

**Carbon monoxide bound red blood cells protect red blood cell transfusion-induced hepatic cytochrome P450 impairment in hemorrhagic-shock rats**

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**Abbreviation:** **RBC**, red blood cell; **CYP**, cytochrome P450s; **CO**, carbon monoxide; **CO-RBC**, carbon monoxide bound red blood cell; **rHSA**, recombinant human serum albumin; **PaO<sub>2</sub>**, arterial blood oxygen tension; **PaCO<sub>2</sub>**, arterial blood carbon dioxide tension; **HPLC**, high-performance liquid chromatography; **AUC**, area under the blood concentration-time curve; **ROS**, reactive oxygen species; **IL-6**, interleukin-6; **TNF- $\alpha$** , tumor necrosis factor- $\alpha$ .

## Abstract

Red blood cell (RBC) transfusions for massive hemorrhage induce systemic ischemic-reperfusion, and influence the disposition and pharmacological activity of drugs as a result of a reduction in the level of expression and activity of cytochrome P450s (CYP). It was reported that when organ preserving solutions are exposed to carbon monoxide (CO), the treatment was effective in suppressing the post-reperfusion reduction in renal CYP levels in cases of kidney transplantation. Therefore, we hypothesized that transfusions with RBC that contain bound CO (CO-RBC) would protect the hepatic level of rat CYP during a massive hemorrhage compared to plasma expanders and RBC resuscitation. To achieve this, we created 40% hemorrhagic-shock model rats followed by resuscitation using recombinant human serum albumin, RBC and CO-RBC. At 1 hr after resuscitation, the expressions of hepatic CYP isoforms (1A2, 2C11, 2E1, 3A2) were significantly decreased in the RBC resuscitation group compared to the sham group. Such alterations in hepatic CYP significantly resulted in an increase in the plasma concentrations of substrate drugs (caffeine (1A2), tolbutamide (2C11), chlorzoxazone (2E1) and midazolam (3A2)) for each CYP isoform and, hence, the hypnotic action of midazolam could be significantly prolonged. Interestingly, the reductions in hepatic CYP activity observed in the RBC group were significantly suppressed by CO-RBC resuscitation, and consequently the pharmacokinetics of substrate drugs and the pharmacological action of midazolam remained at levels similar to those under sham conditions. These results indicate that CO-RBC resuscitation has considerable potential in terms of achieving safe and useful drug therapy during massive hemorrhages.

## Introduction

Red blood cell (RBC) transfusions are the first administered care in cases of patients who are in a state of hemorrhagic-shock and has greatly contributed to human health and welfare. However (and unfortunately), resuscitation from a massive hemorrhage by RBC transfusion causes systemic ischemia-reperfusion (Allan et al., 2011), a major deleterious factor that affects the maintenance of homeostasis. Several recent investigations have clearly shown that a warm hepatic ischemia-reperfusion induced a decrease in hepatic Cytochrome P450 (CYP) levels and metabolic activity (Izuishi et al., 2000; Eum and Lee, 2004). CYP is a heme protein that has a broad range of functions, including the metabolism and detoxification of endogenous and exogenous substances (Morgan, 2001). Since the majority of CYP is located in the liver, any alteration in hepatic CYP levels or activity would constitute a major influence on the disposition and pharmacological activities of drugs. In emergencies, where RBC transfusions are needed, CYP-metabolized drugs, such as corticosteroid hormones, cardiac stimulants and anesthetics, are frequently used to maintain general status; therefore, a decrease in CYP levels and activity due to ischemia-reperfusion as the result of a RBC transfusion, can result in altered blood levels of these drugs, and consequently could have an effect on their pharmacological characteristics. In fact, Harbrecht et al. reported that patients undergoing RBC transfusion experienced elevated blood concentrations of CYP-metabolized drugs, such as flurbiprofen, mephenytoin, chlorzoxazone probably as a result of suppressed metabolism (Harbrecht et al., 2005). Hence, maintaining reasonable levels of CYP during RBC transfusions is generally believed to be critical in minimizing changes in the disposition of CYP substrate drugs and their pharmacological effects, but drugs or treatments designed to achieve these conditions are not currently available.

Carbon monoxide (CO) possesses anti-inflammatory, anti-apoptotic and anti-oxidant activities, suggesting that it could be used to prevent organ damage caused by ischemia-reperfusion injury (Otterbein et al., 2000; Brouard et al., 2002; Zuckerbraun et al.,

2005; Stec et al., 2008; Motterlini and Otterbein, 2010). Nakao et al. recently reported an interesting observation, in which the exposure of organ preservation solutions to CO was effective in suppressing the post-reperfusion reduction in renal CYP levels in a rat model of kidney transplantation (Nakao et al., 2008). This suggests that the use of CO might be useful in reducing CYP levels, even in the case of resuscitation from hemorrhagic-shock via a RBC transfusion. However, it should be noted that a carrier for controlling the disposition of CO would be essential in successful clinical applications of CO. Fortunately, since CO binds easily and stably to hemoglobin in RBC, RBC represents a leading candidate for use as a carrier for delivering CO. In fact, Cabrales et al. recently reported that when CO-RBC is injected into hemorrhaging hamsters, it is as effective as the systemic or microhemodynamic administration of RBC and its cytoprotective effect in the subcutaneous microcirculation is higher than RBC (Cabrales et al., 2007). In addition, Sakai et al. reported that CO-RBC showed a resuscitative effect in hemorrhagic-shocked rats and that oxidative damage to organs was reduced in comparison to RBC (Sakai et al., 2009). These findings led us to the hypothesis that CO-bound RBC (CO-RBC) has the potential for use as a novel RBC transfusion preparation with a CYP protein protective effect.

The objective of this study was to determine (i) whether CO-RBC transfusion represents a promising resuscitative fluid and (ii) to what extent would CO-RBC transfusions influence the hepatic levels of various rat CYP isoforms (1A2, 2C11, 2E1, 3A2) and the disposition and pharmacological action of CYP-metabolized drugs compared with recombinant human serum albumin (rHSA) and a RBC transfusion using 40% exsanguinated model rats.

