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Carbon Monoxide Rescues the Developmental Lethality of Experimental Rat Models of Rhabdomyolysis-Induced Acute Kidney Injury[S]

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ABSTRACT

Many victims, after being extricated from a collapsed building as the result of a disaster, suffer from disaster nephrology, a term that is referred to as the crush syndrome (CS). Recommended treatments, which include dialysis or the continuous administration of massive amounts of fluid are not usually easy in cases of such mass natural disasters. In the present study, we examined the therapeutic performance of a biomimetic carbon monoxide (CO) delivery system, CO-enriched red blood cells (CO-RBCs), on experimental animal models of an acute kidney injury (AKI) induced by traumatic and nontraumatic rhabdomyolysis, including CS and rhabdomyolysis with massive hemorrhage shock. A single CO-RBC treatment was found to effectively suppress the pathogenesis of AKI with the mortality in these model rats being improved. In addition, in further studies using glycerol-induced rhabdomyolysis model rats, the pathogenesis of which is similar to that for the CS, AKI and mortality were also reduced as the result of a CO-RBC treatment. Furthermore, CO-RBCs were found to have renoprotective effects via the suppression of subsequent heme protein–associated renal oxidative injury; the oxidation of myoglobin in the kidneys, the generation of reactive oxygen species by free heme produced from degraded-cytochrome P450 and hemoglobin-associated renal injury. Because CO-RBCs can be prepared and used at both hospitals and at a disaster site, these findings suggest that CO-RBCs have the potential for use as a novel cell therapy against both nontraumatic and traumatic rhabdomyolysis including CS-induced AKI.

SIGNIFICANCE STATEMENT

After mass natural and man-made disasters, people who are trapped in collapsed buildings are in danger of acute kidney injury (AKI), including crush syndrome (CS)-related AKI. This paper reports that carbon monoxide–enriched red blood cells (CO-RBCs), which can be prepared at both hospitals and disaster sites, dramatically suppressed the pathogenesis of CS-related AKI, thus improving mortality via suppressing heme protein–associated renal injuries. CO-RBCs have the potential for serving as a practical therapeutic agent against disaster nephrology associated with the CS.

Introduction

Large-scale natural disasters, such as earthquakes, hurricanes, and volcanic eruptions, occur worldwide, are completely unpredictable, and are beyond human control. Building collapses frequently occur in these mass disasters, and numerous lives of many citizens are threatened by rubble or are buried under collapsed buildings. The most common causes of death in such cases are direct trauma, massive bleeding, and asphyxia, whereas victims who survived but remain trapped in a collapsed building are still in danger of disaster-related nephrology, which is referred to as the crush syndrome (CS)-related acute kidney injury (AKI) (Sever et al., 2015).

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ABBREVIATIONS: 4-HNE, 4-hydroxy-2-nonenal; AKI, acute kidney injury; BUN, blood urea nitrogen; CO, carbon monoxide; CO-RBC, carbon monoxide–enriched red blood cell; CPK, creatine phosphokinase; Cr, creatinine; CS, crush syndrome; P450, cytochrome P450; HO-1, heme oxygenase-1; Mb, myoglobin; NAG, N-acetyl-β-D-glucosaminidase; NO_2-Tyr, nitrotyrosine; PAS, periodic acid-Schiff; RBC, red blood cell; ROS, reactive oxygen species; SCr, serum creatinine.

The underlying pathogenesis of CS-related AKI is similar to that of AKI after rhabdomyolysis. CS-related AKI develops soon after a person is extricated from a collapsed building. A major factor that contributes to this syndrome is damage to myocytes and the release of the contents of these cells into the circulation. Myoglobin (Mb), a major component of myocytes, causes the onset and subsequent development of AKI. Other cell contents, including potassium and uric acid, also cause deleterious systemic effects, such as hyperkalemia and acidosis (Gibney et al., 2014), and unfortunately also aggravate the pathology AKI. Based on current medical knowledge, vigorous fluid administration (isotonic saline and sodium bicarbonate) or renal replacement therapy (hemodialysis) from the earliest time are recognized as the only effective therapy for treatment of most crush victims (Better and Abassi, 2011; Sever and Vanholder, 2013) because such therapy can restore renal function resulting in the prevention of AKI, electrolyte abnormality, acidosis, and a reduced mortality (Smith and Greaves, 2003; Zarbock et al., 2016). However, massive fluid administration can cause hypervolumenia and edema and can worsen the compartment syndrome, particularly in the elderly and children (Greaves et al., 2003). In addition, it is difficult to perform hemodialysis after such disasters due to the chaotic conditions, including interruptions in electric power and water and damage to hospitals and dialysis centers that are located nearby. Hence, developing an effective and practical medication for the treatment of CS-related AKI that can be performed at both a hospital and a prehospital situation would be highly desirable.

It is well known that heme oxygenase-1 (HO-1) functions as a cytoprotective protein via the modulating reactive oxygen species (ROS) and inflammation that occur in various disorders (Bolisetty et al., 2017). Previous studies based on rodent models of rhabdomyolysis have clearly shown that HO-1 is a major factor in renoprotection against rhabdomyolysis-associated AKI (Nath et al., 1992; Wei et al., 2011). However, iron produced by HO-1 in the process of the heme metabolism also represents a source of ROS via the Fenton reaction, which can sometimes worsen the degree of AKI (Zager et al., 1995). Carbon monoxide (CO), a byproduct of the HO-1 reaction, is thought to mediate the therapeutic effects of HO-1 due to fact that the biologic functions are common to both CO and HO-1. Considering these collective factors, we hypothesized that a CO supplement instead of HO-1 induction could exert therapeutic effects against CS-related AKI.

We previously developed a CO delivery system using CO-enriched red blood cells (CO-RBCs), in which CO is bound to nearly all (100%) of the hemoglobin molecules in red blood cells (RBCs), as a novel cell therapy (Ogaki et al., 2013, 2014). CO-RBCs can be easily and rapidly prepared by bubbling CO gas through an RBC preparation for 5 min in ex vivo conditions. This system mimics the following biologic relationship between RBCs and CO; RBCs act as an endogenous CO storage system and delivers cargo due to the fact that approximately 75% of the CO in the body is found in RBCs in the form of carboxyhemoglobin, and CO stored in the blood pool is supplied to tissues when needed (Coburn, 1970). Fortunately, CO-RBCs can also function as an original O_2-RBC after releasing CO, so CO-RBCs would be expected to be beneficial for the simultaneous treatment of both the CS and hemorrhages because most crush victims would require O_2-RBC transfusions due to hemorrhages caused by falling rubble. Therefore, a CO-RBC treatment would be expected to be a practical approach for the treatment of patients with the CS after a mass disaster.

The present study was designed to verify the hypothesis that CO has the potential for limiting the intensity of CS-related AKI. For this purpose, we used CO-RBCs as a biomimetic CO donor and first evaluated the effects of CO-RBCs on the mortality and AKI in CS model rats. We then investigated the contribution of the versatile biologic function of CO for suppressing the pathogenesis of rhabdomyolysis-associated AKI using glycerol-induced rhabdomyolysis model rats.

Methods

Animals. All male Sprague-Dawley rats (6 weeks old; Kyudou Co., Kumamoto, Japan) were housed in a room with the temperature maintained at 18–24°C and 40%–70% relative humidity, with a 12-hour light/dark cycle, and allowed free access to food and drinking water. Maintenance of the animals and the experimental procedures performed on them were carried out in accordance with National Institutes of Health guidelines. All animal experiments were reviewed and approved by the Animal Care and Use committee of Kumamoto University.

