Akt-eNOS signaling

**Running title: The Effects of Dexamethasone on Crush Syndrome**

Isamu Murata, PhD, Kazuya Ooi, PhD, Shingo Shoji, MS, Yohei Motohashi, BS, Miwa Kan, BS, Kazuo Ohtake, PhD, Soichiro Kimura, PhD, Hideo Ueda, PhD, Genya Nakano, MS, Kunihiro Sonoda, MS, Yutaka Inoue, PhD, Hiroyuki Uchida, PhD, Ikuo Kanamoto, PhD, Yasunori Morimoto PhD, and Jun Kobayashi, MD, PhD

## SDC Method 1: Experimental design

On reperfusion following ischemia, the vascular endothelium becomes swollen and the vessel lumens are plugged and packed with erythrocytes, leukocytes, and platelets, resulting in the occurrence of a no-reflow phenomenon [1]. However, if the duration of ischemia is not long enough to cause no-reflow phenomenon, cytosolic toxic mediators including myoglobin derived from lysed skeletal muscle cells are released to the circulation following reflow phenomenon, leading to systemic inflammations with high mortality. However, the shorter

ischemia duration (less than 4 hours in this model) is, the less cell damages are, resulting in less systemic inflammations with low mortality. We previously reported that a 5-hour bilateral hindlimb ischemia exhibited the highest mortality rate (75%) at 24 hours after reperfusion compared with other durations of ischemia (0% mortality in less than 4-hour ischemia and only 10% mortality in more than 6-hour ischemia) [2], thereby providing a critical time window to cause reflow and rhabdomyolysis leading to serious CS.

Based on these reports, a 5-hour compression by the tourniquet was chosen for the crush interval in the current study [2,3] which provides a more practical animal model closely similar to lethal human CS than other animal models.

**SDC Method 2: Histology**

All sections from tissues were scored by a blinded observer according to the semiquantitative histological scoring systems of skeletal muscle, lung and kidney described in the previous reports [4,5,6].

**SDC Table 1 and 2 : Effects of the conventional therapies on plasma biochemical markers, mean arterial pressure and blood gas of CS rat**

CS control rats showed significant increases in the markers of rhabdomyolysis, such as CPK, $K^+$, and LDH in the plasma as early as one hour after reperfusion. CS control rats also

showed a significantly decreased MAP throughout the 24-hour experimental period, which consequently induced metabolic acidosis shown in the arterial blood gas analysis (pH, base excess, $HCO_3^-$ and lactate). However, fluid resuscitation (30mL/kg/hr[7,8], starting immediately after reperfusion for 3 hours) of saline or bicarbonated Ringer's solution (BICARBON®), significantly suppressed rhabdomyolysis and subsequent hypovolemic hypotension (SDC Table 2). The circulatory improvement also decreased the plasma levels of renal damage markers (creatinine and BUN, SDC Table 1). These beneficial effects were more observed in the bicarbonated Ringer's solution than saline (SDC Table 1 and 2).

**SDC Figure 1: Effects of dexamethasone on the survival rate of CS rat.**

The survival rates among the four groups from 0 to 48 h after reperfusion. Open circle: sham (n=10), closed circle: CS control (n=29), open diamond: CS+DEX-0.1(n=14), closed diamond: CS+DEX-5.0 groups (n=10), respectively. DEX treatment showed dose-dependent improvement of survival rates from 24% (CS control group) to 24% (CS+DEX-0.1 group) and 100% (CS+DEX-5.0 groups) after a 48 h experimental period. **: $p<0.01$ vs. CS control group, ††: $p<0.01$ vs. CS+DEX-0.1 group. Survival curves were calculated by the Kaplan-Meier method and compared by a log-rank test.

**SDC Figure 2: Effects of the conventional therapies on the survival rate of CS rat.**

The survival rates among the four groups treated with or without conventional therapies from 0 to 48 h after reperfusion. Open circle: sham (n=10), closed circle: CS control (n=29), open square: CS+saline (n=10), closed square: CS+BICARBON$^{R}$ groups (n=10). Aggressive saline resuscitation showed a high survival rate (90%) until 6 hours after reperfusion, although it ultimately ended in failure 48 hours after reperfusion with a final mortality rate (30%) similar to the CS control group. CS+BICARBON$^{R}$ group showed a higher survival rate (60%) at 48-hour experimental period, however it does not appear nearly so good as the high-dose DEX-treated group. #: $p<0.01$ vs. CS control group. Survival curves were calculated by the Kaplan-Meier method and compared by a log-rank test.

**SDC Figure 3: Effects of DEX on the total eNOS expression in the injured muscles.**

The ratio of protein levels of eNOS to α-tubuline. The ratios are all represented by arbitrary units. The lower panels show Western blots of eNOS and α-tubuline in four groups at each experimental period (data are representative of three independent experiments). White bar: sham, black bar: CS control, gray bar: CS+DEX-0.1, shaded bar: CS+DEX-5.0 groups, respectively. The values are shown as the means ± SEM with n=3 per group. These result calculated by a one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test (not significant).