## Materials and Methods

### *Preparation of resuscitative fluids*

The three resuscitative fluids used for this study were prepared as follows. (i) To prepare the rHSA resuscitative fluid, a 25% rHSA (Nipro Co, Osaka, Japan) solution was diluted to 5% with a saline solution. (ii) The RBC resuscitative fluid was prepared as described in a previous report (Taguchi et al., 2011b); blood samples from donor male Sprague-Dawley (SD) rats were withdrawn into heparinized syringes (ca. 0.15 mL of 10,000 IU/mL heparin to 10 mL of blood) and centrifuged; the pellet was then washed three times to remove plasma components by resuspension in saline followed by centrifugation (3000 g, 10 min, 4°C). The hemoglobin concentration of a washed RBC suspension was determined with a hemoglobin B test kit (Wako Chemicals, Saitama, Japan), and was adjusted to 10 g/dL. A washed RBC suspension (8.6 mL) was mixed with a solution of rHSA (25%, 1.4 mL) to regulate [rHSA] in the suspending medium to 5 g/dL and the colloid osmotic pressure to approximately 20 torr. (iii) CO gas was gently bubbled through the RBC resuscitative fluid, prepared as described above, for 5 min. This solution designated as the CO-RBC resuscitative fluid (Sakai et al., 2009).

### *Preparation of hemorrhagic-shock model rats and resuscitation from hemorrhagic-shock*

All animal experiments were performed according to the guidelines, principles, and procedures for the care and use of laboratory animals of Kumamoto University. Eighty-eight male SD rats (180-210 g, Kyudou Co, Kumamoto, Japan) were anesthetized and polyethylene catheters (PE 50 tubing, outer diameter equal to 0.965 mm, and inner diameter equal to 0.58 mm; Becton Dickinson and Co., Tokyo, Japan) containing saline and heparin were then introduced into the left femoral artery. Hemorrhagic-shock was induced by removing 40% of the total blood volume according to a previous report (Taguchi et al., 2009; Taguchi et al., 2011a). Systemic blood volume was estimated to be 56 mL/kg (Sakai et al., 2004). Under this experimental condition, the mean arterial pressure remained at less than 40 mm Hg for 30 min.

The hemorrhagic-shock rats were resuscitated by an infusion of rHSA (n=20), RBC (n=20) or a CO-RBC (n=20) resuscitative fluid at a rate of 1 mL/min. The volume of the infused resuscitative fluids was equal to 40% of total blood volume at the baseline. After resuscitation from shock, the femoral artery was ligated, and the skin was sutured with a stitch. In addition, thirteen hemorrhagic-shock rats were not resuscitated with any fluid, and fifteen rats were subjected to a sham operation. All animals were maintained in a temperature-controlled room with a 12-hr dark/light cycle and *ad libitum* access to food and water. The resuscitated rats were selected at random from each group, and were used at 1 and 24 hr after resuscitation in the experiments outlined below.

#### *Measurement of systemic responses*

Blood gases were evaluated before hemorrhage (baseline), after hemorrhage, and 1 hr after resuscitation. Blood samples were collected from the right femoral artery. A blood gas analyzer (i-STAT; Fuso Pharmaceuticals Industries, Tokyo, Japan) was used for analyses of arterial blood oxygen tension (PaO<sub>2</sub>), arterial blood carbon dioxide tension (PaCO<sub>2</sub>), pH, base excess and lactate.

#### *Western blot analysis*

At 1 hr or 24 hr after resuscitation from hemorrhage, the vena cava of the rats was cannulated with a polyethylene catheter (diameter, 0.9 mm), and the liver was perfused with 1.15% KCl. The liver was homogenized (1000 rpm, 5 min, on ice) using a homogenizer (Ikemoto Scientific Technology, Tokyo, Japan) in lysis buffer (0.23M sucrose, 5 mM Tris-HCl, 2 mM EDTA, 50x protease inhibitor cocktail, pH7.4). After centrifuging (550 g, 5 min, 4 °C) the homogenized liver preparation, the supernatant was used as the liver extract. After measurement of the protein content using a Bradford assay, each sample was suspended in loading buffer (2% SDS, 125 mM Tris-HCl (pH 7.4), 20% glycerol, 5% 2-mercaptoethanol) and

heated at 100 °C for 2 min. The samples were separated by 12.5% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA) by wet electroblotting. The blots were blocked with 5% ECL advance blocking agents (GE Healthcare Bio-sciences Corp., Piscataway, NJ) in TBS containing 0.3% Tween 20 (TBS-T). The blots were washed once with TBS-T and incubated for 2 hr at room temperature with primary antibodies specific for CYP1A2, CYP2C11, CYP2E1 and CYP3A2 (polyclonal goat anti-rat CYP1A2, CYP2C11, CYP2E1 and polyclonal rabbit anti-rat CYP3A2, NOZAN, Kanagawa, JAPAN) in TBS-T. The blots were washed three times with TBS-T and incubated with secondary antibodies (horseradish peroxidase-linked anti-goat and anti-rabbit IgG (Invitrogen, Eurogen, OR)) for 1 hr at room temperature. The blots were washed three times with TBS-T and the immunoblots were visualized using an ECL system (ECL Advance Western Blotting Detection Kit; GE Healthcare Bio-sciences Corp.) with LAS-3000mini (Fujifilm, Tokyo, Japan). The band intensities were quantified using the NIH Image analysis software (<http://rsbweb.nih.gov/ij/>).

#### *The pharmacokinetic experiment protocol with CYP cocktail*

The pharmacokinetic experiments using the CYP cocktail were performed as described in a previous report (Liu et al., 2009). The CYP cocktail consisted of four probe drugs, caffeine (Nacalai tesque Co, Kyoto, Japan) for CYP1A2, tolbutamide (Alexis Co, Lausen, Switzerland) for CYP 2C11, chlorzoxazone (Oxford Biomedical Research Inc, Oxford, U.S.A) for CYP 2E1 and midazolam (Sigma Chemicals Inc (St Louis, MO, U.S.A) for CYP3A2. At 1 hr after resuscitation from hemorrhage, the CYP cocktail (2.5 mg of each drug/kg) was administered to each rat via the tail vein. Blood samples were collected at multiple time points after injection of the CYP cocktail (5, 15, 30 min, 1, 2, 4, 6, 12, and 24 hr) and the plasma was separated by centrifugation (3000 g, 5 min, 4°C). The concentration of drugs in plasma was determined by high-performance liquid chromatography (HPLC) according to a previous report



(Liu et al., 2009). The HPLC system consisted of a Hitachi L-6000 pump, a Hitachi L-4000 UV detector operated at 230 nm and Hitachi D-2500 Chromato integrator. LC analyses were carried out on a Cadenza-CD-C18 column (250 mm  $\times$  3.0 mm i.d., particle size 3  $\mu$ m).