Experimental Protocol for CS Model Rats. CS model rats was prepared as follows: the hind limbs of the rats were compressed by a rubber tourniquet that was applied by wrapping five turns around a metal cylinder under a 2 kg load (Murata et al., 2011). At 5 h after starting the compression, the compression was released by cutting and removing the rubber tourniquets. At 1 h after decompression, rats were injected with either saline (22.4 ml/kg, $n = 16$), O_2-RBCs (1400 mg Hb/kg, 22.4 ml/kg, $n = 16$), or CO-RBCs (1400 mg Hb/kg, 22.4 ml/kg, $n = 16$) from tail vein. Ten rats of each group were used for monitoring survival rate. Remained rats ($n = 6$ per each group) and control rats ($n = 5$) were sacrificed at 5 h after test samples administration and collected blood and kidneys for further biochemical and histologic analysis. All procedures were carried out under pentobarbital anesthesia (intraperitoneal injection, 50 mg/kg). The O_2-RBC and CO-RBC solution were prepared in ex vivo as described in a previous report (Ogaki et al., 2013, 2014).

Experimental Protocol for the Glycerol-Induced Rhabdomyolysis Model Rats. Rhabdomyolysis model rats were prepared by the intramuscular injection of a 50% glycerol solution (8 ml/kg) in both hind limbs (Nishida et al., 2015). Water was withheld for 24 h before the glycerol injection. The rats were randomized to receive saline (22.4 ml/kg, $n = 24$), O_2-RBCs (1400 mg Hb/kg, 22.4 ml/kg, $n = 24$), or CO-RBCs (1400 mg Hb/kg, 22.4 ml/kg, $n = 24$) at 3 h after glycerol injection. Ten rats of each group were used for monitoring survival rate. At 6 h after the glycerol injection, glycerol-induced rhabdomyolysis model rats ($n = 3$ per each group) and control rats ($n = 3$) were sacrificed and collected kidneys for evaluation of oxidized Mb in kidney. At 24 h after the glycerol injection, glycerol-induced rhabdomyolysis model rats ($n = 11$ per each group) and control rats ($n = 8$) were sacrificed and collected blood and organs. Samples collected from six glycerol-induced rhabdomyolysis model rats of each group and five control rats were used for biochemical and histologic analysis. Remaining samples (glycerol-induced rhabdomyolysis model rats: $n = 5$, control rats: $n = 3$) were used for the evaluation of Mb, free heme, and cytochrome P450 (CYP) content, hematology, and electrolytes. Urine samples were collected using a metabolic cage for 24 h. All procedures were carried out under pentobarbital anesthesia (intraperitoneal injection, 50 mg/kg).

Experimental Protocol for Traumatic Rhabdomyolysis Model Rats. At 1 h after glycerol injection, a massive hemorrhage was induced by removing 40% of the total blood volume (22.4 ml/kg) as previously reported (Taguchi et al., 2009). Under these experimental conditions, the mean arterial pressure remained at less than 40 mm Hg for 30 min. The rats were resuscitated by an infusion of saline (22.4 ml/kg, $n = 10$), O_2-RBCs (1400 mg Hb/kg, 22.4 ml/kg, $n = 10$), or

CO-RBCs (1400 mg Hb/kg, 22.4 ml/kg, $n = 10$) at a rate of 1 ml/min. After resuscitation, the survival rate was monitored for 24 h. All procedures were carried out under pentobarbital anesthesia (intraperitoneal injection, 50 mg/kg).

Biochemical Analysis of Blood, Organ, and Urine Samples. The collected blood and kidney samples were pretreated as previously described (Ogaki et al., 2015). Blood urea nitrogen (BUN), serum creatinine (SCr), and urinary creatinine (Cr) concentrations were measured using assay kits (Wako Pure Chemical, Osaka, Japan). The activity of urinary N-acetyl-β-D-glucosaminidase (NAG) in urine was determined using a commercial assay kit (Shionogi Pharmaceutical, Osaka, Japan). Creatine phosphokinase (CPK) concentrations were measured by means of a Creatine Kinase Fluorometric Assay kit (Cayman Chemical Co., Ann Arbor, MI). The amount of Mb was determined using an ELISA kit (Life Diagnostics, PA). Free heme was determined by means of a Heme Assay Kit (Cayman Chemical Company). The hemoglobin concentration in urine was determined by a Hemoglobin test (Wako Pure Chemical). Blood pH, base excess, lactate concentrations, Na^+, and K^+ were determined using an iSTAT analyzer (Fuso Pharmaceutical Industries, Osaka, Japan). For each measured item for urianalysis, a test stick was dipped into the collected urine and the level was estimated by visual examination of the color after a specific time of exposure according to the instructions.

Histologic Evaluation. The collected left kidneys were fixed in 4% phosphate buffered formalin and embedded in paraffin. Kidney sections (4 μm) were subjected to periodic acid-Schiff (PAS) staining for morphologic analysis. The primary antibodies for immunostaining for nitrotyrosine (NO_2-Tyr) (cat#: AB5411; Chemicon International, Temecula, CA) and 4-hydroxy-2-nonenal (4-HNE) (cat. no. bs-6313R; Bioss, Boston, MA) were diluted 50-fold prior to use. In the case of NO_2-Tyr, Alexa Fluor 546 goat anti-rabbit IgG (H + L) (cat. no. AB11010; Invitrogen, Eugene, OR) was diluted 200 times prior to use. In case of 4-HNE, the tissue sections were reacted with Histofine Simple Stain MAX PO (R) (Nichirei Biosciences, Tokyo, Japan), followed by reaction with 3,3′-Diaminobenzidine solution. After the reaction, slides were observed using a microscope (BZ-8000; Keyence, Osaka, Japan). TUNEL staining were performed using an in situ cell death detection kit, Fluorescein (Roche, Basel, Switzerland), and treatment with DAPI (Dojin Chemical, Kumamoto, Japan). All kidney sections were viewed by light microscopy (BZ-8000 Keyence Corp.). NO_2-Tyr–positive or 4-HNE–positive areas were normalized by field with an area of 270 × 360 μm (original magnification, ×400). The number of TUNEL-positive cells were counted in the field with an area of 540 × 720 μm (original magnification, ×200). At least 10 randomized fields of kidney specimens from mice were used in the analysis for NO_2-Tyr, 4-HNE–positive areas, and TUNEL-positive cells.

Evaluation of P450 Content in Kidney. The collected kidneys were pretreated as previously described (Ogaki et al., 2015). P450 contents in kidney and liver were determined spectrophotometrically as described in a previous report (Omura and Sato, 1964).

Evaluation of Oxidative Stress. Malondialdehyde was determined by means of a TBARS Assay Kit (Cayman Chemical Company). The free radical activity in plasma was evaluated measuring hydroperoxide levels by the d-ROMs test (Diacron, Grosseto, Italy). The results of the d-ROMs test are expressed in arbitrary units, the so-called Carratelli units, where 1 Carratelli unit corresponds to 0.08 mg/100 ml H_2O_2 (Trotti et al., 2001).

Spectroscopy Analysis of Oxidant Mb in the Kidney. Oxidized Mb was evaluated as previously reported with minor modifications (Moore et al., 1998). In short, the kidney was homogenized on ice using a homogenizer in PBS (pH 7.4). The supernatant was collected after centrifuging (6000 rpm, 10 min, 4°C). The absorbance (380–540 nm) of supernatant was monitored using UV-visible spectrophotometer.

Cell Experiments. LLC-PK1 cells (American Type Culture Collection, Manassas, VA) were cultured at 37°C in 5% CO_2 in Medium 199 containing 10% FBS. The LLC-PK1 cells were incubated in 96-well plates with 100 μM CO-RBCs for 2 h and then incubated with 1 μg/ml Mb for 1.5 h (ROS production) or 24 h (cell viability) with or without 250 μM H_2O_2. ROS production and cell viability were determined by CM-H_2DCFDA (Thermo Fisher Scientific, MA) and a WST-8 solution (Dojindo Laboratories, Kumamoto, Japan), respectively.

Statistics. The means for groups were compared by analysis of variance followed by Tukey's multiple comparison. The survival rates were compared using Kaplan-Meier survival curves and the log-rank test. A probability value of $P < 0.05$ was considered to be significant.

Results

CO-RBCs Decreased the Mortality and AKI in CS Model Rats. It has been reported that an elevation in CPK levels is a prognostic marker of AKI after rhabdomyolysis (Safari et al., 2016). In our CS model, the CPK level in plasma reached 20,000 U/l at 1 h after decompression (Supplemental Fig. 1).

