

## Basic Study

**Effects of mineralocorticoid receptor antagonists on responses to hemorrhagic shock in rats**

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**Abstract****AIM**

To evaluate the effects of mineralocorticoid receptor (MR) antagonists on mortality and inflammatory responses after hemorrhagic shock (HS) in rats.

**METHODS**

One hundred and two male Sprague-Dawley rats were randomly assigned to one of the following three groups: Control, spironolactone (SPL), and eplerenone (EP) groups. HS was induced by the removal of blood. One half of rats were evaluated to determine mortality, hemodynamics, plasma tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) concentrations, and arterial blood gas at 8 h after

HS recovery. In the remainder of rats, the expression levels of genes encoding cytokines were evaluated in liver tissue samples at 1 h after HS recovery.

### RESULTS

The survival rates 8 h after HS recovery were 71%, 94%, and 82% in the control, SPL, and EP groups, respectively. There were no significant differences in survival rates among the three groups ( $P = 0.219$ ). Furthermore, there were no significant differences in gene expression levels in the liver or plasma TNF- $\alpha$  concentrations among the three groups ( $P = 0.888$ ).

### CONCLUSION

Pretreatment with MR antagonists did not improve mortality or cytokine responses in the liver after HS recovery in rats.

**Key words:** Hemorrhagic shock; Mortality; Inflammatory response; Mineralocorticoid receptor antagonist; Cytokine

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**Core tip:** Mineralocorticoid receptor (MR) antagonists have anti-inflammatory effects in models of ischemic and reperfusion injury, suggesting potential clinical value in patients with hemorrhagic shock. However, our findings indicate that pretreatment with MR antagonists does not improve mortality rates or cytokine responses in the liver after recovery from hemorrhagic shock in rats.

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## INTRODUCTION

Hemorrhagic shock (HS), a frequent and dangerous complication of trauma and massive intraoperative bleeding, is associated with high mortality and morbidity. HS causes ischemic injury in vital organs and tissues, and resuscitation for HS causes reperfusion injury in these tissues. Ischemic and reperfusion injury (IRI) causes the release of numerous pro-inflammatory mediators, such as cytokines and nitric oxide, and results in multiple organ dysfunction (MOD), a leading cause of death in HS patients<sup>[1-5]</sup>. Mineralocorticoid receptor (MR) antagonists, such as spironolactone (SPL) and eplerenone (EP), have anti-inflammatory effects *in vitro*<sup>[6-8]</sup>. In particular, SPL inhibits inflammatory responses, such as the attenuation of cytokine and NF-kappa B responses, *in vitro*<sup>[6-8]</sup>. Moreover, in several animal models, MR antagonists

protect against IRI in various organs, including the kidney, liver, intestine, heart, and brain<sup>[9-13]</sup>. These observations suggest that MR antagonists have beneficial effects during HS and after recovery from HS. In clinical settings, MR antagonists are often administered to hypertensive patients to control blood pressure (BP)<sup>[14,15]</sup>. However, it is not clear whether MR antagonists have beneficial effects when administered before reaching the HS state caused by trauma and massive intraoperative bleeding. We hypothesized that pretreatment with MR antagonists has beneficial effects on MOD after HS. Accordingly, we evaluated the effects of pretreatment with SPL and EP on mortality and inflammatory responses after HS in rats.

## MATERIALS AND METHODS

All procedures involving animals were reviewed and approved by the Committee on Animal Experimentation of Kanazawa University (AP-153421).

### Experimental protocol

**Effect of MR antagonists on mortality and inflammatory responses in HS rats:** Fifty-one male Sprague-Dawley (SD) rats (body weight, 350–400 g) were randomly divided into the following 3 groups ( $n = 17$  per group): Control (no medication), SPL (oral administration of SPL at 10 mg/kg per day for 5 d), and EP (oral administration of EP at 100 mg/kg per day for 5 d). The rats received SPL and EP with food. Rats in the SPL and EP groups received powder medicine with powder feed for 5 d. The doses of EP and SPL were selected based on previous studies in rats<sup>[16,17]</sup>.