**SDC Figure 4: Effects of DEX on the histological findings in the gastrocnemius muscle.**

In comparison with sham (a), the gastrocnemius muscles of the CS control rats showed degenerated and atrophic muscle fibers with significant edema and a mild dilation of the interstitial vessels (b). These findings were much less pronounced in the low-dose DEX (c), and were largely abrogated in the high-dose DEX (d). Hematoxylin-eosin staining of the gastrocnemius muscles of sham (A), CS control (B), CS+DEX-0.1 (C), and CS+DEX-5.0 groups (D), respectively. Microscopic photos are representative of 3 independent experiments (×200, scale bars=100 μm).

**SDC Figure 5: Effects of DEX on the histological findings in the lung.**

The most remarkable pathological features were found in the lungs, which showed prominent intra-alveolar and interstitial edema in the CS control rats (f) in comparison with the normal alveolar wall with a thin alveolar septum in the sham-injured rats (e). These pathological changes were dose-dependently improved by DEX (g and h). Hematoxylin-eosin staining of the lung of sham (A), CS control (B), CS+DEX-0.1 (C), and CS+DEX-5.0 groups (D), respectively. Microscopic photos are representative of 3 independent experiments (×200, scale bars=100 μm).

**SDC Figure 6: Effects of DEX on the histological findings in the kidney.**

The cortex of the kidneys of the CS control rats exhibited a moderate dilation of the distal tubules and a flattening of epithelial cells due to acute renal failure in comparison with the sham-injured rats (i and j). DEX also improved these pathological changes (k and l). Hematoxylin-eosin staining of the kidney of sham (A), CS control (B), CS+DEX-0.1 (C), and CS+DEX-5.0 groups (D), respectively. Microscopic photos are representative of 3 independent experiments (×200, scale bars=100 μm).

## SDC REFERENCES


(1) Blaisdell FW. The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. *Cardiovasc. Surg.* 2002; **10**: 620-630.

(2) Murata I, Ooi K, Sasaki H, Kimura S, Ohtake K, Ueda H, Uchida H, Yasui N, Tsutsui Y, Yoshizawa N, Hirotsu I, Morimoto Y, and Kobayashi J. Characterization of systemic and histologic injury after crush syndrome and intervals of reperfusion in a small animal model. *J. Trauma.*2011; **70**: 1453-1463.

(3) Murata I, Nozaki R, Ooi K, Ohtake K, Kimura S, Ueda H, Nakano G, Sonoda K, Inoue Y, Uchida H, Kanamoto I, Morimoto Y, and Kobayashi J. Nitrite reduces ischemia/reperfusion-induced muscle damage and improves survival rates in rat crush injury

model. *J. Trauma*. 2012; **72**: 1548-1554.

(4) Kalns J, Cox J, Baskin J, Santos A, Odland R, Fecura S Jr. Threshold Model for Extremity Compartment Syndrome in Swine. *J Surg Res*. 2011; **167**: e13–e19

(5) Rotta AT, Gunnarsson B, Hernan LJ, Fuhrman BP, Steinhorn DM. Partial liquid ventilation influences pulmonary histopathology in an animal model of acute lung injury. *J Crit Care*. 1999; **14**: 84-92.

(6) Hu H, Zou C, Xi X, Shi Z, Wang G, Huang X. Protective effects of pioglitazone on renal ischemia-reperfusion injury in mice. *J Surg Res*. 2012; **178**: 460–465.

(7) Better OS, Rubinstein I. Management of shock and acute renal failure in casualties suffering from the crush syndrome. *Renal Failure*. 1997; **19**: 647-653.

(8) Sever MS, Vanholder R, Lameire N. Management of crush-related injuries after disasters. *N Engl J Med*. 2006; **354**:1052-1063.