We therefore treated CS model rats with each solution at 1 h after decompression (Fig. 1A). All of the CS model rats that were treated with saline and O_2-RBCs died within 24 h after decompression, whereas all of the rats that had been treated with CO-RBCs survived for periods of up to 24 h after decompression (Fig. 1B). In addition, based on the results of biochemical analyses, the CS model rats induced AKI at 6 h after decompression, whereas the administration of CO-RBCs significantly ameliorated CS-induced AKI (Fig. 1, C and D). In addition, as evidenced by a histologic evaluation of kidneys, the CS model rats that had been treated with saline and O_2-RBCs showed tubular damage such as foamy and hyaline droplet degeneration (Fig. 1E). It is noteworthy that such histologic changes were largely inhibited by the CO-RBC treatment.

CO-RBCs Prevented Oxidative Injury and Apoptosis in Kidneys in CS Model Rats. As evidenced by immunostaining and a quantitative analysis of oxidation products (4-HNE and NO_2-Tyr) in kidneys, both of these oxidation products had accumulated in the kidney at 6 h after decompression (Fig. 2, A, C, and D). On the other hand, the CO-RBC treatment resulted in a significant suppression in the accumulation of 4-HNE and NO_2-Tyr after decompression, indicating that CO-RBCs effectively inhibited oxidative injury in the kidney but that the O_2-RBC treatment did not. Along with the results for the accumulation of oxidative products, CS model rats that were treated with CO-RBCs showed the less apoptosis-positive cells compared with those that were treated with saline and O_2-RBCs (Fig. 2, B and E).

CO-RBCs Improved the Mortality of Traumatic and Nontraumatic Rhabdomyolysis Model Rats Induced by Glycerol Treatment. CS model rats would not be suitable for further investigations related to how CO provides therapeutic effects in CS-related AKI because of their short survival period (Fig. 1B). The underlying mechanism of CS-related AKI is the same as that of traumatic rhabdomyolysis (Better and Abassi, 2011). Based on this fact, we next evaluated the effects of CO-RBCs on the survival in glycerol-induced traumatic rhabdomyolysis model rats with combined massive hemorrhage and showed that CO-RBC resuscitation improved the mortality of these animals compared with saline and O_2-RBC resuscitation (Fig. 3, A and B). However, this model is also not suitable for further studies due to their short survival period.

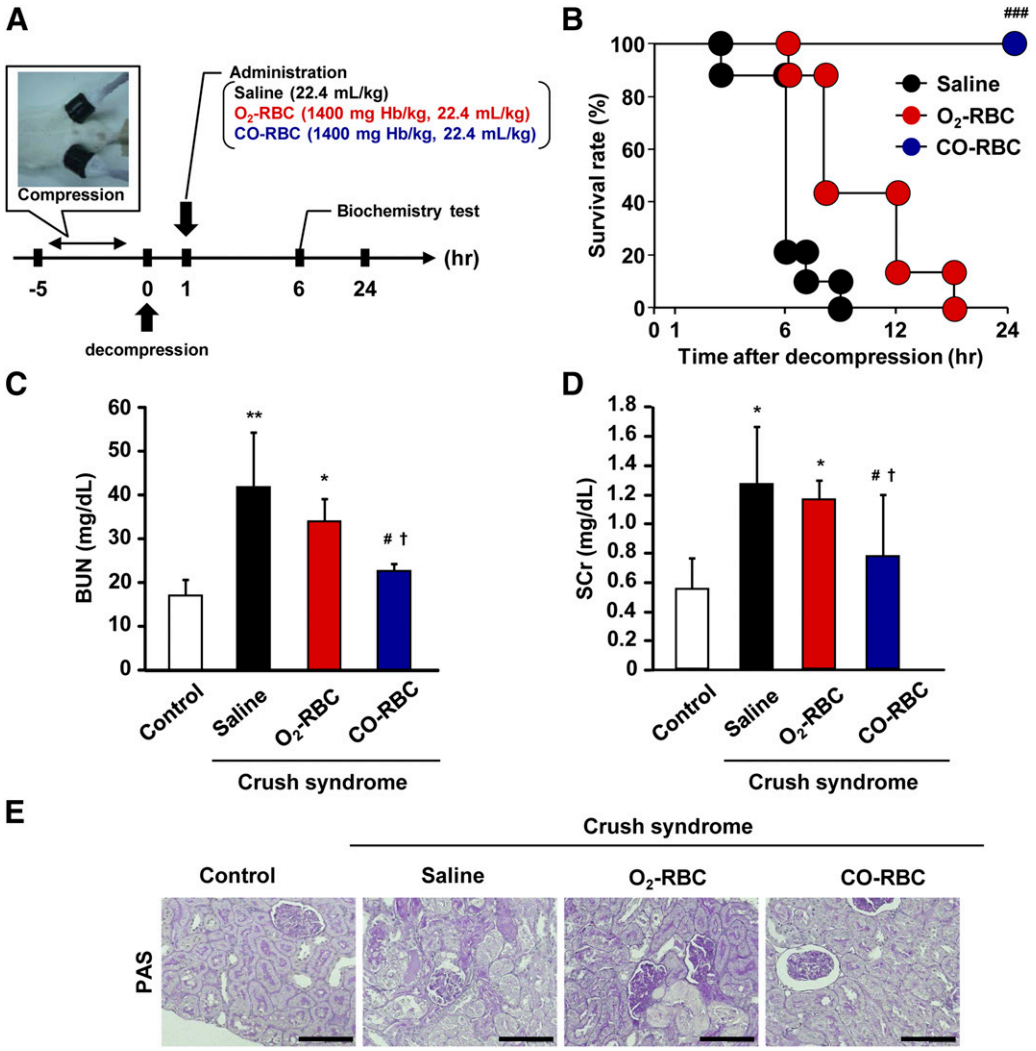

Fig. 1. Therapeutic effects of saline, O_2-RBC, and CO-RBC treatment on AKI in CS model rats. (A) Experimental protocol for CS model rats. (B) Survival rate of CS model rats after saline, O_2-RBC, and CO-RBC treatment. The survival rate was compared using Kaplan-Meier survival curves and the log-rank test ($n = 10$). Changes in the levels of BUN (C) and SCr (D) at 5 h after saline, O_2-RBC, and CO-RBC treatment of the CS model rats. Each column represents the mean ± S.D. ($n = 5$ to 6). $^*P < 0.05$; $^{**}P < 0.01$ vs. control, $\#P < 0.05$; $\#\#P < 0.01$; $\#\#\#P < 0.001$ vs. saline, $\dagger P < 0.05$; $\dagger\dagger P < 0.01$ vs. O_2-RBC. (E) Representative PAS-stained kidney sections at 5 h after saline, RBC, and CO-RBC treatment of the CS model rats. Scale bar, 100 μm.

Hence, we next evaluated the survival rate of glycerol-induced rhabdomyolysis model rats after each treatment (Fig. 3C). In this model, the CPK and plasma Mb levels were significantly increased at 3 h after the glycerol injection (Supplemental Fig. 2). Thus, we administered each sample at 3 h after the glycerol injection. The saline-treated and O_2-RBC–treated rats survived for 2 days but gradually died during a 5-day period after the glycerol injection, whereas the CO-RBC–treated rats all survived throughout observation period (Fig. 3D). Based on these findings, we used the glycerol-induced rhabdomyolysis model rats to investigate the renoprotective mechanism of CO-RBCs against rhabdomyolysis-induced rhabdomyolysis.