After medication for 5 d, all rats were anesthetized with pentobarbital sodium (intraperitoneal injection of 50 mg/kg)<sup>[18]</sup>. Rats underwent tracheostomy, and a cannula was inserted into the trachea. The tracheal cannula was attached to a respirator after a cannula was inserted into the carotid artery. Ventilation was performed by administering oxygen (100%, 1 L/min) at a frequency of 32 breaths/min with an inspiratory/expiratory ratio of 1:1 using a small animal respirator. Then, the femoral artery and vein were cannulated. After the operation, lactate Ringer's solution containing muscle relaxant (rocuronium bromide 0.75 mg/mL) and an anesthetic (pentobarbital sodium 0.98 mg/mL) were continuously infused through the cannula of the femoral vein at 10 mL/kg per hour. The femoral artery catheter was connected to a pressure transducer to monitor the arterial blood pressure and heart rate (HR). Rats were placed on a warming pad and maintained at 36–38 °C, as measured using a rectal thermometer.

After the stabilization of rats for 30 min, their blood was drawn *via* the carotid artery cannula to induce HS. Systolic arterial pressure (SAP) was maintained at less than 40 mmHg for 40 min. Removal blood volume were  $13 \pm 0.4$  mL,  $13 \pm 0.5$  mL, and  $13 \pm 0.4$  mL in the control group, SPL group, and EP group, respectively. The

removed blood was diluted two-fold in lactate Ringer's solution and an equal volume was returned through the femoral vein cannula. The methods for this experiment were described in a previous study<sup>[19]</sup>.

The survival rate, SAP, and HR were observed for up to 8 h after HS recovery. The arterial blood sample (0.25 mL) was obtained before HS and at 0, 1, 2, 3, 4, and 5 h after HS recovery. And then PH, Lactate, BE and Hb were measured immediately by The ABL800 FLEX blood gas analyzer. Furthermore, arterial blood samples (1.5 mL) were obtained before HS and at 2, 4, and 5 h after HS recovery to measure plasma tumor necrosis factor (TNF)- $\alpha$ . The TNF- $\alpha$  concentrations were measured using enzyme-linked immunosorbent assay (ELISA) kits (Boster Biological Technology, Pleasanton, CA, United States).

### Effects of MR antagonists on gene expression in the liver after recovery from hemorrhagic shock:

An additional 51 male SD rats were randomly divided into the following 3 groups ( $n = 17$  per group): Control group (no medication), SPL group (oral administration of SPL at 10 mg/kg per day for 5 d), and EP group (oral administration of EP at 100 mg/kg per day for 5 d).

As described above, all animals were anesthetized and HS was induced. At 1 h after recovery from HS, the organization of the liver was examined in the three groups. Liver tissue samples were obtained at 1 h after recovery from HS; the time was established based on previous studies<sup>[20]</sup>. Each sample was placed in a container, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ .

Total RNA was isolated from liver tissues using the High Pure RNA Tissue Kit (Roche Diagnostics, Mannheim, Germany). The quality and quantity of RNA was determined using a NanoDrop (NanoDrop Technologies, Wilmington, DE, United States). The RNA was reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States). Quantitative real-time detection polymerase chain reaction (RTD-PCR) was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The following primers and TaqMan probes (Applied Biosystems) were used: interleukin (IL)-6 (Rn01410330\_m1), TNF (Rn01525859\_g1), IL-1 $\beta$  (Rn00580432\_m1), intercellular adhesion molecule (ICAM) 1 (Rn00564227\_m1), and 18S rRNA (18S,4319413E). The following standard protocol was followed for all reactions: 30 s at  $95^{\circ}\text{C}$  (initial denaturation), 40 cycles of 5 s at  $95^{\circ}\text{C}$  and 30 s at  $60^{\circ}\text{C}$ . mRNA levels were standardized against 18S rRNA expression levels<sup>[21]</sup>.