#### *The pharmacodynamic experiments protocol*

The pharmacodynamic experiments for midazolam involved the method developed by Wang et al, with minor modifications (Wang et al., 2010). The hemorrhagic-shock rat model was prepared under ether anesthesia and resuscitated as described above. All rats were awake within 30 min after resuscitation. At 1 hr after resuscitation from hemorrhage, midazolam (5 mg/kg) was administered to each rat via the tail vein. After the administration of midazolam, the hypnotic effect index was recorded. The experiments were carried out in a quiet room at room temperature. The time lapse between the administration of midazolam and the onset of losing the righting reflex was recorded as sleep latency. The interval between the loss and recovery of the righting reflex was recorded as the sleeping time.

#### *Data analysis*

A two-compartment model was used to determine the pharmacokinetic parameters after administering the CYP cocktail. Pharmacokinetic parameters were estimated by curve-fitting using MULTI, a normal least-squares program (Yamaoka et al., 1981). Data are shown as the means  $\pm$  SD for the indicated number of animals. Significant differences among each group were examined using the Student's t-test. The Spearman test was used for the correlation analysis. A probability value of  $p < 0.05$  was considered to be statistically significant.

## Results

### *Systemic response to hemorrhagic-shock and resuscitation by RBC and CO-RBC*

All rats that received RBC or CO-RBC as a resuscitative fluid survived until their planned death. In contrast, all non-resuscitated rats (n=13) died within 1.5 hr after the induction of hemorrhagic-shock, and 6 of the 20 rats that had been resuscitated by rHSA died during the experiment (Fig. 1). Changes in blood gas parameters were not significantly different among the rHSA, RBC and CO-RBC resuscitative fluid groups (Table 1). Moreover, the CO-RBC resuscitative fluid group showed no signs of hypoxia or abnormal behavior after resuscitation. These data indicate that CO-RBC resuscitation did not present any toxic liability and would be expected to function as a resuscitation fluid, analogous to RBC resuscitation.

### *Expression of isoforms of the hepatic CYP protein*

To evaluate the effect of resuscitative fluids on hepatic CYP levels, the expression levels of the hepatic protein CYP isoforms, CYP1A2, 2C11, 2E1 and 3A2, were determined by Western blot analysis. At 1 hr after resuscitation, the protein expressions of all CYP isoforms in the RBC resuscitation group were significantly decreased compared to the sham group, while those in the rHSA and CO-RBC resuscitation group were maintained at constant levels (Fig. 2). Furthermore, these changes were maintained in all groups during the entire period of the experiment (24 hr after resuscitation). These data suggest that CO-RBC resuscitation protects against at least four hepatic CYP isoforms.

### *Plasma concentration of CYP cocktail*

We next addressed the issue of whether changes in the levels of CYP isoform proteins directly affected the pharmacokinetics of the process using four probe drugs, namely, caffeine (1A2), tolbutamide (2C11), chlorzoxazone (2E1) and midazolam (3A2) that are specifically metabolized by the CYP isoforms. Fig. 3 shows the time course for the plasma concentration of

the four probe drugs in the sham, rHSA, RBC and CO-RBC resuscitation groups. The plasma concentrations of the four probe drugs were substantially elevated in the RBC resuscitation group, while the concentrations in the rHSA and CO-RBC resuscitation group showed a similar profile to that for the sham group. Compared to the sham group, the area under the blood concentration-time curve (AUC) for the four probe drugs in the RBC resuscitation group were significantly increased, while the results for the rHSA and CO-RBC resuscitation group were similar to those for the sham group (Table 2).

Moreover, as shown in Fig. 4A, B and D, the AUC for caffeine, tolbutamide and midazolam were negatively correlated with the expression of CYP1A2, CYP2C11 and 3A2 protein, respectively (caffeine:  $r=0.99$ ,  $p=0.042$ , tolbutamide:  $r=0.99$ ,  $p=0.046$ , midazolam:  $r=0.99$ ,  $p=0.028$ ). Similarly, tendencies for negative correlations between the AUC and the expression of CYP2E1 were observed for chlorzoxazone (Fig. 4C;  $r=0.98$ ,  $p=0.086$ ). In addition, considering all of the data shown in Fig. 4A-D, a significantly negative correlation exists between them (Fig. 4E;  $r=0.93$ ,  $p=0.017$ ), indicating that such a relation was common among the CYP isoforms. These data confirm that the significant alternations in hepatic CYP expressions by hemorrhaging and resuscitation were major contributors to the changes in the AUC for the CYP metabolized drugs.

#### *Pharmacological effect of midazolam*

To evaluate whether the alteration in CYP protein expression has an effect on pharmacological parameters, sleep latency and sleeping time induced by the administration of midazolam were measured. As shown in Fig. 5A, sleeping latency remained essentially unchanged among all groups. However, sleeping time in the RBC resuscitation group was significantly increased compared to that in the sham group, while sleeping time for the rHSA and CO-RBC resuscitation group was similar to that for the sham group (Fig. 5B).

As shown in Fig. 5C, sleeping time induced by midazolam was negatively correlated

with the levels of CYP3A2 expression ( $r=0.99$ ,  $p=0.007$ ). These data indicate that the changes in the pharmacological effect of midazolam are largely dependent on the expression of the CYP3A2 protein, even under conditions of hemorrhaging and resuscitation.

## Discussion

The clinical indications for RBC transfusion, the utility of which is unquestionable, include an emergency administration to combat massive bleeding. During such RBC transfusions for a massive hemorrhage, ischemia-reperfusion injury occurs systemically in many organs, especially in the liver, which, in turn, can change the dispositions of therapeutic drugs and their pharmacological effects as a result of a reduction in the level of expression and activity of CYP. The present study focused on this issue using hemorrhagic-shock model rats, and further examined the effect of CO-RBC on hepatic CYP protection and resuscitative action. The major findings of the present study are as follows; (i) CO-RBC appears to be as effective as RBC as a resuscitation fluid, (ii) CO-RBC significantly suppressed reductions in CYP isoform levels that occur during RBC resuscitation from a massive hemorrhage, (iii) consequently such an inhibition in the alteration of CYP isoform levels by CO-RBC significantly suppressed the changes in the Pharmacokinetic-Pharmacodynamic properties for CYP-metabolizing drugs.