Renoprotective Effects of CO-RBCs on AKI in Glycerol-Induced Rhabdomyolysis Model Rats. A CO-RBC treatment significantly suppressed the increase in the level of BUN and SCr compared with saline and O_2-RBC treatment (Fig. 4, A and B). In addition, the CO-RBC treatment significantly suppressed the decrease in Cr clearance and the increase in the level of an AKI marker (NAG activity) (Fig. 4, C and D). Based on the results of histologic evaluations, severe damage was observed in the renal cortico-medullary boundary zone rather than in the inner stripe of the outer medulla or inner medulla by glycerol injection. However, the CO-RBC treatment suppressed such histologic damages induced by glycerol (Fig. 4E). Along with the slight dark-tea–colored urine in CO-RBC treatment, the level of Mb in urine was significantly decreased in the case of the CO-RBC treatment (Fig. 4, F and G). Moreover, the results of blood and urinary parameters indicated the induction of acidosis in this model rat, but the CO-RBC treatment attenuated these changes in acidosis parameters (Table 1).

CO-RBCs Attenuated Oxidative Injury in Glycerol-Induced Rhabdomyolysis Model Rats. The pathogenesis of AKI after rhabdomyolysis includes oxidative injury in the kidney. The accumulation of oxidative products (4-HNE) in the kidney and ROS markers (malondialdehyde and hydroperoxide) in plasma in the CO-RBC treatment group were significantly decreased compared with that in saline and O_2-RBC treatment group (Fig. 5, A–D), indicating that CO-RBC suppressed oxidative damage in the kidney.

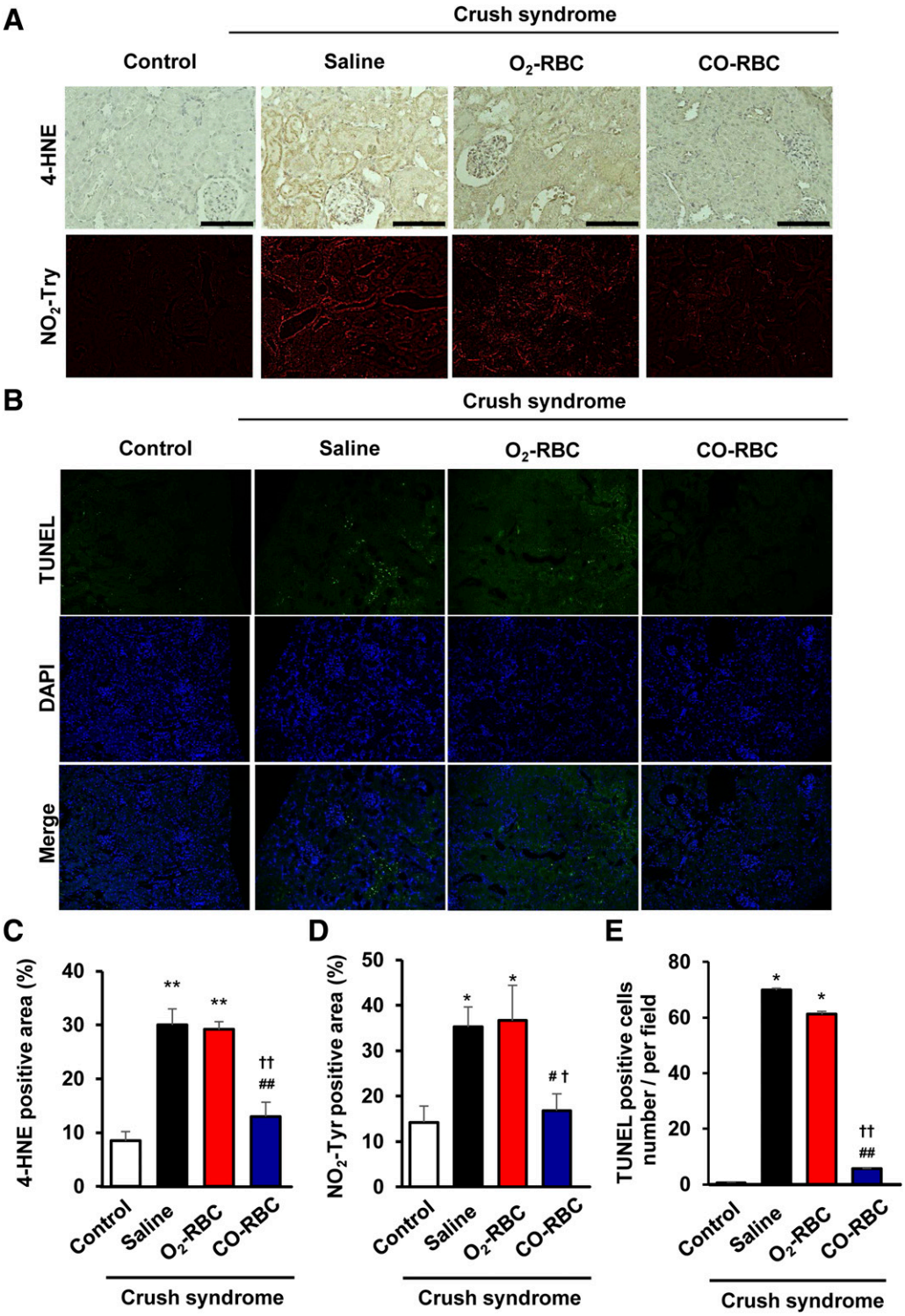

Fig. 2. Histologic evaluations of kidneys after each treatment in CS model rats. (A) Immunostaining of renal 4-HNE (upper panels) and NO_2-Tyr (lower panels) of kidneys of CS model rats after saline, O_2-RBC, and CO-RBC treatment. (B) TUNEL staining of the kidneys of CS model rats after saline, O_2-RBC, and CO-RBC treatment. Scale bar, 100 μm. 4-HNE–positive areas (C), NO_2-Tyr–positive area (D), and TUNEL-positive cells (E) number in kidney sections collected from control rats and CS model rats after saline, O_2-RBC, and CO-RBC treatment. Each column represents the mean ± S.D. (n = 5 to 6). *$P < 0.05$; **$P < 0.01$ vs. control, #$P < 0.05$; ##$P < 0.01$ vs. saline, †$P < 0.05$; ††$P < 0.01$ vs. O_2-RBC.

Effects of CO-RBCs on Oxidative Injury Induced by Mb and Oxidized Mb. Mb itself initiates the generation of ROS and peroxidation via a redox cycle between different oxidation states of Mb [ferric (Fe^{3+}) and ferryl (Fe^{4+}) Mb] (Moore et al., 1998). Thus, the effect of CO-RBCs on cellular ROS levels and cell viability induced by Mb was examined using LLC-PK1 cells and porcine kidney epithelial cells. The presence of Mb with or without H_2O_2 caused a significant increase in ROS production, but a CO-RBC pretreatment significantly suppressed the extent of cellular ROS production

