Constructing a continuous hemodiafiltration-type circulatory model of acute kidney injury in pigs

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Abstract

Introduction: Animal-model experimental systems capable of reflecting the effects of devices for continuous renal replacement therapy (CRRT) on living organisms are limited; thus, aimed to construct an animal model of AKI-CRRT using pigs.

Methods: Pigs were subjected to renal artery ischemia–reperfusion injury (IRI) and then to a maximum of 24 h of continuous hemodiafiltration (CHDF)-type CRRT.

Results: Post-IRI, pigs' creatinine levels rose threefold, and they exhibited 24 h of anuria and clear aggravation of oxidative stress, demonstrating successful induction of AKI for CRRT. Post-CRRT, no significant changes in their vital signs or hematological parameters were observed. Creatinine and blood urea nitrogen clearance, as well as suppression of increases in oxidative stress, were also confirmed.

Conclusion: We believe that the use of our model can enable the preclinical evaluation of the effects of under-development CRRT devices on living organisms under conditions similar to those encountered in an actual clinical setting.

KEYWORDS
acute kidney injury, animal model, continuous renal replacement therapy, oxidative stress, renal artery ischemia–reperfusion injury

1 | INTRODUCTION

Acute kidney injury (AKI) exhibits high mortality rates in acute-stage patients [1, 2], and the need for advancements in AKI therapy is a very important issue in clinical nephrology. In 2012, the KDIGO classification system was proposed as an index for diagnosing AKI [3]. Continuous renal replacement therapy (CRRT) is indicated in patients diagnosed with stage III AKI using the KDIGO system. The therapy seeks to supplement and relieve the kidneys and promote water clearance [3]. The performance of CRRT devices is primarily evaluated using in vitro experimental systems; very few reports of animal-model experimental systems capable of reflecting...
the effects of these devices on living organisms exist. Consequently, we believe that if one were able to evaluate such devices in animal models, that closely resemble the pathology of actual clinical patients, device manufacturers would be able to launch products whose real-world clinical performance deviates very little from findings obtained during R&D testing.

Pigs are very similar to humans in terms of their size, anatomy, and physiology; they are therefore of great importance to medical and biological research, and in recent years have come to be widely used in a variety of fields, including basic AKI research [4, 5]. While a few reports of the development of AKI models or 24-h extracorporeal circulation models in pigs have been made [6–8], to the extent of our knowledge no model that incorporates both AKI and 24-h extracorporeal circulation exists. Thus, we report a study in which we created a porcine AKI model, subjected it to a maximum of 24 h of continuous diafiltration (CHDF)-type CRRT circulation, and evaluated its performance as an animal AKI-CRRT model.

2 | MATERIALS AND METHODS

2.1 | Animal experiment protocol

This animal experiment was carried out after receiving approval from the Asahi Kasei Medical Animal Experiment Committee and the Japan SLC Animal Experiment Committee (Contract_SLC_19-002, Contract_SLC_20-002, SLC; 19044, SLC; 20037). A total of twelve 3-month-old female Large White pigs were used. Nine animals were used for the AKI model study, and three animals were used for CRRT after the AKI model was created. For the habituation period, animals were reared at 30–80% humidity and 18–28°C for 2 weeks. They were provided with 2000 g/day of Nexel Breed (Nosan Corp., Japan) and free water. After habituation, we performed a renal artery ischemia–reperfusion injury operation. CRRT-group animals were subjected to a maximum of 24 h of CRRT 1 day after surgery (Day 1). Animals used to investigate our AKI model were autopsied 7 days after surgery (Day 7), whereas CRRT animals were autopsied 5 days after surgery (Day 5). The organs obtained by dissection were fixed with 10% neutral buffered formalin solution.