Harbrecht et al. examined the disposition of drugs metabolized by CYP isoforms in 23 critical ill trauma patients after RBC transfusions, and reported that their metabolic activities by CYP were decreased and were associated with ischemia-reperfusion injury (Harbrecht et al., 2005). In this study, we also found marked reductions in the levels of hepatic CYP isoforms associated with RBC resuscitation and interestingly, this was significantly inhibited in the case of CO-RBC resuscitation close to the sham levels (Fig. 2). Correspondingly, after CO-RBC resuscitation, the dispositions of some CYP substrate drugs (caffeine, tolbutamide, chlorzoxazone and midazolam) for the respective CYP isoforms and the hypnotic action of midazolam remained at levels similar to those found for the sham group (Fig. 3, 5 and Table 2). In those observations, changes in the AUC of the substrate drugs of each CYP isoform or the pharmacological effect of midazolam were negatively correlated with the expression levels of the respective CYP isoforms (Fig. 4 and 5). These results suggest that such alternations in pharmacokinetic properties and the pharmacodynamic action of CYP substrates is due to

metabolic inhibition and not the suppression of renal excretions. CYP-metabolizing drugs are frequently employed in the treatment of hemorrhagic-shock. For example, fentanyl and propofol are frequently used as surgical narcotics. Therefore, maintaining drug metabolism using CO-RBC is of great significance from the viewpoint of achieving a safe and effective drug therapy during a massive hemorrhage. Such protective effects of CO-RBC against hepatic CYP isoforms might be exerted in pathological situations involving not only systemic hemorrhagic-shock but also various local ischemia, such as liver transplantation, in which CYP metabolizing immunosuppressive agents (e.g., tacrolims, cyclosporine) are frequently used (Tamura et al., 1997; Abu-Amara et al., 2010). Since the results reported herein involved the use of a rat model of hemorrhagic-shock, it is not entirely clear that the results would be directly applicable to humans. However, it should be noted that similar results were reported for human subjects by Harbrecht et al., such as changes in the activities of CYP isoforms (CYP2C9, 2C19, 2E1) and hepatic injury in patients with RBC transfusions (Harbrecht et al., 2005). Therefore, this shared aspect between human and animal models supports the hypothesis that the results reported herein may well be applicable to humans.

Another finding of note in the present study was the early manifestation of the effect of CO, at 1 hr after resuscitation. Ischemia-reperfusion injury in the liver has been demonstrated to show a biphasic pattern, consisting of both acute- and subacute-phase responses (Izuishi et al., 2000; Eum and Lee, 2004; Aitken and Morgan, 2007). The former is characterized by hepatocellular injury after 3-6 hr of reperfusion, while the latter peaks after 18-24 hr of reperfusion. In ischemic rats, hepatic CYP levels were significantly decreased after both 5 and 24 hr of reperfusion. However, no information is available concerning the effect of hepatic CYP at the more acute-phase of ischemia-reperfusion, such as at 1 hr after reperfusion. Present findings clearly show that, at least 1 to 24 hr after RBC resuscitation, the hepatic CYP levels of all isoforms and their activities were significantly decreased by RBC transfusion-induced ischemia-reperfusion during hemorrhagic-shock, and this was effectively attenuated by the

CO-RBC treatment. Therefore, it would be expected that CO-RBC treatment would also exert a protective effect against hepatic CYP levels and activity during the acute- and subacute-phase of ischemia-reperfusion.

Details of the mechanism by which CO-RBC ameliorates hepatic CYP content induced by ischemia-reperfusion are currently unclear. There are two possibilities that explain the decrease in CYP expression after RBC transfusion; 1) degradation of CYP enzymes by ischemia-reperfusion, 2) inhibition of the biosynthesis of CYP enzymes by ischemia-reperfusion. There is now a substantial body of evidence to support the notion that many of the pathophysiological events triggered by ischemia-reperfusion injury are mediated through the production of reactive oxygen species (ROS), and to the subsequent secretion of inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Carcillo et al., 2003). Previous studies clearly showed that these cytokines and ROS cause a decrease in CYP expression due to an enhancement in CYP degradation (Thelen and Dressman, 2009; Vee et al., 2009; Weaver, 2009). These findings lead us to hypothesize that the reduction in CYP expression after RBC resuscitation may be caused by the degradation of CYP enzymes rather than the inhibition of CYP synthesis, and that CO-RBC may inhibit CYP degradation via the suppression of ROS production or the expression of IL-6 and TNF- $\alpha$  because it has been demonstrated that CO suppresses both the production of ROS and inflammatory cytokines (Mottterlini and Otterbein, 2010). In fact, at 1 hr after resuscitation, the mRNA levels of each CYP isoform remained relatively unchanged among the sham, rHSA, RBC, and CO-RBC resuscitation groups (data not shown). We plan to investigate this mechanism in more detail in a future study.

Cabrales et al. and Sakai et al. prepared CO-RBC under the same conditions as were used in the present study, and compared its resuscitative effect and safety with RBC transfusion using a rodent model of 50% hemorrhagic-shock (Cabrales et al., 2007; Sakai et al., 2009). As a result, they concluded that CO-RBC could be as effective as RBC in recovering from

hemorrhagic-shock, in terms of its influence on blood pressure, laboratory parameters and microhemodynamics, and it suppressed tissue damage induced by reperfusion, an effect not seen in the case of RBC resuscitation. The present experimental conditions also show that CO-RBC possesses the same resuscitative effect as RBC, including survival and blood gas parameters (Fig.1 and Table 1). However, from a clinical perspective, the toxicity of CO-RBC is of concern due to the potential for CO poisoning (Weaver, 2009). In this study, no symptoms of CO poisoning were observed with 40% hemorrhagic-shock rats. Similarly, no adverse or toxic effects of CO were evident in the aforementioned study with 50% hemorrhagic-shock hamsters and rats (Cabrales et al., 2007; Sakai et al., 2009). Such little toxicity of CO treatment during ischemia-reperfusion is probably due to the rapid elimination of CO. In fact, HbCO levels in blood, an indicator of CO poisoning, were found to rise transiently just after CO-RBC administration, but then fell to baseline levels within 1 hr. Judging from these comprehensive findings, it could be concluded that the risk of CO poisoning due to CO-RBC resuscitation is low, and that CO-RBC has the potential for use as a promising resuscitative fluid.

Although our results demonstrated the utility of CO-RBC, some limitations must also be taken into account. First, the bleeding volume was fixed at 40% in the present study. In actual clinical settings involving RBC transfusions, more than 40% of the total blood supply is frequently lost, which can necessitate the transfusion of blood to an extent that the entire circulating blood volume is exceeded. For this reason, it will be necessary to assess the resuscitation and hepatic CYP protective effects of CO-RBC under conditions involving more than a 40% blood loss. Second, present study did not examine the changes in CYP levels in other organs. Although the majority of CYP is located in the liver, it is well known that intestinal CYP also plays an important role in the bioavailability of orally administered drugs (Provenzani et al., 2011). Therefore, it will be necessary to investigate the changes in CYP levels in the gastrointestinal tract, and evaluate the suppressive effects of CO-RBC thereon.