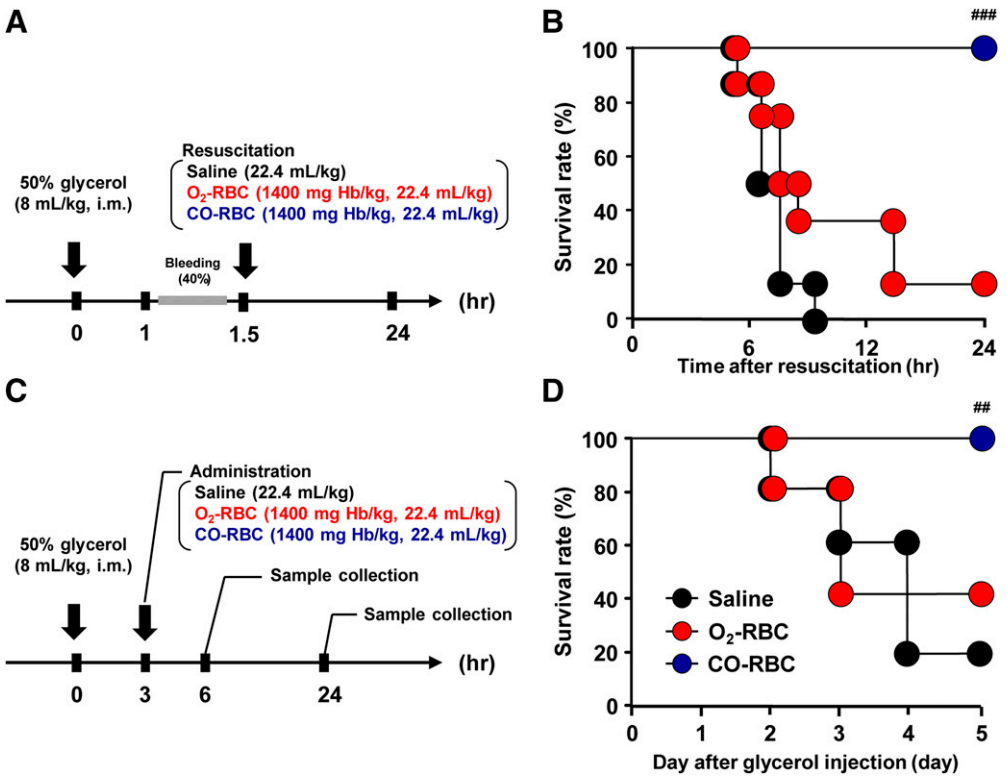

Fig. 3. Effects of saline, O_2-RBC, and CO-RBC treatment on survival period in traumatic and nontraumatic rhabdomyolysis model rats. (A) Experimental protocol for the traumatic rhabdomyolysis model rats induced by glycerol injection. (B) Survival rate of traumatic rhabdomyolysis model rats induced by glycerol injection after saline, O_2-RBC, and CO-RBC resuscitation. (C) Experimental protocol for the nontraumatic rhabdomyolysis model rats induced by glycerol injection. (D) Survival rate of nontraumatic rhabdomyolysis model rats induced by glycerol injection after saline, O_2-RBC, and CO-RBC resuscitation. The survival rate was compared using Kaplan-Meier survival curves and the log-rank test. (n = 10) $^{**}P < 0.01$; $^{***}P < 0.001$ vs. saline.

(Fig. 6A). CO-RBCs also significantly increased cell viability compared with PBS and the O_2-RBC treatment (Fig. 6B).

Furthermore, in in vivo conditions, the accumulation of Mb in the kidney was decreased in the CO-RBC treatment compared with the saline and O_2-RBC treatments (Fig. 6C). In addition, we qualitatively evaluated the level of oxidized Mb in kidneys in rhabdomyolysis model rats by spectroscopic analysis. As shown in Fig. 6D, the CO-RBC treatment resulted in a relative decrease in the production of Mb oxidant compared with the saline and O_2-RBC treatment.

Effects of CO-RBCs on Free Heme Production in Plasma and Kidney in Glycerol-Induced Rhabdomyolysis Model Rats. The level of free heme in kidneys and plasma, which cause the production of ROS via the Fenton reaction, was significantly decreased by the CO-RBC treatment compared with the saline and O_2-RBC treatment (Fig. 7, A and B). Although heme proteins, including P450 and hemoglobin, are the main sources of free heme in the kidney, the renal P450 content was significantly decreased in the saline-treated and O_2-RBC–treated rhabdomyolysis model rats, but such a decrease was suppressed in the CO-RBC treatment group (Fig. 7C). Interestingly, the P450 content in the liver, which contains the most abundant levels of CYP, was the same among all groups (Supplemental Fig. 3). Furthermore, urinary hemoglobin was also suppressed by the CO-RBC treatment (Fig. 7D). The results for urea hemoglobin, anemia, and hemolysis were also suppressed by the CO-RBC treatment (Supplemental Fig. 4).

Discussion

The major finding of the present study is that CO-RBCs ameliorated rhabdomyolysis-induced AKI with a high survival rate in three types of experimental nontraumatic and traumatic rhabdomyolysis model rats. It is especially noteworthy that the CO-RBC treatment exerted these renoprotective effects, even after plasma CPK values had increased significantly, which is a parameter for predicting the risk of rhabdomyolysis-induced AKI (Safari et al., 2016). Therefore, a CO-RBC treatment would be expected to be effective for both the prevention of and the treatment of rhabdomyolysis-induced AKI. The renoprotective effect of CO-RBCs could be attributed to the release of CO from CO-RBCs, causing a suppression in oxidative toxicity derived from the three kinds of heme proteins, Mb, CYP, and hemoglobin.

The accumulated body of evidence indicates that Mb that is released from damaged myocytes plays a crucial role in the pathogenesis of rhabdomyolysis-related AKI. Because the molecular weight of Mb is 17.8 kDa, the release of excessive amounts of Mb easily filtered by the glomerular apparatus and interacts with the Tamm-Horsfall protein, results in the formation of Mb-Tamm-Horsfall protein complexes in the renal tubules. This complex formation is one of the characteristics of rhabdomyolysis-associated AKI and results in the obstruction in renal tubules, leading to the development of AKI. In particular, the formation of the Mb-Tamm-Horsfall protein complex is occurred readily in the acidic urine conditions (Zager, 1989). Based on this fact, patients with rhabdomyolysis are administered fluids that contain sodium bicarbonate to increase urinary pH in a clinical situation. In addition, the accumulation of Mb also induced the vasoconstriction of renal medullary arteries in the kidney by scavenging nitric oxide, leading to renal ischemia. The renal ischemic condition (hypoxic condition) is one of the risk factors for the onset and progression of AKI due to fact that renal hypoxia is a common feature of AKI, drives signaling cascades and leads to renal pathology (Ow et al., 2018). In the present study, the CO-RBC treatment clearly suppressed the accumulation of Mb in the