2.2 | Creation of AKI model

AKI model was created using renal artery ischemia–reperfusion injury (IRI). Pigs were immobilized by intramuscular administration of a mixture of midazolam and medetomidine, and anesthetized with isoflurane. After anesthesia, the medial and posterior peritoneum were incised, and the tissue around the left kidney was removed to expose the left renal artery. We subjected the left renal artery to ischemia using a vascular clip, and visually confirmed that the color of the kidney faded. The ischemic exposure time was 60, 120, 180 min (IRI 60, IRI 120, IRI 180). After ischemia, the clip was removed, and reperfusion was performed. For the right kidney, the right renal artery was exposed, then ligated, and the ligated site was cut. After cutting, the incision was sutured and disinfected.

2.3 | CRRT circulation

On Day 1, a double lumen catheter (COVIDIEN Japan Co., Ltd., Japan) was inserted into the jugular vein under isoflurane anesthesia and connected to the circuit. ACH-Σ was used as the blood purification device, and CHDF-FSA (Asahi Kasei Medical Co., Ltd., Japan) was used as the circuit. The CRRT hemofilter used was CUREFLO-A (Asahi Kasei Medical Co., Ltd., Japan) made of polysulfone membrane. Circuits and columns were primed with saline prior to treatment. The treatment was CHDF-type, and circulation was performed for up to 24 h under the conditions of $Q_b = 100 \text{ mL/min}$, $Q_d = 500 \text{ mL/h}$, $Q_f = 1500 \text{ mL/h}$, and $Q_s = 1440 \text{ mL/h}$. In accordance with the activated clotting time (ACT), heparin sodium was continuously administered at 50–100 U/kg/h as an anticoagulant. Anesthesia during circulation consisted of inhaled isoflurane and intravenous medetomidine at 1.5 μL/kg/h, and propofol at 4–10 mg/kg/h. Subblood-BSG (Fuso Pharmaceutical Industries Ltd., Japan) was used as the dialysate and replacement fluid. Furthermore, as an infusion solution, Fructlact injection (Otsuka Pharmaceutical Factory, Japan) was intravenously administered at a rate of 60 mL/h.

2.4 | Biochemical blood tests

Blood was collected over time with a heparin anticoagulant. The supernatant was collected by centrifugation at 2000g, 4°C for 10 min to obtain plasma. A Drychem (NX700V, Fujifilm Co., Ltd., Japan) unit was used as the measuring device, and creatinine (CRE), urea nitrogen (UN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), sodium (Na), potassium (K), chlorine (Cl), and phosphorus (P) were measured.

2.5 | Hematological tests

Blood was collected over time with an EDTA-2K anticoagulant, and the red blood cell count, white blood cell
count, and platelet count were measured with an automatic blood cell counter (ProCyte Dx research, IDEXX Laboratories, Inc., USA).

### 2.6 Oxidative stress markers

Blood was collected over time with an EDTA-2K anticoagulant, and the supernatant was collected by centrifugation at 2000g, 10 min, and 4°C to obtain plasma. Advanced oxidation protein products (AOPP) were measured with Kit (Nikken Zile Co., Ltd.) as an index of oxidative stress.

### 2.7 Vital signs

A vital signs monitor (BSM-3952, Nihon Kohden Co., Ltd., Japan) was used to measure pulse rate, noninvasive blood pressure, saturation of percutaneous oxygen (SpO₂), and body temperature.

### 2.8 Urine volume and biochemical testing of urine

Urine was collected in a metabolic cage for 23 h, and urine volume was measured. Neutrophil Gelatinase-Associated Lipocalin (NGAL) and L-type Fatty Acid-Binding Protein (L-FABP) were measured as AKI markers. For biochemical examination, urine was collected over time with a urinary catheter, centrifuged at 2000g, 10 min, and 4°C, and then the supernatant was used as a sample. NGAL and L-FABP were measured using an ELISA Kit (BIOPORTO Diagnostics, Inc., USA and LifeSpan Biosciences, Inc., USA).

### 2.9 Histopathology

After dissection, the kidney, liver, heart, and lung were sampled and then fixed with 10% neutral buffered formalin to prepare slide sections. H&E staining was performed by a standard method.

### 2.10 Statistical analysis

Results are expressed as mean ± standard deviation. Statistical analysis of these data was performed using a Student's t test or paired t test, using BellCurve for Excel.