### Animal care and use statement

The protocol was designed to minimize pain or discomfort. Before operation all rats were anesthetized with pentobarbital sodium (intraperitoneal injection of 50 mg/kg). Ventilation was performed by administering oxygen (100%, 1 L/min) using a small animal respirator during experiment. Lactate Ringer's solution containing muscle

relaxant (rocuronium bromide 0.75 mg/mL) and an anesthetic (pentobarbital sodium 0.98 mg/mL) were continuously infused through the cannula of the femoral vein at 10 mL/kg per hour during experiment. All rats were euthanized by intravenous injection KCl under general anesthesia for tissue collection.

### Statistical analysis

At a sample size analysis, one-sided Fisher's exact tests with a significance level of 5% and a power of 85% showed that a minimum of 17 rats per group was needed to detect a difference in the survival ratio of at least 40% between control and treatment groups. It was based on the result of the preliminary experiment. All data are expressed as means  $\pm$  standard error (SE). At survival rate analysis, the death was defined as an event. The observation period was defined as eight hours since HS recovery. If an event did not occur at the time of the end during an observation period, it was censored. Survival rates for the three groups were compared using the Kaplan-Meier and Log Rank (Mantel-Cox) tests. Significance was defined as  $P < 0.01$ . Differences between groups in hemodynamic properties, including blood gas analysis (BGA) and plasma TNF- $\alpha$ , were analyzed using two-way repeated measure ANOVA, followed by post-hoc tests (Bonferroni's method). Differences in gene expression levels between groups were analyzed by one-way ANOVA. Significance was defined as  $P < 0.01$ . Data analyses were implemented in SPSS v.23 (SPSS Inc., Chicago, IL, United States).

## RESULTS

### Mortality rate and hemodynamics

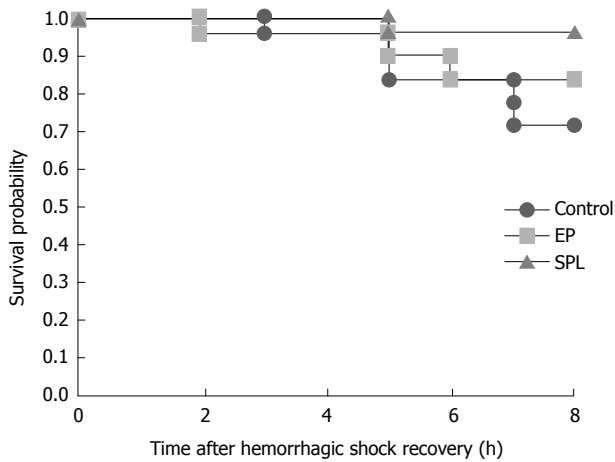
The survival rates 8 h after recovery from HS were 71%, 94%, and 82% in the control group, SPL group, and EP group, respectively. There were no significant differences in survival rate among the three groups (Figure 1).

SAPs gradually decreased after HS recovery in all groups. SAPs of the control group decreased more in comparison with SAPs of the MR antagonists treatment group. SAPs of the EP treatment group did not decrease very much in comparison with the SAP of the control group. There were significant differences among three groups in SAP ( $P < 0.01$ ). There were significant differences between EP group and control group in SAP at 5-8 h after the HS recovery (EP vs control;  $P < 0.01$ , Figure 2).

### Inflammatory responses

The lactate concentrations increased immediately after HS, but recovered in all groups. There were no significant differences among the three groups in lactate concentration, Hb, PH, and BE (Figure 3).

The plasma TNF- $\alpha$  concentration in all groups increased slightly after the recovery from HS. There were no significant differences in plasma TNF- $\alpha$  concentrations among the three groups (Figure 4).



**Figure 1 Kaplan–Meier survival analysis.** The survival rates 8 h after recovery from HS were 71%, 94%, and 82% in the control group, SPL group, and EP group, respectively. There were no significant differences in survival rate among the three groups ( $P = 0.219$ ). HS: Hemorrhagic shock; EP: Eplerenone; SPL: Spironolactone.