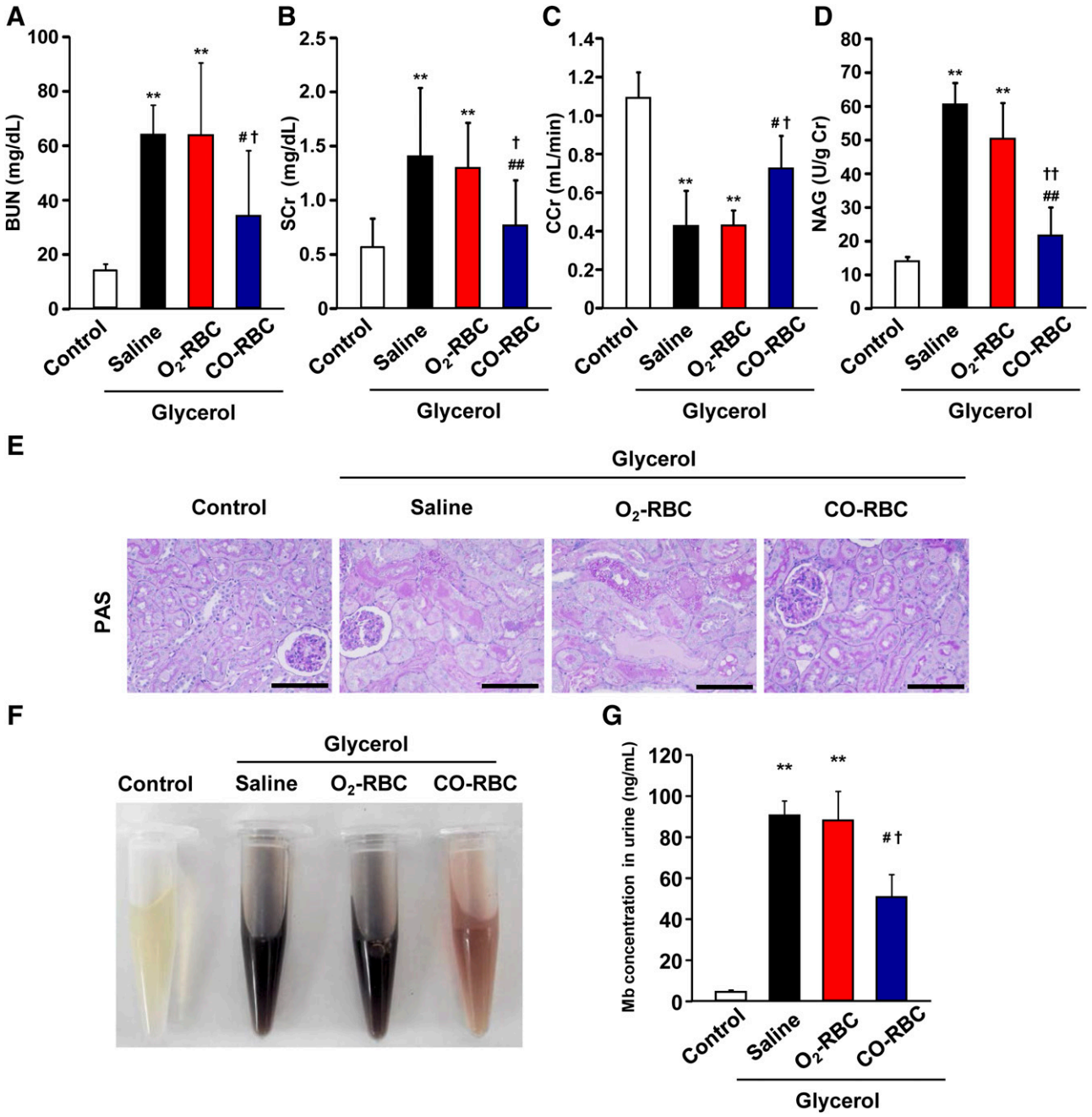

Fig. 4. Therapeutic effects of saline, O_2-RBC, and CO-RBC treatment on AKI in glycerol-induced rhabdomyolysis model rats. Changes in the levels of BUN (A), SCr (B), Cr clearance (CCr) (C), and urinary NAG activity (D) after saline, O_2-RBC, and CO-RBC treatment in glycerol-induced rhabdomyolysis model rats. NAG activity was expressed as units per gram of urinary Cr. CCr during 24 h after the injection of glycerol was calculated as ml/min. (E) Representative PAS-stained kidney sections in control and saline, O_2-RBC, and CO-RBC treatment rats at 24 h after glycerol injection. The appearance of urine (F) and urinary Mb (G) concentration in glycerol-induced rhabdomyolysis model rats treated with saline, O_2-RBCs, or CO-RBCs. Each column represents the mean ± S.D. (n = 5 to 6). $^{**}P < 0.01$ vs. control, $\#P < 0.05$; $\#\#P < 0.01$ vs. saline, $\dagger P < 0.05$; $\dagger\dagger P < 0.01$ vs. O_2-RBC. Scale bar, 100 μm.

kidney and subsequently inhibited tubular cast formation in CS and rhabdomyolysis model rats (Fig. 1E; Fig. 4E; Fig. 6C). CO-RBCs also restored the reduction in urinary pH that was observed in rhabdomyolysis rats (Table 1). Such a skewing of the urinary pH toward the alkaline side could also contribute to the suppression of Mb precipitation in renal tubules and consequently ameliorate the rhabdomyolysis-associated AKI.

The ferrous heme (Fe^{2+}) inside the Mb can be oxidized to the ferric heme (Fe^{3+}) and ferryl heme (Fe^{4+}) under certain pathologic conditions, which initiates oxidative injury (Boutaud et al., 2010). In the case of rhabdomyolysis, the accumulation of Mb in kidneys is also known to be a source of heme redox cycling, leading to oxidative injury (Bosch et al., 2009). In fact, acetaminophen was found to inhibit renal damage in a glycerol-induced rhabdomyolysis model via inhibiting the lipid peroxidation catalyzed by heme redox cycling (Boutaud et al., 2010). In the present study using an LLC-PK1 cell line, CO-RBCs suppressed ROS production and subsequently increased cell viability (Fig. 6, A and B). Furthermore, the amount of oxidized Mb in the kidney was greatly decreased in the CO-RBC treatment group compared

TABLE 1

Analysis of blood gas parameters, electrolytes, and urinalysis parameters at 24 h after saline, O_2-RBC, or CO-RBC treatment of glycerol-induced rhabdomyolysis model rats

Proteinuria and pH were evaluated using urine test paper. Scores 0, 1, 2, 3, 4, and 5 for proteinuria correspond to negative, false negative, 30, 100, 300, and 1000 mg/dl of protein, respectively. Scores 5–9 directly correspond to urinary pH 5–9. All unianalyses were performed under blind conditions. Values are shown as the mean ± S.E. (n = 3–5).

	pH	Base Excess (mmol/l)	Lactate (mmol/l)	K^+ (mEq/l)	Na^+ (mEq/l)	Hemoglobin (g/dl)	Urinary Protein	Urinary pH
Control	7.41 ± 0.02	1.8 ± 0.7	0.9 ± 0.1	4.46 ± 0.02	134 ± 1.0	13.8 ± 0.9	1.7 ± 1.2	7.66 ± 0.58
Saline	7.31 ± 0.02*	−2.1 ± 1.1**	2.8 ± 1.2**	7.12 ± 0.28**	117 ± 3.0*	11.4 ± 1.9*	3.7 ± 0.8*	5.66 ± 0.58**
O_2-RBC	7.33 ± 0.01*	−2.7 ± 1.9**	2.7 ± 1.1**	6.78 ± 0.22**	118 ± 2.4*	15.6 ± 0.9##	4.0 ± 1.0*	6.33 ± 0.58**
CO-RBC	7.38 ± 0.04#	−1.3 ± 1.7*#	1.2 ± 1.2##	5.52 ± 0.18*#	124 ± 2.0	13.6 ± 1.1#	1.3 ± 0.8#	7.00 ± 1.00*#

*$P < 0.05$; **$P < 0.01$ vs. control. #$P < 0.05$; ##$P < 0.01$ vs. saline.

with the saline and O_2-RBC treatment group in glycerol-induced rhabdomyolysis model rats (Fig. 6D). Sher et al. previously demonstrated that CO diminished the oxidative toxicity of Mb by binding to ferrous heme inside the Mb (Sher et al., 2014). This finding leads to the possibility that CO released from CO-RBCs would inhibit the auto-oxidation of heme in Mb via direct binding to heme, resulting in the CO-RBC treatment suppressing the Mb oxidant-induced oxidative injury in the kidney. In addition, urinary pH appears to be related to the redox signaling of Mb. Moore et al. reported that the redox cycling of Mb was inhibited by urine alkalinization in a rhabdomyolysis model animal, resulting in ameliorating Mb-induced oxidative injury, especially lipid peroxidation (Moore et al., 1998). As mentioned above, a higher urinary pH was observed in the CO-RBC treatment group compared with the saline and RBC treatment group in glycerol-induced rhabdomyolysis model rats (Table 1). Therefore, CO may also indirectly inhibit Mb toxicity via skewing urinary pH toward the alkaline side.

CYP is thought to be related to the pathogenesis of other renal diseases based on evidence collected using experimental model animals (Liu et al., 2002b) because P450 is one of the sources of free heme P450 2E1 in the kidney and appears to be a particularly key P450 isoform for the induction of AKI. In fact, it has been reported that free heme that is released by the degradation of P450 2E1 in the kidney caused the production of ROS and cytotoxicity in a rhabdomyolysis-associated AKI (Wang et al., 2014). Furthermore, Liu et al. reported that treatment with a P450 2E1 inhibitor suppressed cisplatin-induced renal injury in P450 2E1 null mice ((Liu et al., 2002a);