**Table 1 Effects of I/R injury and DEX on plasma biochemical markers**

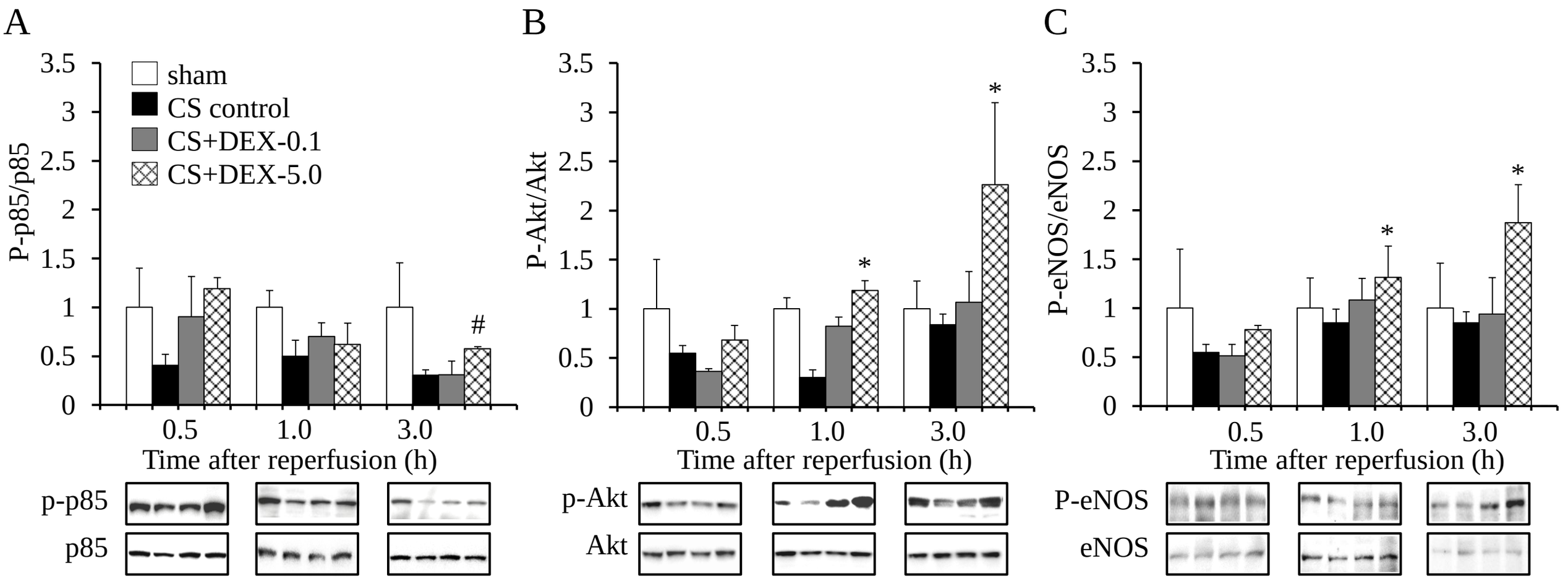
| Time after reperfusion (h) | | 1 | | 3 | | 6 | | 24 | |
|---|---|---|---|---|---|---|---|---|---|
| $K^+$ (mEq/L) | sham | 4.2 ± 0.1 | ## | 3.9 ± 0.1 | ## | 3.7 ± 0.1 | ## | 3.9 ± 0.1 | ## |
| | CS control | 7.3 ± 0.2 | | 8.9 ± 0.1 | | 9.2 ± 0.5 | ** | 6.6 ± 0.5 | |
| | Dex-0.1 | 6.4 ± 0.3 | | 7.6 ± 0.1 | ** | 6.5 ± 0.3 | ** | 5.9 ± 1.0 | ** |
| | Dex-5.0 | 6.1 ± 0.2 | | 6.4 ± 0.2 | | 6.5 ± 0.3 | | 4.3 ± 0.4 | |
| BUN (mg/dL) | sham | 13.3 ± 2.2 | | 13.5 ± 1.3 | ## | 11.7 ± 0.9 | ## | 20.4 ± 1.2 | ## |
| | CS control | 24.8 ± 0.5 | | 41.3 ± 3.7 | | 56.6 ± 2.1 | | 37.0 ± 1.6 | * |
| | CS+Dex-0.1 | 25.4 ± 1.4 | | 40.0 ± 4.3 | | 51.6 ± 4.0 | | 24.2 ± 2.1 | * |
| | CS+Dex-5.0 | 23.0 ± 2.0 | | 39.8 ± 1.3 | | 49.1 ± 3.4 | | 27.0 ± 1.2 | |
| creatinine (mg/dL) | sham | 0.16 ± 0.01 | | 0.17 ± 0.02 | ## | 0.20 ± 0.01 | ## | 0.24 ± 0.01 | # |
| | CS control | 0.30 ± 0.05 | | 1.51 ± 0.33 | ** | 0.79 ± 0.07 | ** | 0.53 ± 0.05 | |
| | CS+Dex-0.1 | 0.20 ± 0.01 | | 0.37 ± 0.01 | ** | 0.27 ± 0.04 | ** | 0.24 ± 0.03 | |
| | CS+Dex-5.0 | 0.20 ± 0.01 | | 0.31 ± 0.03 | | 0.27 ± 0.03 | | 0.21 ± 0.02 | |
| CPK (IU/L) | sham | 1371 ± 244 | ## | 1034 ± 218 | ## | 322 ± 81 | ## | 121 ± 13 | ## |
| | CS control | 21,220 ± 2,229 | ** | 24,320 ± 1,930 | | 23,187 ± 1,494 | | 15,240 ± 1,335 | |
| | CS+Dex-0.1 | 8,960 ± 1,620 | ** | 24,867 ± 3,351 | * | 26,080 ± 4,688 | * | 7,593 ± 1,902 | * |
| | CS+Dex-5.0 | 8,893 ± 924 | | 18,227 ± 2,715 | | 14,487 ± 2,517 | | 5,743 ± 322 | |
| LDH (IU/L) | sham | 224 ± 32 | # | 309 ± 18 | ## | 181 ± 20 | ## | 69 ± 7 | ## |
| | CS control | 1,860 ± 57 | | 3,190 ± 650 | | 3,466 ± 198 | | 3,633 ± 583 | |
| | CS+Dex-0.1 | 739 ± 99 | | 2,488 ± 420 | | 2,603 ± 539 | * | 3,717 ± 666 | * |
| | CS+Dex-5.0 | 679 ± 94 | | 2,009 ± 301 | | 2,328 ± 301 | | 2,324 ± 214 | |

Values are shown as the means ± SEM. n= 3 per group. #: $p<0.05$ vs. sham group, ##: $p<0.01$ vs. sham group, *: $p<0.05$ vs. CS control group, **: $p<0.01$ vs. CS control group by a one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test.