### 3 RESULTS

#### 3.1 Creation of AKI model

Figure 1 shows blood chemistry, urine volume, and histopathological examination of the kidney up to Day 7. Blood CRE and UN concentrations continued to increase at both IRI 120 and IRI 180, showing more than 3 times the values from Day 1 to presurgery (pre), there was little variance (Figure 1A). In addition, urine volume remained below 0.3 mL/kg/h over Day 1–4 postsurgery, after which an increase in urine volume was confirmed (Figure 1B).
**Figure 2**  Histopathological testing of kidney tissue. (A, B) H&E-stained images of IRI 60 kidneys. (B) was taken at a higher magnification. Asterisk indicates infiltration of inflammatory cells into the interstitium. (C, D) H&E-stained images of IRI 120 kidneys. (D) was taken at a higher magnification. Arrowheads indicate dilation of the renal tubules. Dilation of Bowman’s capsule and proliferation of interstitial fibroblasts can also be seen. (E, F) H&E-stained images of IRI 180 kidneys. (F) was taken at a higher magnification. Arrows indicate urinary casts. Asterisk indicates infiltration of inflammatory cells into the interstitium. Dilation of renal tubules and shedding/regeneration of epithelial cells can also be seen.

**Table 1**  Biochemical values up until Day 7

|        | Pre       | Day 1     | Day 2     | Day 3     | Day 5     | Day 7     |
|--------|-----------|-----------|-----------|-----------|-----------|-----------|
| **AST (IU/L)** |           |           |           |           |           |           |
| IRI 60 | 24.0 ± 9.5| 64.7 ± 9.7*| 41.0 ± 6.9*| 30.3 ± 4.5| 21.7 ± 0.6| 24.0 ± 4.4|
| IRI 120| 20.0 ± 3.5| 208.3 ± 49.7*| 68.0 ± 9.8*| 31.0 ± 5.6*| 21.7 ± 6.4| 19.0 ± 2.6|
| IRI 180| 19.3 ± 2.1| 186.0 ± 48.1*| 60.3 ± 16.4*| 32.3 ± 2.9*| 17.7 ± 2.9| 14.3 ± 4.0|
| **ALT (IU/L)** |           |           |           |           |           |           |
| IRI 60 | 35.0 ± 13.5| 36.0 ± 10.1| 32.3 ± 9.3| 33.0 ± 11.5| 27.0 ± 6.6| 26.7 ± 5.1|
| IRI 120| 39.0 ± 2.6| 38.7 ± 1.2| 34.0 ± 3.0| 32.3 ± 2.1*| 29.3 ± 2.3*| 29.0 ± 3.6*|
| IRI 180| 40.3 ± 4.2| 43.7 ± 5.5| 34.3 ± 3.2| 30.7 ± 3.1*| 25.7 ± 1.2*| 22.0 ± 2.6*|
| **Na (mEq/L)** |           |           |           |           |           |           |
| IRI 60 | 142.3 ± 1.1| 138.4 ± 1.3*| 137.9 ± 1.5*| 141.3 ± 2.6| 141.2 ± 1.5| 142.4 ± 1.5|
| IRI 120| 142.8 ± 2.3| 135.2 ± 1.9| 132.7 ± 1.1*| 130.2 ± 1.3*| 128.2 ± 1.5*| 127.3 ± 2.4*|
| IRI 180| 142.9 ± 0.2| 137.2 ± 2.1*| 134.8 ± 1.8*| 132.9 ± 0.1*| 126.5 ± 6.5*| 125.4 ± 9.0*|
| **K (mEq/L)** |           |           |           |           |           |           |
| IRI 60 | 4.1 ± 0.2| 3.9 ± 0.4| 3.9 ± 0.3| 3.7 ± 0.3| 3.7 ± 0.0*| 4.0 ± 0.1|
| IRI 120| 3.9 ± 0.2| 4.9 ± 0.2*| 4.7 ± 0.4*| 5.0 ± 0.8| 5.0 ± 1.2| 3.5 ± 0.3|
| IRI 180| 4.1 ± 0.1| 4.9 ± 0.3*| 4.6 ± 0.4| 4.9 ± 0.5*| 5.1 ± 1.0| 4.1 ± 1.1|
| **Cl (mEq/L)** |           |           |           |           |           |           |
| IRI 60 | 102.8 ± 1.9| 97.7 ± 0.9| 98.0 ± 0.5| 101.4 ± 2.7| 102.4 ± 0.9| 102.8 ± 1.2|
| IRI 120| 101.9 ± 0.4| 94.8 ± 2.3| 90.6 ± 0.4| 88.2 ± 1.0| 85.3 ± 1.2| 83.6 ± 2.0|
| IRI 180| 101.9 ± 1.3| 96.2 ± 1.9| 92.2 ± 3.0| 90.6 ± 1.3| 85.5 ± 2.9| 82.8 ± 6.0|
| **P (mg/dL)** |           |           |           |           |           |           |
| IRI 60 | 6.4 ± 0.4| 7.8 ± 0.3*| 7.3 ± 1.2| 6.4 ± 0.8| 5.4 ± 0.9| 5.9 ± 0.7|
| IRI 120| 6.6 ± 0.3| 9.7 ± 1.1*| 9.8 ± 0.6*| 9.2 ± 0.6*| 10.2 ± 1.2*| 13.3 ± 0.4*|
| IRI 180| 7.0 ± 0.3| 9.0 ± 0.4*| 9.1 ± 0.6*| 8.8 ± 0.6*| 9.1 ± 0.4*| 11.3 ± 0.8*|