In conclusion, we provide the first demonstration that RBC resuscitation-induced ischemia-reperfusion causes a decrease in the levels of isoforms of hepatic CYP and that this significantly influences the disposition of CYP-metabolizing drugs and their pharmacological action, from at least 1 to 24 hr after resuscitation. In addition, CO-RBC is as effective as RBC as a resuscitation fluid for hemorrhagic-shock, but also minimizes the unfavorable effects of drug therapy associated with RBC-induced ischemia-reperfusion by protecting against decreases in hepatic CYP isoforms. These findings show that CO-RBC has the potential for serving as a novel blood transfusion medium that improves the limitations associated with the currently used RBC transfusions.

## **AUTHORSHIP CONTRIBUTION**

*Participated in research design:* Ogaki, Taguchi, Otagiri, and Maruyama.

*Conducted experiments:* Ogaki, and Taguchi.

*Contributed new reagents or analytic tools:* Otagiri, and Maruyama.

*Performed data analysis:* Watanabe.

*Wrote or contributed to the writing of the manuscript:* Ogaki, Taguchi, and Maruyama.

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## Footnotes

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## Figure Legends

### Figure 1

**Survival rate of hemorrhagic-shock rats without resuscitation (opened circles) and with resuscitation by recombinant human serum albumin (rHSA) (hatched circles), red blood cells (RBC) (closed circles) or carbon monoxide bound red blood cells (CO-RBC) (gray circles).**

The number of rats (n) in non-resuscitation, rHSA, RBC and CO-RBC group was n=13, n=20, n=20 and n=20, respectively. At 1 hr after resuscitation, rHSA (n=8), RBC (n=10) and CO-RBC (n=10) were planned use for experiment and planned kill.

### Figure 2

**Expression of hepatic protein CYP isoforms (A: CYP1A2, B: CYP2C11, C: CYP2E1, D: CYP3A2) in sham and hemorrhagic-shock rats at 1 and 24 hr after resuscitation by recombinant human serum albumin (rHSA), red blood cells (RBC) or carbon monoxide bound red blood cells (CO-RBC).**

Protein expression of each CYP isoform/ $\beta$ -actin in sham rat defined as 100 %. In the rHSA group, three of ten rats died during the experiment. Therefore, the number of rats (n) in rHSA group was four and three at 1 hr and 24 hr, respectively. The other group of rats all survived during experiment (n=5). The values are means  $\pm$  S.D.. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. sham rats group.

<sup>##</sup>  $p < 0.01$  vs. RBC resuscitation group.

### Figure 3

**Plasma concentration profile for the CYP cocktail (A: caffeine (CYP1A2), B: tolbutamide (CYP2C11), C: chlorzoxazone (CYP2E1), D: midazolam (CYP3A2)) in sham (opened circles) and hemorrhagic-shock rats at 1 hr after resuscitation by recombinant human serum albumin (rHSA) (hatched circles), red blood cells (RBC) (closed circles) or carbon**



**monoxide bound red blood cells (CO-RBC) (gray circles).**

In the rHSA group, two of five rats died during experiment. Therefore, the number of rats (n) in rHSA group was three. The other group rats all survived during experiment (n=5).

Each points represents the means  $\pm$  S.D. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. sham rats group. #  $p < 0.05$ , ##  $p < 0.01$  vs. RBC resuscitation group.

#### Figure 4

**Relationship between the expression of hepatic CYP isoform proteins and the area under the blood concentration-time curve (AUC) for (A) caffeine, (B) tolbutamide, (C) chlorzoxazone, (D) midazolam and (E) group figure 3A-D together after an injection of the CYP cocktail to sham (opened circle) and hemorrhagic-shock rats at 1 hr after resuscitation by recombinant human serum albumin (rHSA) (hatched circle), red blood cells (RBC) (closed circle) or carbon monoxide bound red blood cells (CO-RBC) (gray circle).**

The linear regression of logarithmic values was calculated using the least-squares method (A;  $y = -5.0122x + 557.88$ ,  $r = 0.99$ ,  $p = 0.042$ , B;  $y = -4.4273x + 494.45$ ,  $r = 0.99$ ,  $p = 0.046$ , C;  $y = -5.7324x + 589.91$ ,  $r = 0.98$ ,  $p = 0.086$ , D;  $y = -3.7556x + 524.6$ ,  $r = 0.99$ ,  $p = 0.028$ , E;  $y = -4.62x + 681$ ,  $r = 0.93$ ,  $p = 0.017$ ).

#### Figure 5

**Effect of recombinant human serum albumin (rHSA), red blood cells (RBC) or carbon monoxide bound red blood cell resuscitation (CO-RBC) on (A) sleep latency and (B) sleeping time induced by midazolam in hemorrhagic-shock rats. (C) Relationship between the expression of hepatic CYP3A2 protein and sleeping time induced by midazolam in sham (open circle) and hemorrhagic-shock rats at 1 hr after resuscitation by rHSA (hatched circle), RBC (closed circle) or CO-RBC (gray circle).**

In the rHSA group, one of five rats died during experiment. Therefore, the number of rats (n) in rHSA group was four. The other group rats all survived during experiment (n=5).

Each points represents the means  $\pm$  S.D. \*\*  $p < 0.01$

The linear regression of logarithmic values was calculated using the least-squares method ( $y = -0.42x + 106.86$ ,  $r = 0.99$ ,  $p = 0.007$ ).