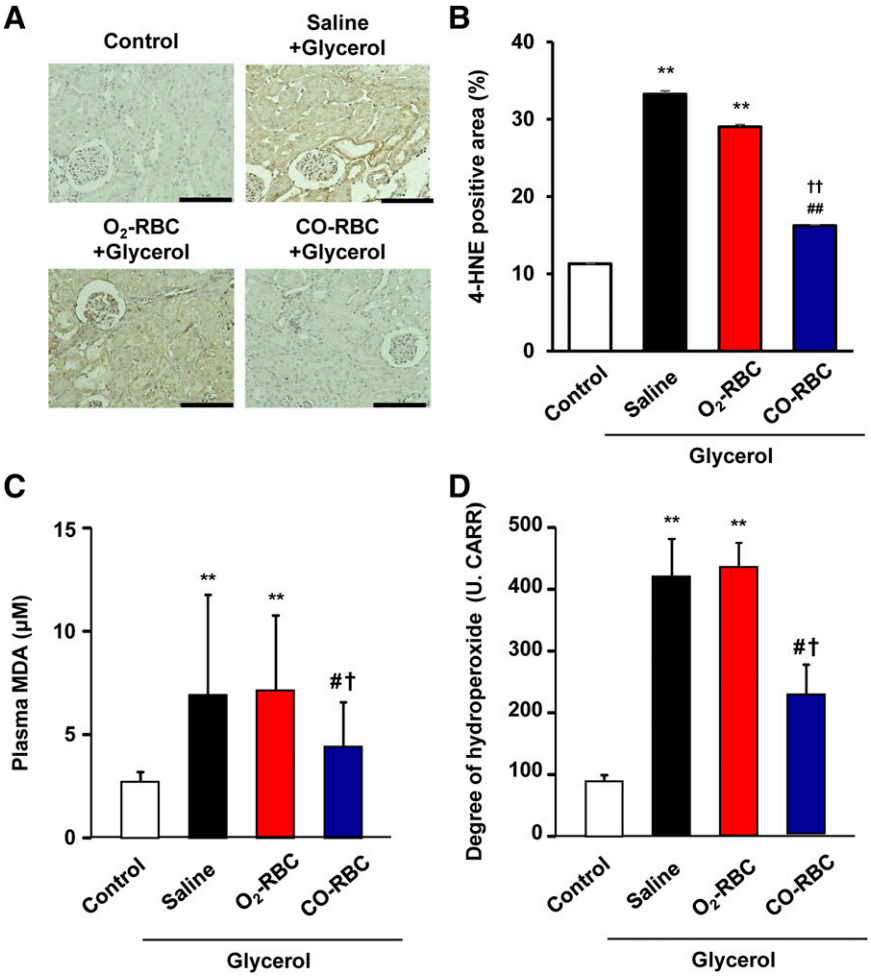

Fig. 5. Evaluation of oxidative injury in glycerol-induced rhabdomyolysis model rats. (A) Immunostaining of renal 4-HNE of kidneys of glycerol-induced rhabdomyolysis model rats after saline, O_2-RBC, and CO-RBC treatment. (B) 4-HNE–positive areas in kidney sections collected from control rats and CS model rats after saline, O_2-RBC, and CO-RBC treatment. Changes in the levels of malondialdehyde (MDA) (C) and hydroperoxide (D) in plasma in glycerol-induced rhabdomyolysis model rats treated with saline, O_2-RBCs, or CO-RBCs. Each column represents the mean ± S.D. (n = 5 to 6). **$P < 0.01$ vs. control, #$P < 0.05$; ##$P < 0.01$ vs. saline, †$P < 0.05$; ††$P < 0.01$ vs. O_2-RBC. Scale bar, 100 μm.

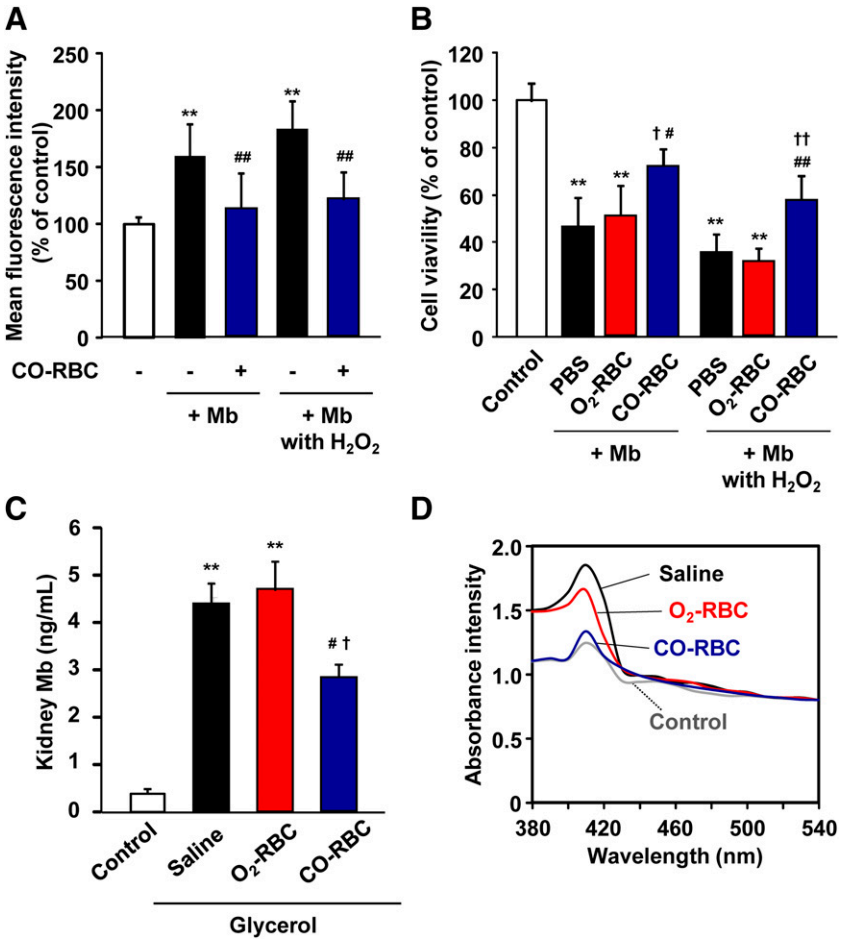

Fig. 6. Effects of CO-RBCs on the Mb redox cycling in vitro and in vivo. (A) Effect of CO-RBCs on cellular ROS levels induced by Mb with or without H_2O_2 in LLC-PK1 cell. Each column represents the mean ± S.D. (n = 5). $^{**}P < 0.01$ vs. nontreatment, $^{\#\#}P < 0.01$, vs. CO-RBC (-). (B) Effect of O_2-RBCs and CO-RBCs on cell viability induced by Mb with or without H_2O_2 in LLC-PK1 cell. Each column represents the mean ± S.D. (n = 5). $^{**}P < 0.01$ vs. control, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$, vs. PBS, $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$ vs. O_2-RBC. (C) Changes in the levels of kidney Mb in glycerol-induced rhabdomyolysis model rats treated with saline, O_2-RBCs, or CO-RBCs. Each column represents the mean ± S.D. (n = 3–5). $^{**}P < 0.01$ vs. control, $^{\#}P < 0.05$ vs. saline, $^{\dagger}P < 0.05$ vs. O_2-RBC. (D) Absorbance spectrum of oxidized Mb in rat kidney collected from normal and glycerol-induced rhabdomyolysis model rats treated with saline, O_2-RBCs, or CO-RBCs. The kidneys were collected at 6 h after an intramasucular injection of glycerol. Data are representative of three rats in each group with similar results.

Liu and Baliga, 2003). Hence, P450 protection would be an effective strategy for suppressing the development of rhabdomyolysis-associated AKI. In this study, the CO-RBC treatment resulted in the renal P450 content being maintained and the amount of free heme in the kidney being decreased in glycerol-induced rhabdomyolysis model rats (Fig. 7, A and C). These facts indicate that the protection of renal P450 is likely one of the renoprotective mechanisms of CO-RBCs. Unfortunately, we were not able to specify which P450 isoforms of CO-RBCs had protective functions in this study. However, we previously revealed that a CO-RBC treatment protected hepatic P450 2E1 in a rat model of hemorrhagic-shock and resuscitation (Ogaki et al., 2013). Therefore, CO-RBCs would be expected to suppress the degradation of renal P450 2E1 in the present experimental rats.