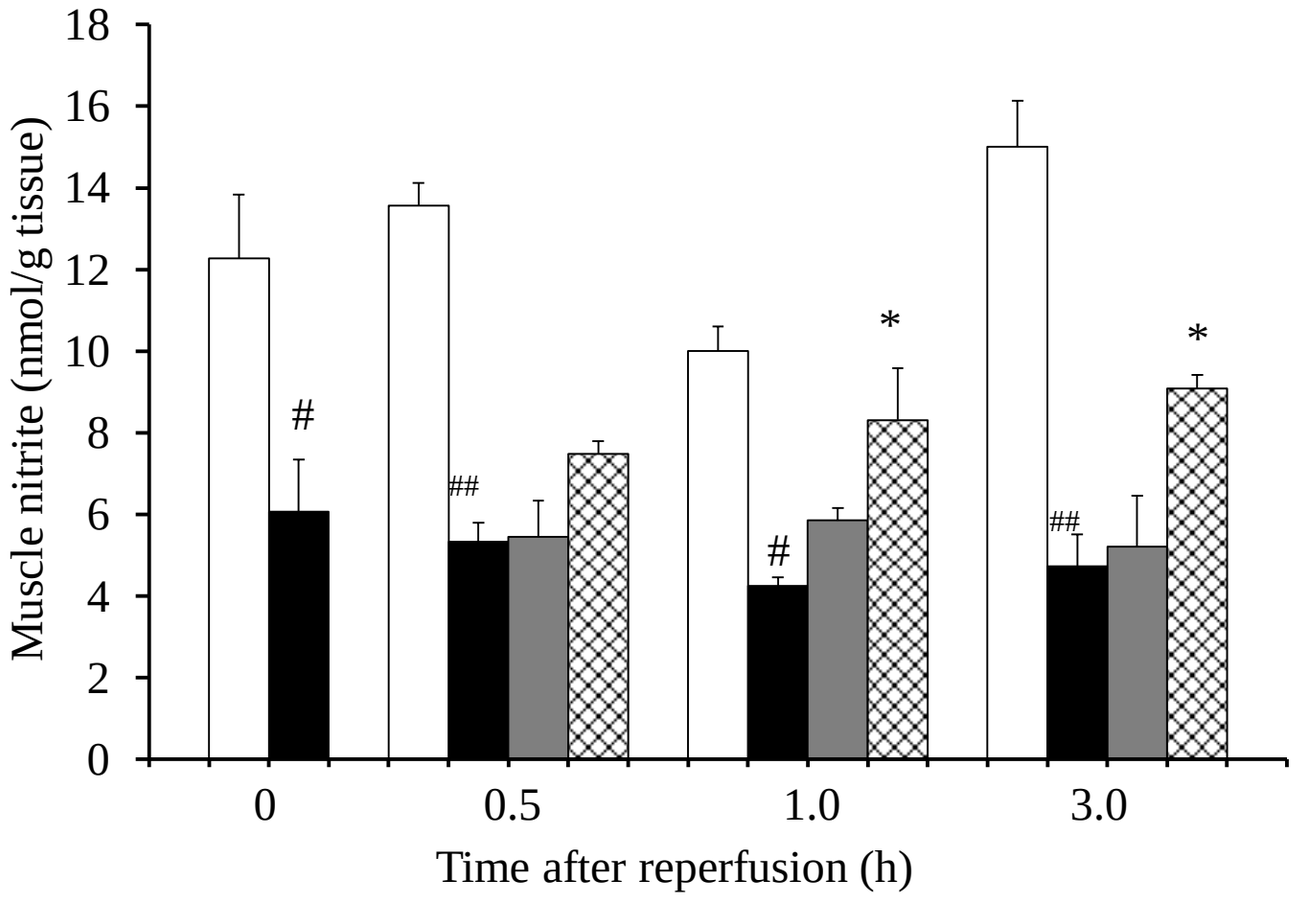
**Table 2 Effects of I/R injury and DEX on MAP and arterial blood gas analysis.**