**Table 1**

**Laboratory variables during hemorrhagic-shock resuscitation by recombinant human serum albumin (rHSA), red blood cell (RBC) or carbon monoxide bound red blood cells (CO-RBC).**

PaO<sub>2</sub>: arterial blood oxygen tension; PaCO<sub>2</sub>: arterial blood carbon dioxide tension; BE: base excess; Lac: lactate

	pH	PaO <sub>2</sub> (torr)	PaCO <sub>2</sub> (torr)	BE (mmol/L)	Lac (mmol/L)	HbCO (%)
before hemorrhage						
rHSA	7.35 ±0.03	75.8 ±4.4	39.1 ±4.2	1.1 ±1.3	0.7 ±0.1	N.D.
RBC	7.35 ±0.02	76.7 ±3.7	39.3 ±3.1	1.7 ±0.7	0.7 ±0.1	N.D.
CO-RBC	7.36 ±0.05	74.3 ±3.5	40.7 ±7.8	1.0 ±1.7	0.7 ±0.2	N.D.
after hemorrhage (0 min)						
rHSA	7.22 ±0.08*	102.1 ±11.3**	24.8 ±6.8**	-4.3 ±1.6**	4.2 ±1.1**	N.D.
RBC	7.21 ±0.03*	101.8 ±9.2**	24.7 ±8.3**	-5.3 ±2.1**	4.4 ±1.3**	N.D.
CO-RBC	7.21 ±0.08*	100.0 ±10.1**	26.7 ±4.5**	-3.7 ±0.6**	4.1 ±1.1**	26.4 ±6.6
after hemorrhage (60 min)						
rHSA	7.21 ±0.03*	84.7 ±8.2	33.4 ±4.9	0.6 ±1.1	0.7 ±0.2	N.D.
RBC	7.21 ±0.01*	78.5 ±6.1	38.3 ±6.1	0.7 ±0.6	0.9 ±0.1	N.D.
CO-RBC	7.20 ±0.07*	75.3 ±2.4	40.3 ±4.7	1.7 ±1.0	0.8 ±0.1	3.2 ±5.0

Values are means ±S.D. (n=6-8). \* p<0.05, \*\* p<0.01 vs baseline.

**Table 2**

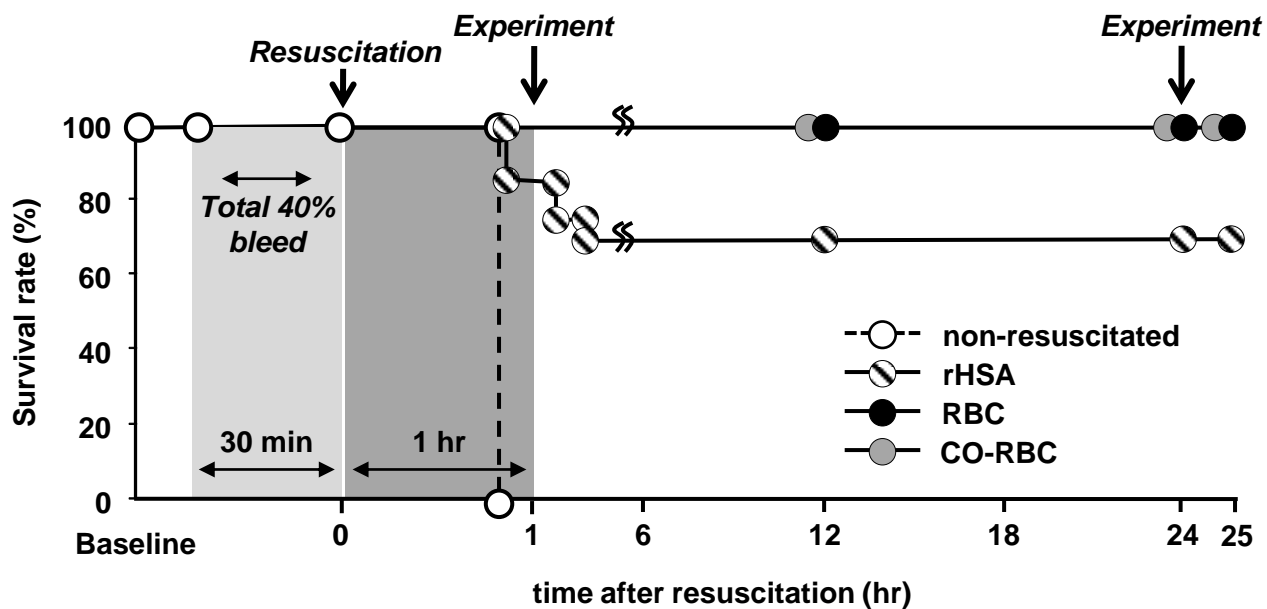
**Pharmacokinetic parameters for the of CYP cocktail after intravenous injection to sham rats and hemorrhagic-shock rats resuscitated by recombinant human serum albumin (rHSA), red blood cell (RBC) or carbon monoxide bound red blood cells (CO-RBC).**

Blood samples were collected at 5, 15, 30 min, 1, 2, 4, 6, 12 and 24 hr after the injection of the CYP cocktail (caffeine (CYP1A2), tolbutamide (CYP2C11), chlorzoxazone (CYP2E1) and midazolam (CYP3A2)), and the drugs were detected by high-performance liquid chromatography.