**Note:** Values expressed as mean ± SD (N = 3).

**Abbreviations:** ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cl, chlorine; K, potassium; Na, sodium; P, phosphorus.

*p < 0.05: significantly different from the pre.
This result corresponds to stage III of the KDIGO classification [3]. H&E staining of the kidney revealed dilation of the renal tubules, fibrosis of the interstitium, inflammatory cell infiltration, and urinary cast in IRI 120 and IRI 180 (Figure 2). IRI 180 showed stronger inflammatory cell infiltration. Regarding other biochemical values, AST increased on Day 1 in IRI 120 and IRI 180, before decreasing on subsequent days. Furthermore, K and P increased over time, while Cl decreased (Table 1).

We also measured urine AKI markers and the degree of oxidative stress (Figure 3). Urinary NGAL behaved differently between IRI 60 and IRI 120/IRI 180. In IRI 60, it peaked on Day 1 but had decreased significantly by Day 7. On the other hand, in IRI 120 and IRI 180, it peaked on Day 2–3 and remained high thereafter. Urinary L-FABP peaked on Day 1 at IRI 60, but was barely detected on Day 3. In IRI 120 and IRI 180, it peaked at Day 2 and remained detectable until Day 7 (Figure 3A). AOPP increased little over time in IRI 60 compared to pre, while in IRI 120 and IRI 180, it increased over time until Day 5 (Figure 3B).

3.2 Application of CRRT to AKI model

CRRT was performed on the more inflammatory IRI 180. In this study, three animals underwent circulation for up to 24 h. One animal completed circulation in 16 h due to the formation of a white thrombus at the tip of the catheter (data not shown). Figure 4 shows blood CRE and UN during CRRT circulation. It was confirmed that the increases in blood CRE and UN were suppressed by CRRT circulation. It was also confirmed that the blood concentration on the “Out” side was significantly lower than that on the column “In” side. For other biochemical
test values, we confirmed that AST and P decreased over time and K increased transiently (Table 2).

During circulation, no reduction in platelet count was observed. Red blood cell count did not show any large changes until 16 h after circulation, but it tended to decrease after 20 h. The white blood cell count did not show any large changes during circulation. On the other hand, lymphocyte count and neutrophil count fluctuated: lymphocyte count increased after circulation, and neutrophil count decreased.