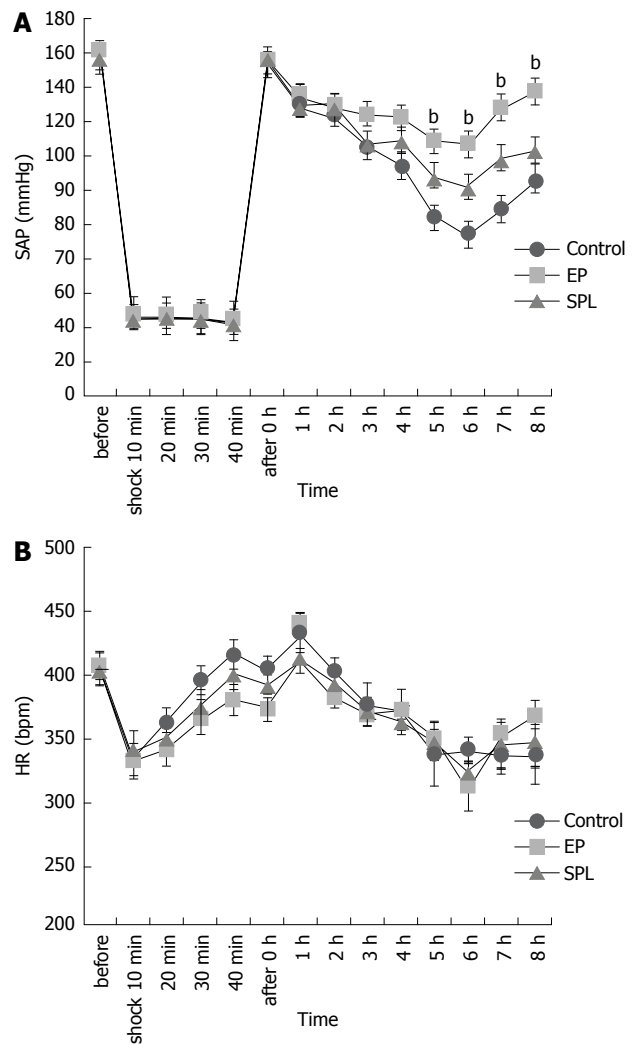
**Gene expression in the liver at 1 h after recovery from hemorrhagic shock**

The mRNA expression levels of *TNF- $\alpha$* , *IL-6*, *IL-1 $\beta$* , and *ICAM-1* did not exhibit significant differences in the liver at 1 h after HS recovery among the three groups (Figure 5).

**DISCUSSION**

HS induced by partial exsanguination in rats caused metabolic acidosis and increased *TNF- $\alpha$*  concentrations immediately after the return of blood. Metabolic acidosis deteriorated immediately after the recovery from HS, but was gradually re-aggravated. As a result, the survival rate was low. HS with the oral administration of SPL and EP, MR antagonists, also resulted in similar to HS of the control. These were no significant differences between the control and MR antagonist-treated groups in the responses after HS recovery. Cytokine gene expression in the liver tissue 1 h after HS recovery did not differ among groups. Thus, our results showed that the oral administration of MR antagonists does not affect the mortality rate and inflammatory responses after HS recovery in rats based on comparisons with control rats after HS recovery.