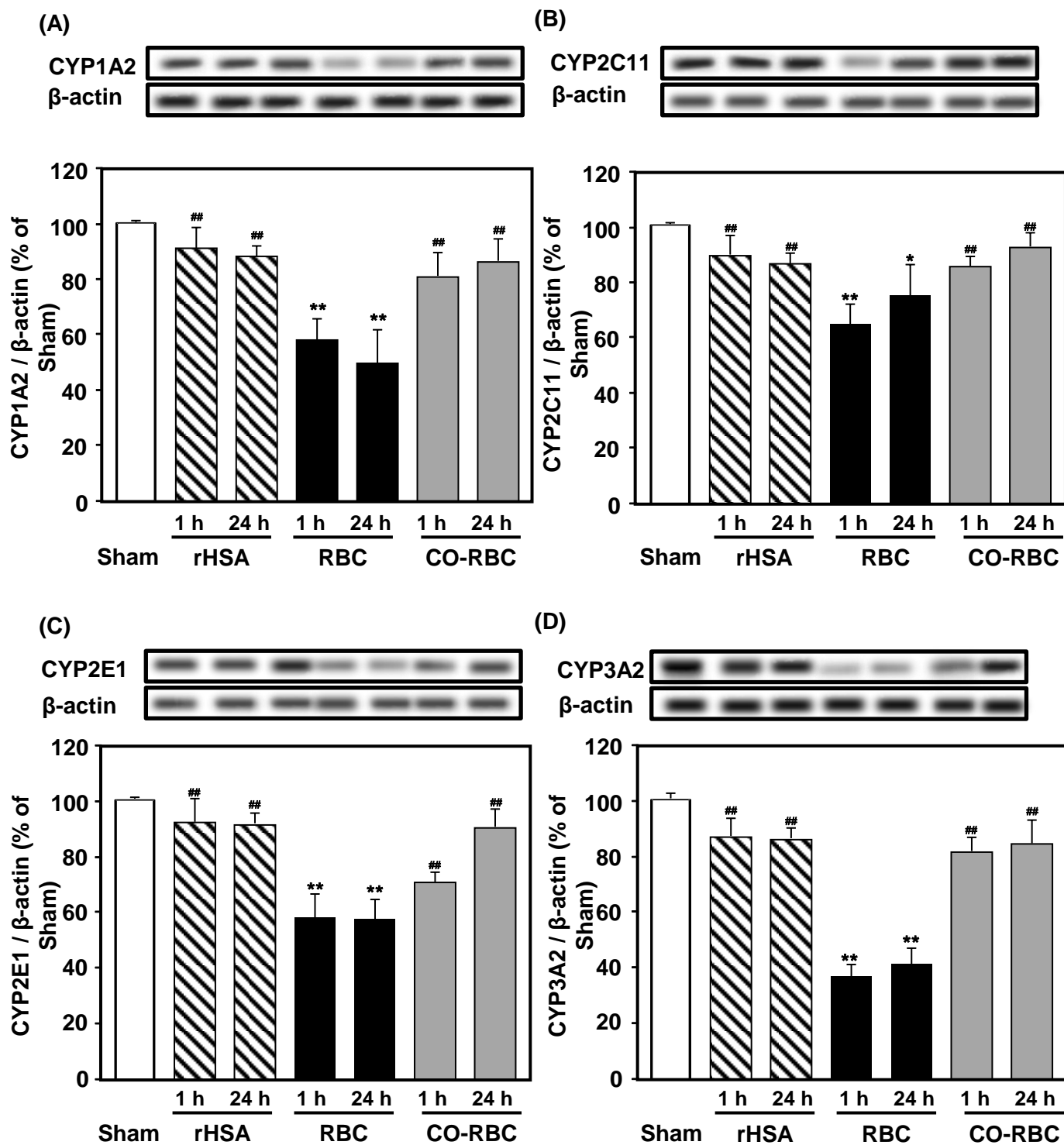
	$T_{1/2}$ (hr)	$K_e$ ( $\times 10^3 \text{ min}^{-1}$ )	AUC (hr $\times$ ng/mL)	CL (mL/hr)
Caffeine (CYP1A2)				
sham	4.8 $\pm$ 2.0	2.7 $\pm$ 1.0	279.7 $\pm$ 25.3	1.86 $\pm$ 0.15
rHSA	5.1 $\pm$ 2.3	2.1 $\pm$ 0.9	384.5 $\pm$ 71.3	1.54 $\pm$ 0.48
RBC	8.2 $\pm$ 3.3*	1.6 $\pm$ 0.8*	509.6 $\pm$ 82.7**	1.04 $\pm$ 0.16**
CO-RBC	5.4 $\pm$ 3.7	1.4 $\pm$ 0.2	406.1 $\pm$ 4.9	1.28 $\pm$ 0.09
Tolbutamide (CYP 2C11)				
sham	2.7 $\pm$ 0.6	4.5 $\pm$ 1.0	222.4 $\pm$ 43.1	2.39 $\pm$ 0.40
rHSA	2.8 $\pm$ 1.4	4.2 $\pm$ 0.7	264.4 $\pm$ 40.2	2.15 $\pm$ 0.56
RBC	6.8 $\pm$ 0.7**	1.7 $\pm$ 0.2**	402.1 $\pm$ 39.0**	1.29 $\pm$ 0.13**
CO-RBC	2.9 $\pm$ 0.6	4.0 $\pm$ 0.7	255.3 $\pm$ 36.9	2.07 $\pm$ 0.31
Chlorzoxazone (CYP 2E1)				
sham	4.8 $\pm$ 1.1	2.5 $\pm$ 0.6	287.3 $\pm$ 61.5	1.87 $\pm$ 0.36
rHSA	3.6 $\pm$ 1.3	3.2 $\pm$ 1.1	304.8 $\pm$ 41.5	1.77 $\pm$ 0.44
RBC	6.7 $\pm$ 0.9**	1.7 $\pm$ 0.2**	593.0 $\pm$ 64.8**	0.95 $\pm$ 0.12**
CO-RBC	3.3 $\pm$ 0.8	3.6 $\pm$ 0.8	315.9 $\pm$ 95.0	1.73 $\pm$ 0.37
Midazolam (CYP 3A2)				
sham	3.2 $\pm$ 0.4	3.7 $\pm$ 0.4	211.3 $\pm$ 19.2	2.47 $\pm$ 0.24
rHSA	3.6 $\pm$ 1.7	3.6 $\pm$ 1.4	316.4 $\pm$ 73.2	1.62 $\pm$ 0.86
RBC	4.5 $\pm$ 1.2*	2.7 $\pm$ 0.6*	496.5 $\pm$ 116.1**	1.08 $\pm$ 0.22**
CO-RBC	3.2 $\pm$ 0.7	3.7 $\pm$ 0.7	293.8 $\pm$ 14.8	1.78 $\pm$ 0.16

Values are means  $\pm$  S.D. (n=3-5). \* p<0.05, \*\* p<0.01 vs sham.