| Time after reperfusion (h) | | 1 | | 3 | | 6 | | 24 | |
|---|---|---|---|---|---|---|---|---|---|
| MAP (mmHg) | sham | 121.3 ± 5.8 | | 116.7 ± 4.7 | | 111.9 ± 7.3 | | 118.1 ± 2.7 | |
| | CS control | 79.6 ± 1.5 | ## | 67.4 ± 2.3 | ## | 54.7 ± 3.0 | ## | 59.7 ± 3.5 | ## |
| | CS+Dex-0.1 | 88.8 ± 2.5 | | 80.5 ± 4.9 | | 76.0 ± 1.7 | * | 72.1 ± 1.4 | |
| | CS+Dex-5.0 | 84.3 ± 5.1 | | 86.1 ± 0.4 | * | 84.6 ± 3.4 | ** | 100.3 ± 1.5 | **,†† |
| pH | sham | 7.42 ± 0.01 | | 7.44 ± 0.00 | | 7.47 ± 0.01 | | 7.42 ± 0.02 | |
| | CS control | 7.37 ± 0.01 | | 7.33 ± 0.01 | # | 7.29 ± 0.03 | ## | 7.28 ± 0.02 | ## |
| | CS+Dex-0.1 | 7.35 ± 0.03 | | 7.33 ± 0.01 | | 7.28 ± 0.02 | | 7.30 ± 0.01 | |
| | CS+Dex-5.0 | 7.41 ± 0.03 | | 7.35 ± 0.04 | | 7.36 ± 0.04 | * | 7.38 ± 0.02 | * |
| $HCO_3^-$ (mEq/L) | sham | 27.3 ± 0.3 | | 27.3 ± 0.3 | | 30.0 ± 0.4 | | 27.0 ± 1.2 | |
| | CS control | 19.9 ± 1.3 | ## | 19.3 ± 0.3 | ## | 16.0 ± 0.6 | ## | 15.7 ± 0.3 | ## |
| | CS+Dex-0.1 | 24.2 ± 0.7 | ** | 21.1 ± 0.2 | | 19.9 ± 0.8 | * | 21.0 ± 1.1 | ** |
| | CS+Dex-5.0 | 26.8 ± 0.6 | ** | 25.0 ± 0.4 | **,† | 23.8 ± 0.8 | **.† | 25.4 ± 0.5 | **.†† |
| base excess (mmol/L) | sham | 3.0 ± 0.6 | | 3.0 ± 0.6 | | 3.6 ± 0.9 | | 1.3 ± 0.3 | |
| | CS control | -5.0 ± 0.8 | ## | -7.0 ± 0.6 | ## | -9.7 ± 0.9 | ## | -6.7 ± 1.2 | ## |
| | CS+Dex-0.1 | -0.3 ± 1.2 | * | -4.7 ± 0.3 | | -6.0 ± 0.0 | | -1.3 ± 0.9 | ** |
| | CS+Dex-5.0 | 2.0 ± 0.6 | ** | 1.0 ± 1.2 | **,† | -1.7 ± 1.5 | **,† | 2.0 ± 0.6 | ** |
| Lactate (mmol/L) | sham | 1.3 ± 0.1 | | 1.1 ± 0.1 | | 1.1 ± 0.3 | | 0.8 ± 0.1 | |
| | CS control | 1.5 ± 0.1 | | 1.1 ± 0.1 | | 2.3 ± 0.1 | ## | 2.9 ± 0.3 | ## |
| | CS+Dex-0.1 | 1.1 ± 0.2 | | 1.2 ± 0.1 | | 1.5 ± 0.1 | | 1.5 ± 0.3 | * |
| | CS+Dex-5.0 | 1.1 ± 0.3 | | 1.2 ± 0.1 | | 1.3 ± 0.2 | * | 0.6 ± 0.2 | ** |

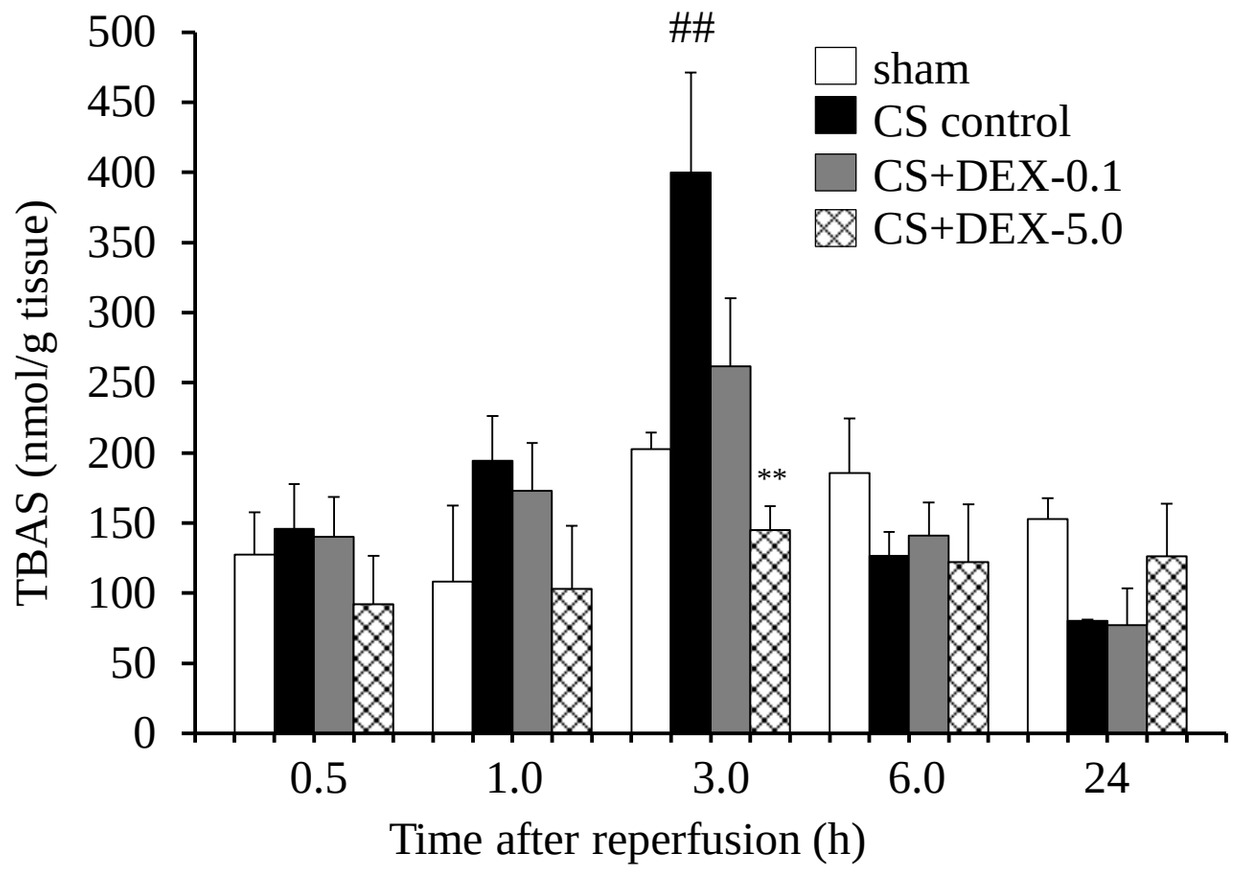
Values are shown as the means ± SEM. n= 3 per group. #: $p<0.05$ vs. sham group, ##: $p<0.01$ vs. sham group, *: $p<0.05$ vs. CS control group, **: $p<0.01$ vs. CS control group, †: $p<0.05$ vs. CS+DEX-0.1 group, ††: $p<0.01$ vs. CS+DEX-0.1 group by a one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test.