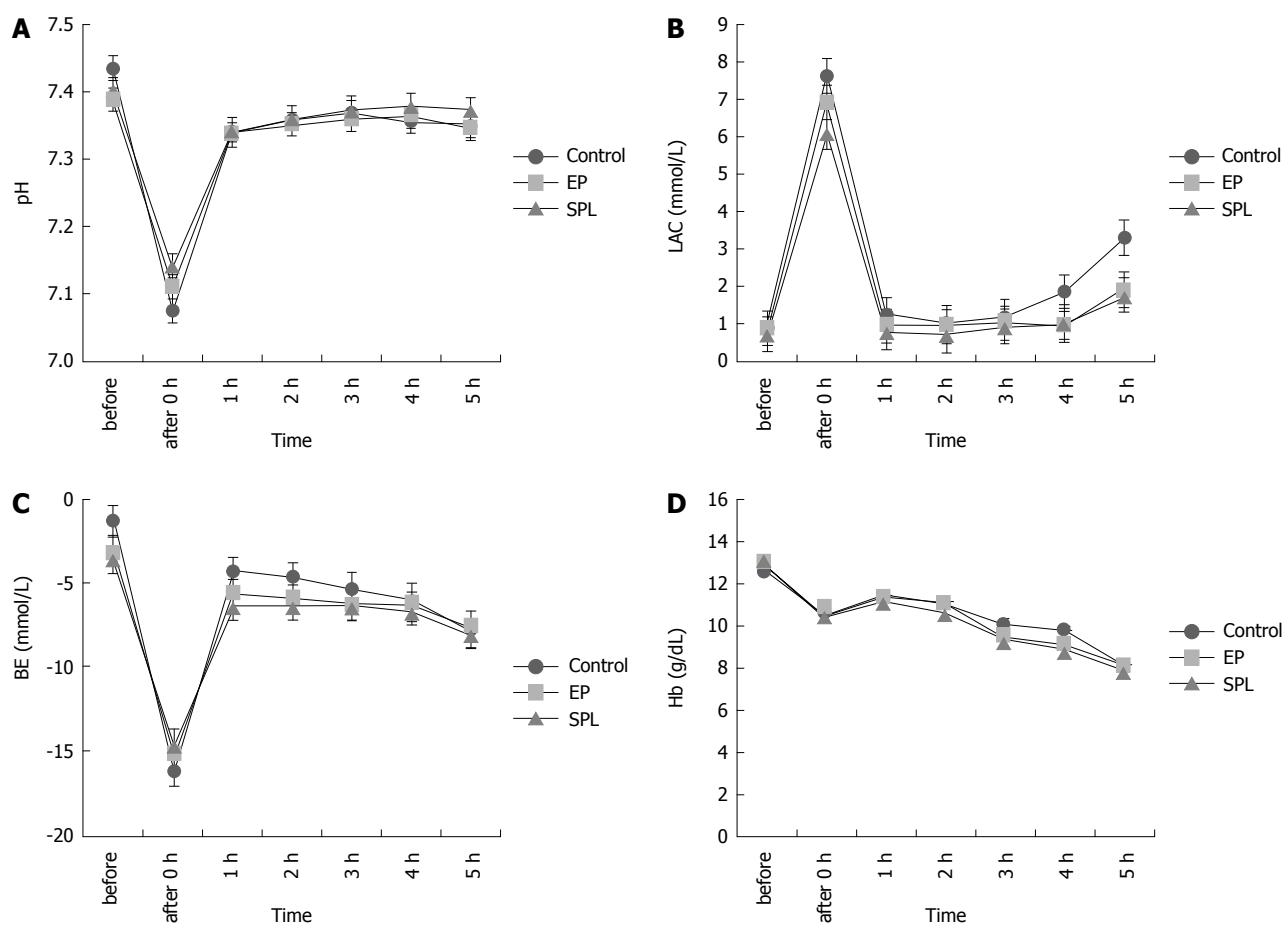
MR antagonists are often used to treat hypertensive patients and have beneficial effects in patients with chronic heart failure and ischemic heart diseases<sup>[14,15,22,23]</sup>. Many recent investigations have demonstrated the beneficial effects of MR antagonists with respect to inflammatory responses and IRI *in vitro* and *in vivo*<sup>[6-13]</sup>. Kato *et al*<sup>[6]</sup> showed that SPL inhibits the production of pro-inflammatory mediators, such as *TNF- $\alpha$*  and nitric oxide, in response to lipopolysaccharides *in vitro*. Ozacmak *et al*<sup>[11]</sup> showed that pretreatment with SPL reduces intestinal injury induced by IR, including the inhibition of inflammatory responses, in rats. These studies suggest that the early and continuous administration of MR antagonists has beneficial effects