Hemoglobin released from RBCs is also a major source of free heme in the kidney and partially contributes to the development of renal damage because hemoglobin easily releases free heme (Sher et al., 2014). In addition, hemoglobin reacts with hydrogen peroxide or nitric oxide, ultimately producing toxic substances such as oxoferryl hemoglobin and transient free radical intermediates (Buehler et al., 2010). In the present study, mild hemolysis and, hence, the accumulation of hemoglobin in urine were observed in CO-RBC treatment group (Fig. 7D; Supplemental Fig. 4). As shown in Fig. 5, B and C, the CO-RBC treatment suppressed the accumulation of oxidation products in both the blood and kidney. Such an anti-oxidative effect of CO could contribute to the suppression of hemolysis because oxidative stress causes RBC rupture, resulting in hemolysis or the leaking of hemoglobin. Thus, CO-RBCs also inhibit hemoglobin-related renal injury.

It is noteworthy that the CO-RBC treatment ameliorated the clinical manifestations associated with rhabdomyolysis, such as hyperkalemia and acidosis (Table 1), which worsens AKI and increases mortality (Sever et al., 2012; Sever and Vanholder, 2013). As described above, acidosis facilitated the Mb-induced AKI. More importantly, it is well known that a high level of potassium in the blood is a life-threatening symptom in patients with CS (Sever et al., 2002) because hyperkalemic cardiac arrest frequently occurred under such a condition (Better and Abassi, 2011). The beneficial effects of CO-RBCs on these clinical systemic features would be derived from the renoprotective effects of CO-RBCs because the kidney is the responsible organ for controlling both blood pH and the systemic electrolyte balance, including potassium, and would lead to a decrease in mortality.

Although CO derived from CO-RBCs provided beneficial effects for the treatment of AKI in this study, the fatal deleterious effects of CO on the body is widely recognized. This contrariety would be due to the concentration and duration of CO exposure (Weaver, 2009). It is generally known that clinical deleterious symptoms derived form CO toxicity are produced when the carboxyhemoglobin level in blood reaches to 40% of total hemoglobin. In separate experiments using healthy mice, we confirmed that the maximum carboxyhemoglobin ratio was 20% after CO-RBC administration at a dose of

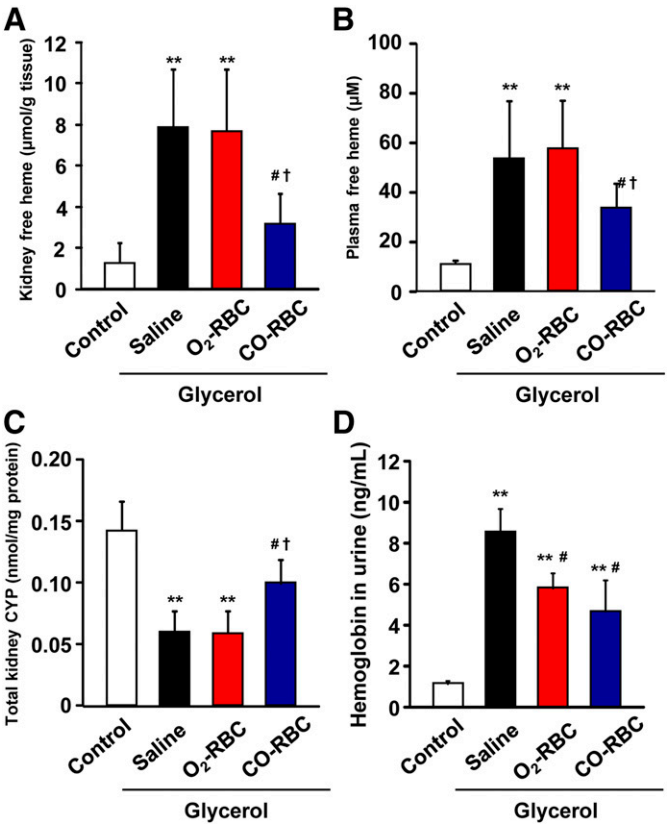

Fig. 7. CO-RBCs attenuate the contents of free heme derived from P450 and hemoglobin. Contents of free heme in kidney (A) and plasma (B) in glycerol-induced rhabdomyolysis model rats treated by saline, O_2-RBCs, or CO-RBCs. Changes in the levels of total renal P450 (C) and hemoglobin (D) in urine in glycerol-induced rhabdomyolysis model rats treated with saline, O_2-RBCs, or CO-RBCs. Each column represents the mean ± S.D. (n = 3–5). $^{**}P < 0.01$ vs. control, $\#P < 0.05$ vs. saline, $\dagger P < 0.05$ vs. O_2-RBC.

1400 mg Hb/kg (Taguchi et al, submitted manuscript). On the other hand, low concentrations of CO can have a variety of positive effects on the body, including anti-apoptosis, anti-inflammatory, anti-oxidative, and anti-proliferative effects (Motterlini and Otterbein, 2010). Considering these collective facts, CO-RBC treatment ameliorated the AKI in the experimental conditions of this study.

When CO is used as a medication, caution is needed concerning respiratory depression that is induced by the reduction in O_2 levels. In the present study, CO-RBCs achieved a 100% survival rate against the rhabdomyolysis-induced AKI in massive hemorrhage shock rats, whereas 90% of the rats that received O_2-RBCs died within 24 h (Fig. 3B). In separate experiments, we confirmed that all of the rats with solely massive hemorrhage shock survived when CO-RBC resuscitation was applied (Ogaki et al., 2013). This implies that the risk of respiratory depression is unlikely because the administration of CO-RBCs does not significantly diminish the level of O_2 in vivo. Furthermore, this also implies that the mortality caused by massive hemorrhage shock was markedly increased by rhabdomyolysis-induced AKI and vice versa and that an additional treatment with O_2-RBCs is necessary to improve their mortalities. In these aspects, CO-RBCs appear to be an adequate therapeutic because they can be used to treat traumatic and nontraumatic rhabdomyolysis-induced AKI without respiratory depression through supplementation with both CO and O_2.

Although the findings reported in this study show that CO-RBCs protected the renal injury *via* the suppression of heme protein–associated renal oxidative injury, other mechanisms could in principle involve in the renoprotection of CO-RBCs. One possible mechanism is the endogenous HO-1/CO system. It was reported that HO-1 induction was associated with renoprotection in rhabdomyolysis-induced AKI (Wei et al., 2011; Uchida et al., 2019). Furthermore, Goebel et al. reported that exogenously derived CO increased HO-1 expression and activity in kidney, resulting in ameliorating AKI (Goebel et al., 2010). When these facts are taken together, the increase in HO-1 expression and activity after CO-RBC administration may contribute to ameliorating rhabdomyolysis-induced AKI in both nontraumatic rhabdomyolysis and CS-induced AKI model rats. Another possibility is that CO would ameliorate renal vasoconstriction, which plays a crucial role in the pathogenesis of rhabdomyolysis-related AKI. It was reported that the levels of endothelin-1, which is the most potent renal vasoconstrictor, increased in the condition of rhabdomyolysis, causing aggravation of the AKI (Karam et al., 1995; Gamkrelidze et al., 2016). Thus, renal vasorelaxation is one possible strategy for the treatment of rhabdomyolysis-related AKI. In a previous study, it was reported that CO reduced vasocontraction induced by various vasoconstrictors including endothelin-1 (Failli et al., 2012). Hence, CO-RBCs may prevent renal vasoconstriction by virtue of the vasorelaxing effects of CO, resulting in the ameliorating rhabdomyolysis-related AKI.

Conclusion

The findings presented herein serve to demonstrate that CO-RBCs have considerable potential as a novel cell therapy for use in the treatment of both nontraumatic and traumatic rhabdomyolysis including CS-induced AKI despite being given after the muscle injury occurs. Beneficially, CO-RBCs can be used in both the hospital and the prehospital including at disaster sites because CO-RBCs can be easily and rapidly prepared by bubbling CO gas through the RBC preparation in ex vivo.

Authorship Contributions

Participated in research design: Taguchi, Fukagawa, Otagiri, Maruyama.

Conducted experiments: Ogaki, Nagasaki, Yanagisawa, Nishida, Maeda, Enoki, Matsumoto, Sekijima, Ooi.

Performed data analysis: Ishima, Watanabe.

Wrote or contributed to the writing of the manuscript: Taguchi, Otagiri, Maruyama.

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