A
B
C
sham
CS control
CS+DEX-0.1
CS+DEX-5.0
P-p85/p85
P-Akt/Akt
P-eNOS/eNOS
3.5
3
2.5
2
1.5
1
0.5
0
0.5
1.0
3.0
Time after reperfusion (h)
*
#
p-p85
p85
p-Akt
Akt
P-eNOS
eNOS

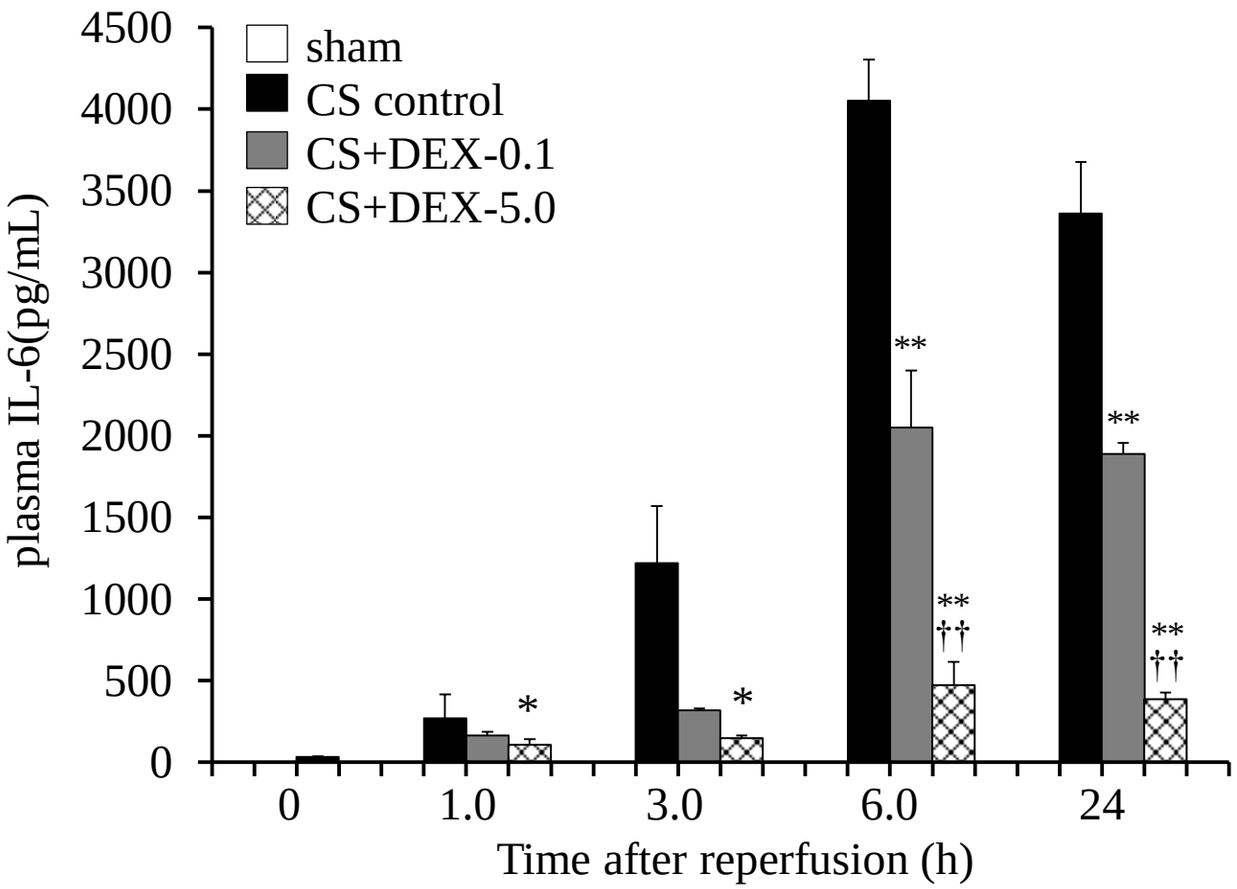
A


Muscle nitrite (nmol/g tissue)
18
16
14
12
10
8
6
4
2
0
#
##
#
##
*
*
0
0.5
1.0
3.0
Time after reperfusion (h)