**Figure 2 Changes in systolic arterial pressure and heart rate over an 8-h period after hemorrhagic shock recovery.** Changes in (A) SAP and (B) HR in three groups. SAPs gradually decreased after HS recovery in all groups. There were significant difference among three groups in a change of the SAP ( $P < 0.01$ ). There were significant difference between EP group and control group in SAP at 5-8 h after the HS recovery. EP vs control;  $P < 0.01$ (5-8 h). Data represent means  $\pm$  SE. <sup>b</sup> $P < 0.01$  compared with controls. SAP: Systolic arterial pressure; HR: Heart rate; HS: Hemorrhagic shock; EP: Eplerenone.

in critical patients with MOD after HS. However, the effects of pretreatment with MR antagonists on MOD after HS are unclear. Therefore, we evaluated the effects of the pretreatment of MR antagonists on mortality and inflammatory responses after HS in rats. Contrary to our expectations, pretreatment with MR antagonists did not have beneficial effects after HS in rats.

Many investigations have demonstrated that activated Kupffer cells release inflammatory cytokines, such as *TNF- $\alpha$*  or *IL-1*, soon after liver IRI. *TNF- $\alpha$*  increases the expression of *ICAM-1* and promotes the adhesion of neutrophils<sup>[24-26]</sup>. Liver failure is recognized after liver IRI, and many other organs (*e.g.*, the myocardium, pancreas, small intestine, kidney, adrenal gland, and lungs) seem to be damaged by inflammatory reactions and oxidation<sup>[27]</sup>. Therefore, it is important to evaluate cytokine production in the liver at an early stage after



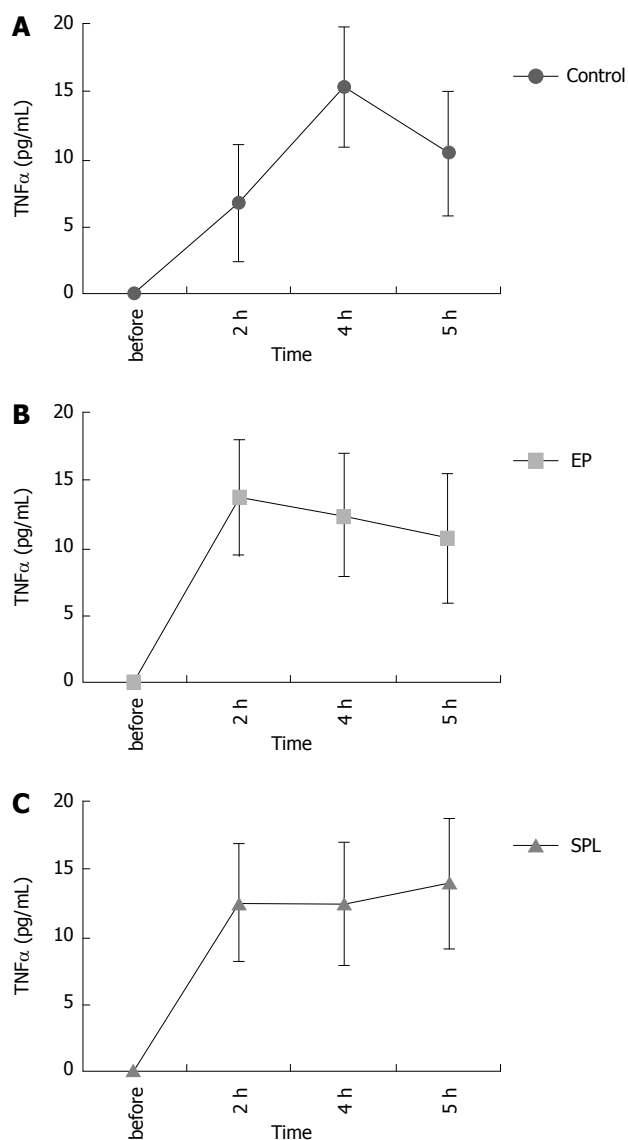
**Figure 3 Blood gas analysis results for a 5-h period after hemorrhagic shock recovery.** Changes in (A) PH, (B) LAC, (C) (BE), and (D) Hb in three groups. The lactate concentrations increased immediately after HS, but recovered in all groups. There were no significant differences among the three groups in PH, LAC, BE, and BE. Data represent means  $\pm$  SE. LAC: Lactic acid; BE: Base excess; Hb: Hemoglobin; HS: Hemorrhagic shock.