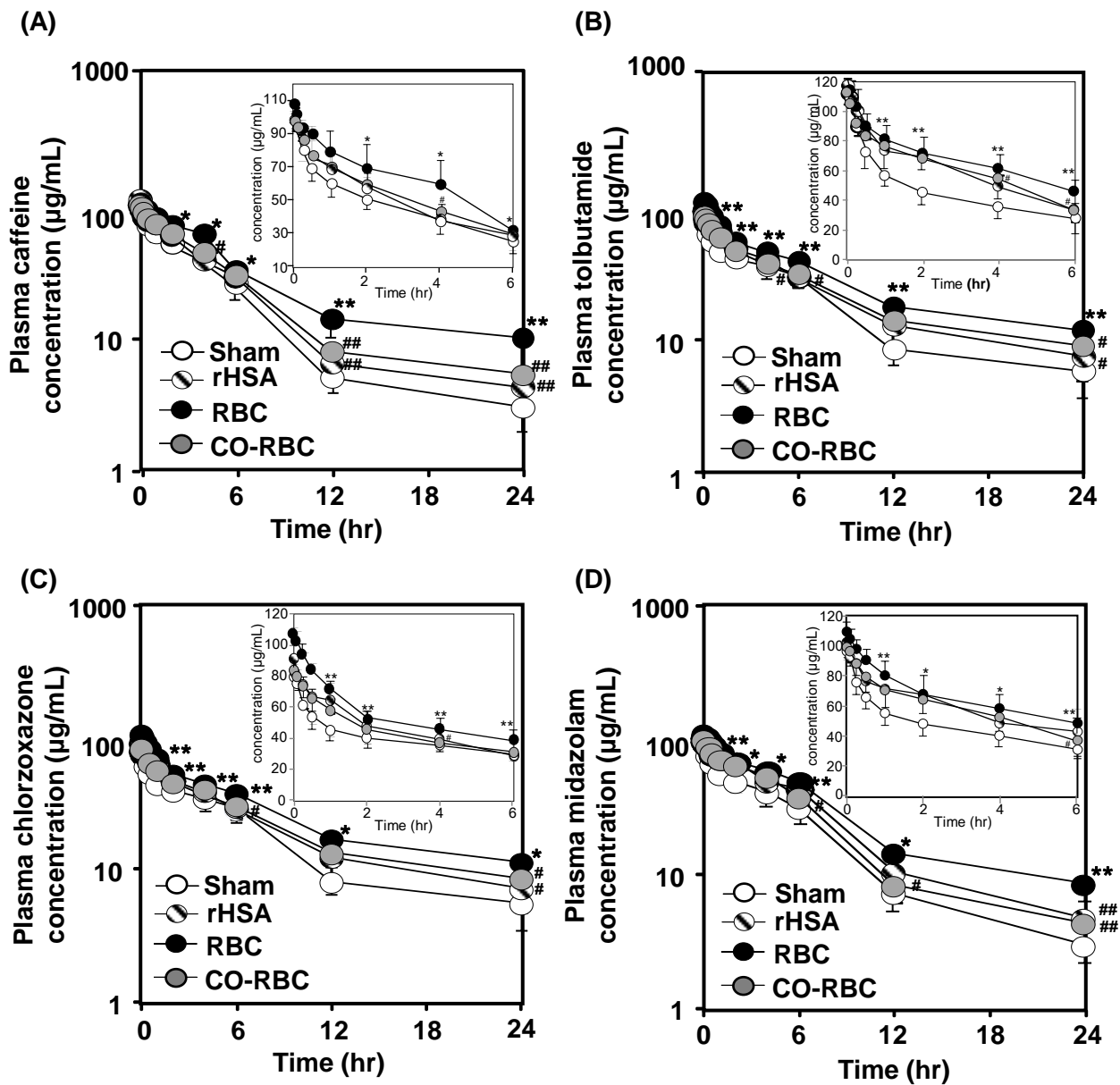
**Fig. 1**



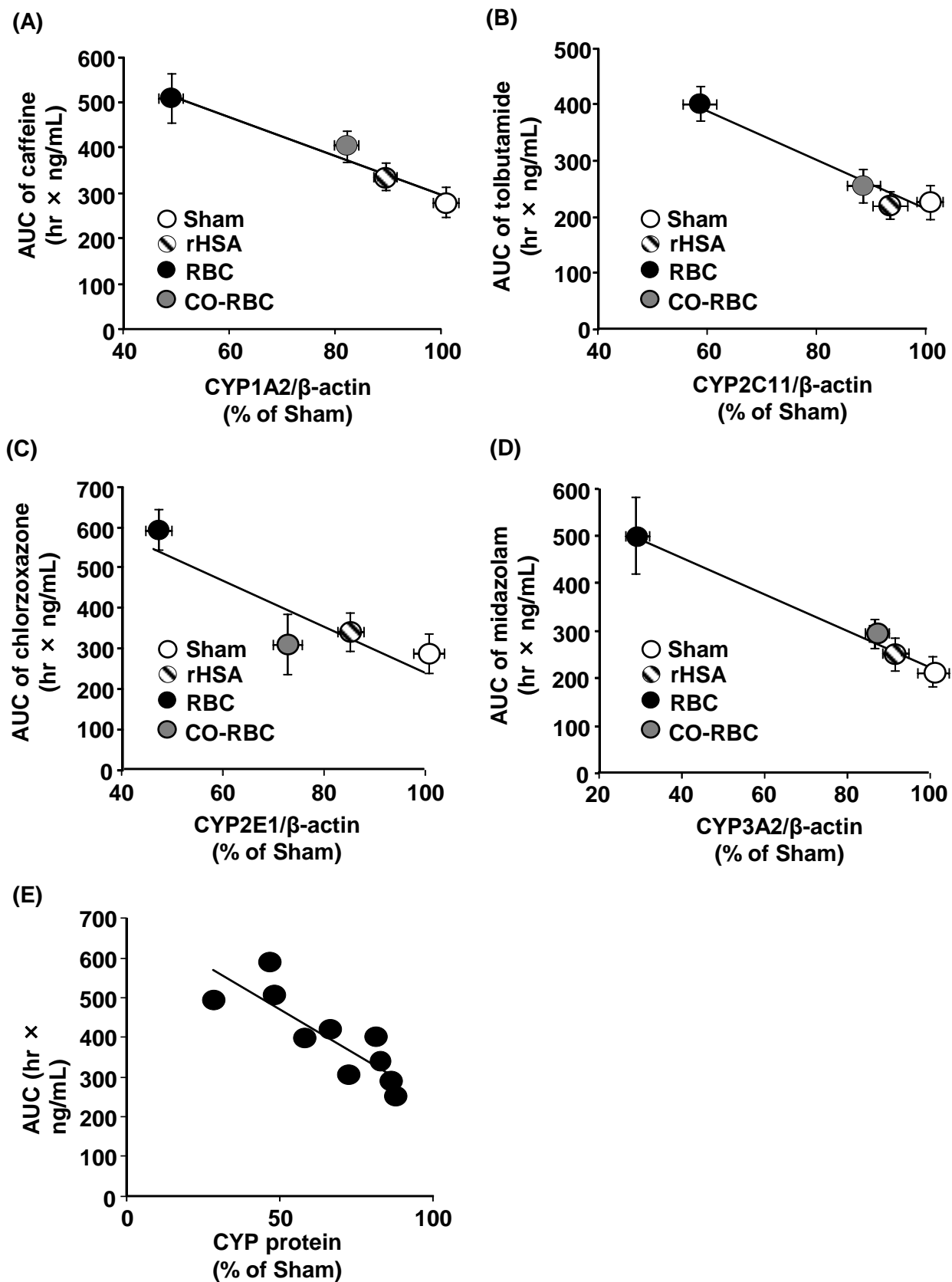
**Fig. 2**



**Fig. 3**



**Fig. 4**





**Fig. 5**