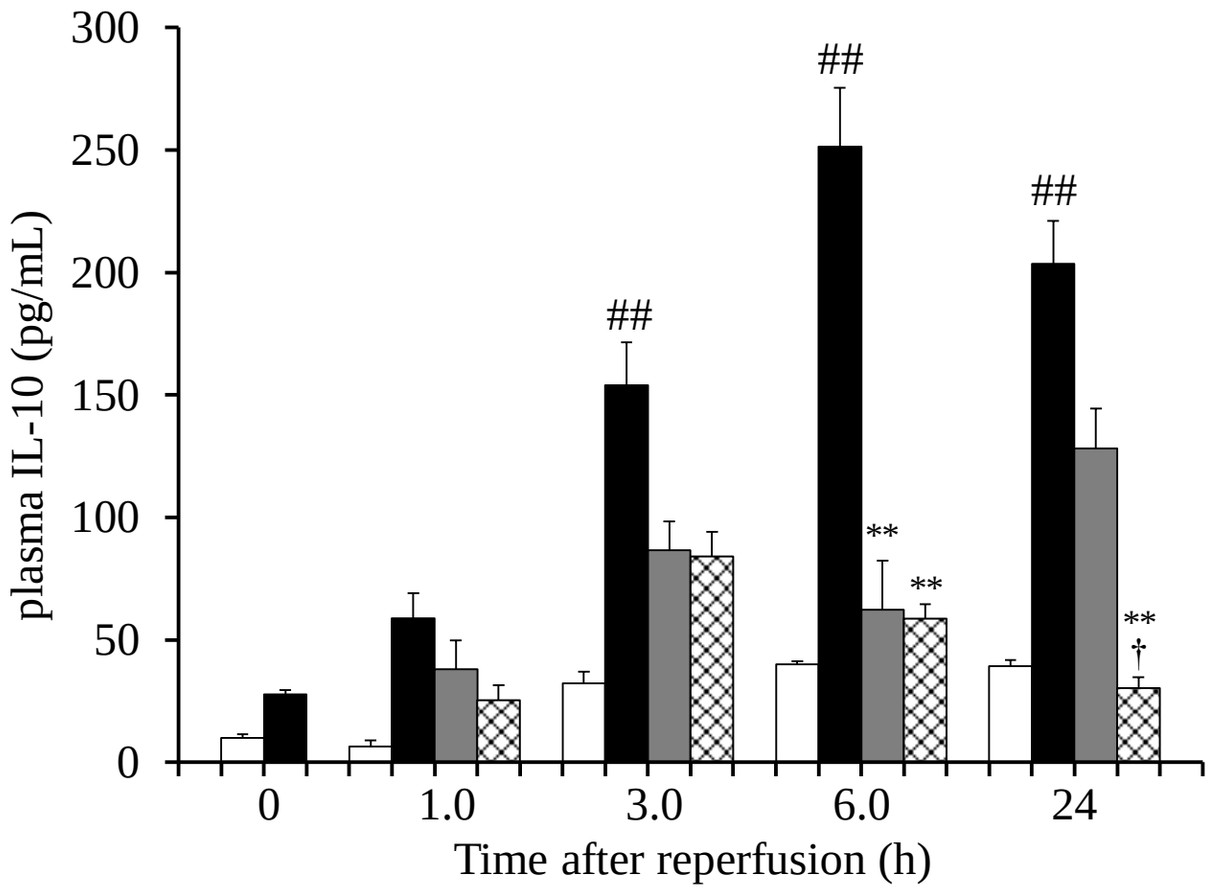
B


TBAS (nmol/g tissue)
500
450
400
350
300
250
200
150
100
50
0
##
**
sham
CS control
CS+DEX-0.1
CS+DEX-5.0
0.5
1.0
3.0
6.0
24
Time after reperfusion (h)