HS recovery. Several reports have suggested that MR antagonists modulate the production of cytokines, such as TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , in various organs after IRI<sup>[9-13]</sup>. Pérez *et al.*<sup>[10]</sup> showed that SPL reduces liver damage caused by IRI induced by increases in catalase activity in the liver. Therefore, we hypothesized that pretreatment with MR antagonists has beneficial effects on the expression of genes encoding cytokines, such as TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and ICAM-1, in the liver after recovery from HS. However, we did not detect beneficial effects of MR antagonist pretreatment on cytokine gene expression after HS in rats.

There are several potential explanations for the differences between our results and those of previous studies. First, it is possible that these differences are explained by the relatively short period of ischemia in this study. Suzuki *et al.*<sup>[28]</sup> reported that the activation of Kupffer cell depends on the duration of liver ischemia. The short duration of ischemia may explain lower plasma TNF- $\alpha$  concentrations in this study than in past reports. Further studies are needed to examine long-term ischemic models. Second, the differences among studies may be explained by differences in the dose and duration of the administration of MR antagonists. Ikeda *et al.*<sup>[9]</sup> used a high SPL dose and long period

of administration (100 mg/kg per day and 12 wk) in rats. Ozacmak *et al.*<sup>[11]</sup> used a high SPL dose and short period of administration (20 mg/kg/day and 3 d) in rats. Further investigations are needed to clarify the dose- and time-dependent effects of MR antagonists. Finally, the present study used normal SD rats. In clinical settings, MR antagonists are generally administered to hypertensive patients. Further investigations of hypertensive rats are needed to establish the general relevance of the results.

In this study, SAPs of the EP treatment group did not decrease in comparison with the SAP of the control group, however it did not affect the survival rate. In a previous study, Kajihara *et al.*<sup>[29]</sup> showed that blood aldosterone and cortisol levels were rapidly increased after hemorrhage in dog. After recovery of hemorrhagic shock, arterial blood pressure and blood cortisol decreased, however the blood aldosterone level remained relatively high. Rushing *et al.*<sup>[30]</sup> showed that serum corticosterone was stimulated in hemorrhagic shock model of rats. The blood corticosterone and aldosterone in rat can be related to the blood pressure change after the hemorrhagic shock. However, we did not evaluate the change of the blood pressure and the relations of blood aldosterone and corticosterone, in this study. The difference between

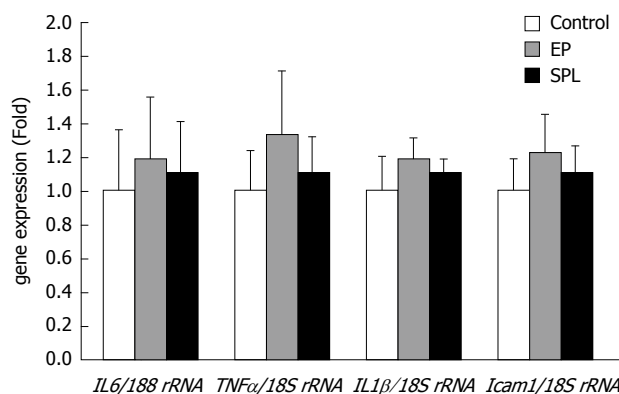


**Figure 4 Concentration of plasma tumor necrosis factor-alpha after hemorrhagic shock recovery.** Changes of plasma TNF- $\alpha$  concentration in (A) control, (B) EP and (C) SPL group. The plasma TNF- $\alpha$  concentration in all groups increased slightly after the recovery from HS. There were no significant differences in plasma TNF- $\alpha$  concentrations among the three groups. Data represent means  $\pm$  SE. SPL: Spironolactone; HS: Hemorrhagic shock; EP: Eplerenone; TNF- $\alpha$ : Tumor necrosis factor-alpha.

EP and SPL is selectivity to MR. So further studies are needed to estimate blood corticosterone and aldosterone in hemorrhagic shock by using rats which MR antagonists were administered.

Clinically, it is important to determine whether MR antagonists have beneficial effects when they are administered before reaching the HS state after trauma and massive intraoperative bleeding. We previously showed that the oral administration of a beta antagonist, carvedilol, increases the mortality rate and worsens inflammatory responses to severe HS in rats<sup>[19]</sup>. Our previous findings suggest that beta antagonists worsen the recovery from severe HS. In the present study, MR antagonists did not worsen HS recovery.

The present study was limited by the number of rats



**Figure 5 Liver gene expression levels 1 h after hemorrhagic shock recovery.** The relative mRNA expression levels of IL-6/18S rRNA, TNF- $\alpha$ /18S rRNA, IL-1 $\beta$ /18S rRNA, and ICAM-1/18S rRNA. These did not exhibit significant differences in the liver at 1 h after hemorrhagic shock recovery among the three groups. Data represent means  $\pm$  SE.

and the method for inducing HS. It may be necessary to evaluate a larger sample of rats to increase power in the analysis of survival rate, but this is difficult in animal experiments. Blood was removed by the carotid artery rapidly and a low blood pressure was maintained. This HS model may not be representative of clinical situations. Accordingly, additional studies should examine the MR antagonists using different methods for HS induction.

In conclusion, the present study showed that pretreatment with MR antagonists, such as SPL and EP, does not decrease the mortality rate and does not attenuate inflammatory responses to HS in rats. These findings suggest that MR antagonists did not worsen HS recovery.

## ARTICLE HIGHLIGHTS

### Research background

Clinically, mineralocorticoid receptor (MR) antagonists such as spironolactone (SPL) and eplerenone (EP) are often administered to hypertensive patients to control blood pressure. However, it is not clear whether MR antagonists have beneficial effects when patients administered MR antagonists become hemorrhagic shock (HS) state caused by trauma and intraoperative bleeding.

### Research motivation

It is very important for perioperative management to clarify the influence of MR antagonist administration before HS state caused by trauma and intraoperative bleeding.

### Research objectives

The effects of pretreatment of MR antagonists on mortality and inflammatory responses after HS were evaluated in rats.

### Research methods

HS was induced by the removal of blood by using rats which MR antagonists were administered or were not administered. The effects of pretreatment of MR antagonists were evaluated by mortality, hemodynamics, plasma TNF- $\alpha$  concentrations, arterial blood gas and liver TNF- $\alpha$ , IL-6, IL-1 $\beta$  and ICAM-1 mRNA expression after HS recovery.

### Research results

There were no significant differences among the three groups in survival rate, plasma TNF- $\alpha$  concentrations, arterial blood gas and liver TNF- $\alpha$ , IL-6, IL-1 $\beta$  and ICAM-1 mRNA expression. Systolic arterial pressure (SAP) after HS

recovery did not decrease in rats of EP group in comparison with control groups. After HS recovery, the reason why blood pressure was maintained in rats of EP group is the problems that remain to be solved, in this research.

### Research conclusions

Pretreatment with MR antagonists did not improve mortality or cytokine responses in the liver after HS recovery in rats. The HS model in the present study was made during general anesthesia after pretreatment of MR antagonists. This model is similar to the clinical situation when patients administered MR antagonists become HS state during operation. The present study suggested that MR antagonists may not be worsen the recovery of HS state and may not need to be withdrawn before the operations.

### Research perspectives

The present study used normal SD rats. In clinical settings, MR antagonists are generally administered to hypertensive patients. Further investigations by using hypertensive rats which MR antagonists were administered will be needed. The present study, SAPs of the EP treatment group did not decrease in comparison with the SAP of the control group, so further studies are needed to evaluate relations of blood corticosterone or aldosterone and blood pressure in hemorrhagic shock by using rats which MR antagonists were administered.

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