A

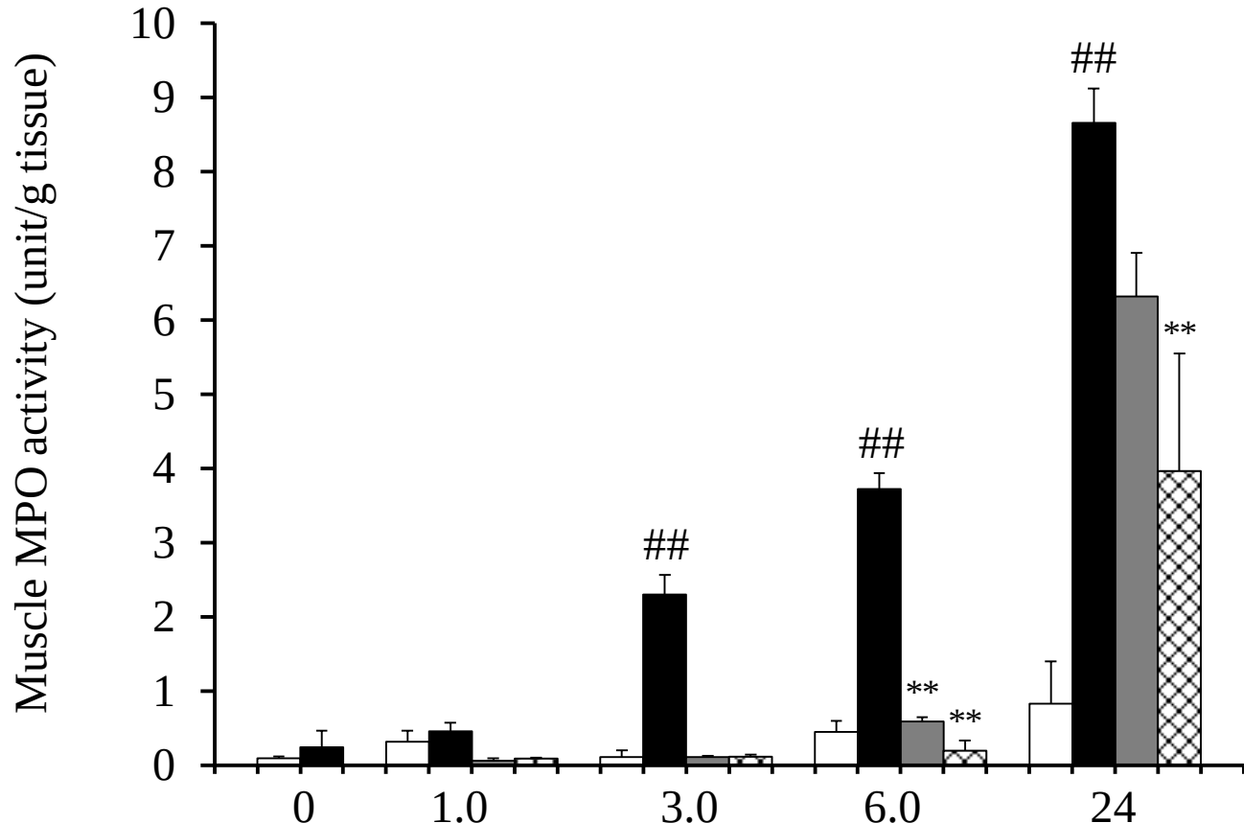

sham
CS control
CS+DEX-0.1
CS+DEX-5.0
plasma IL-6(pg/mL)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
1.0
3.0
6.0
24
Time after reperfusion (h)
*
*
**
**
**
**
††
**
††

B

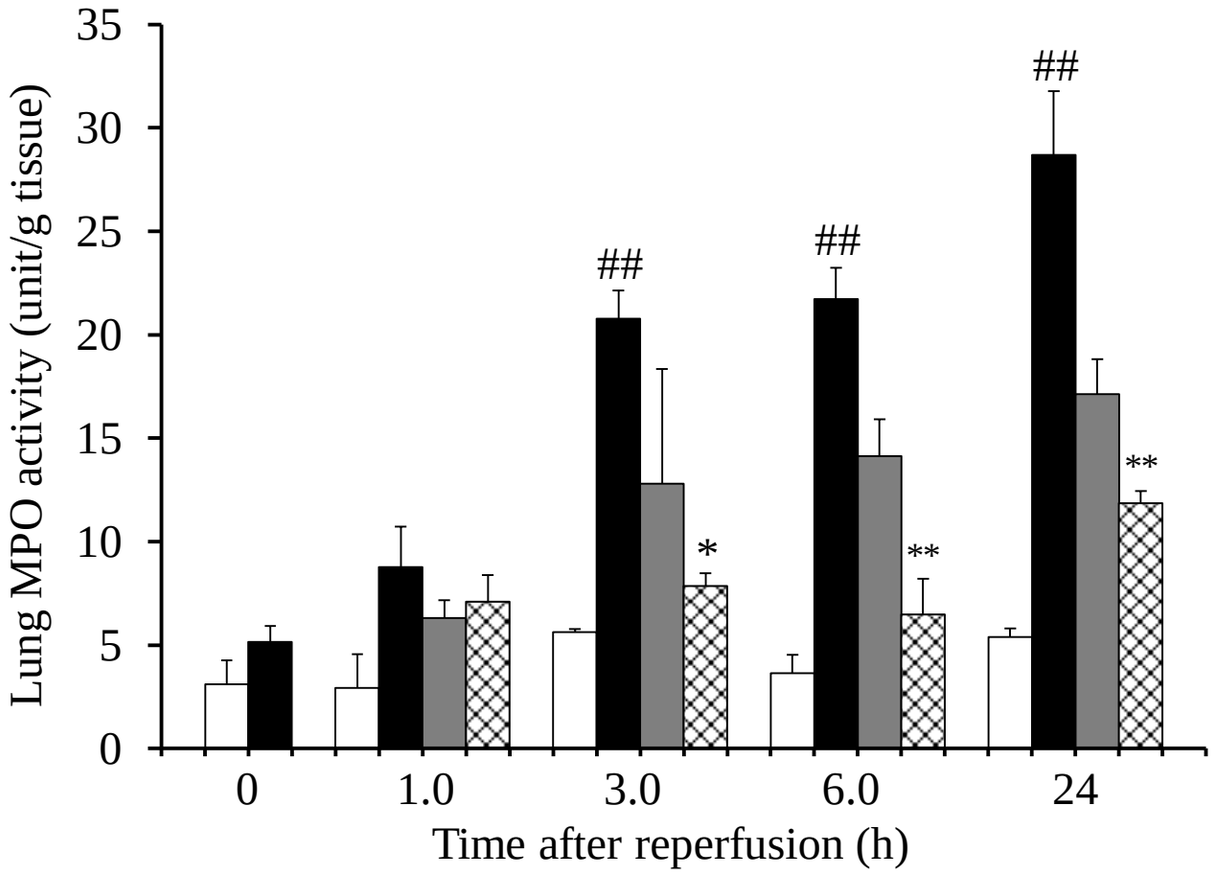

plasma IL-10 (pg/mL)
300
250
200
150
100
50
0
0
1.0
3.0
6.0
24
Time after reperfusion (h)
##
##
##
**
**
**
†

sham
CS control
CS+DEX-0.1
CS+DEX-5.0

A




B




C