Table 3 shows vital signs during circulation. Changes were observed in systolic and diastolic blood pressure at 8 and 12 h of circulation, but no changes were observed in heart rate, body temperature, or SpO₂.
When AOPP, an index of oxidative stress, was measured, subsequent increases in AOPP were suppressed in the group that underwent CRRT (Figure 5).

4 | DISCUSSION

In this study, an AKI model that meets the indication criteria for CRRT was created using IRI. In the IRI 120 and IRI 180 conditions, blood creatinine level more than tripled, and urine volume was <0.3 mL/kg/h, between 1 and 4 days after surgery (Figure 1A,B). These results constitute severe AKI corresponding to stage III of the KDIGO classification. We, therefore, judged our model as meeting the indication criteria for CRRT [3]. Interestingly, blood NGAL levels decreased at the time of dissection in IRI 60 but remained high in IRI 120 and IRI 180 (Figure 3A). Recent reports indicate that sustained high levels of AKI markers, such as NGAL, should serve as indicators of AKI severity, suggesting that individuals with high levels may develop chronic kidney disease (CKD) [9]. It has also been shown that damage to the proximal tubule is a key factor in chronic renal damage [10]. In the model we created in this study, AKI markers, such as NGAL, should serve as indicators of AKI severity, suggesting that individuals with high levels may develop chronic kidney disease (CKD) [9]. It has also been shown that damage to the proximal tubule is a key factor in chronic renal damage [10].

In this study, AKI markers were maintained at a high level until 7 days after surgery, and histopathological examination revealed significant damage to the renal tubules (Figure 2). These results suggest that AKI severe enough to transition to CKD was induced. In addition, oxidative stress may contribute to the progress of AKI [11, 12]; when AOPP, an index of oxidative stress, was measured, there was a remarkable increase was shown in IRI 120 and IRI 180, though no change was observed in IRI 60 (Figure 3B). It has also been reported that AOPP binds to the AGE receptor RAGE and promotes renal damage. [13] These results suggest that, from the viewpoint of oxidative stress, we were further able to induce highly severe AKI that progressed to CKD in this model.

CHDF-type CRRT was performed for up to 24 h from 1 day postsurgery for IRI 180, which was pathologically judged to be the most inflamed. Two out of three pigs achieved 24-h circulation. In the remaining pig, circulation was stopped after 16 h due to increased pressure. An increase in inlet pressure and venous pressure was observed, but there was no change in differential pressure and TMP. It was therefore judged that it was not the clogging of the device itself, but the clogging of the blood return catheter side. This was more evident from the fact that a white thrombus formed in the catheter (data not shown). Although the cause of this is uncertain, it is possible that the blood was stimulated by the air mixed in when the circuit was connected to the catheter and formed a thrombus, or that the blood clot was formed by the blood retention after the catheter was placed. Therefore, it is necessary to investigate a method for further suppressing thrombus formation in catheter placement surgery.

Since blood CRE and UN did not increase during CRRT, it was confirmed that the expected renal replacement therapy was being performed (Figure 4). Interestingly, although there was no significant change in total white blood cell count, hematological tests showed an increase in lymphocyte count and a decrease in neutrophil count (Table 2). Furthermore, CRRT was shown to suppress increases in AOPP (Figure 5). It was suggested that by performing CRRT in a state where inflammatory and oxidative stress are caused by severe AKI and neutrophils and oxidative stress are abundant, blood is purified to a state close to normal. The causes for this are thought to be related to the ability of CRRT to remove inflammatory substances and uremic toxins from the blood, but there is room for further investigation in the future. On the other hand, a decrease in red blood cell count after 20 h of circulation was observed (Table 2). This may have been because CRRT increased blood vessel volume via the extracellular refilling effect seen in dialysis [14], because of a disruption in hematopoietic function due to severe renal damage, thus preventing red blood cells from being produced [15]. Alternatively, this could simply be because of insufficient excretion due to impaired renal function. In addition, a mild decrease in blood pressure was observed during circulation (Table 3). In this study, isoflurane was mainly used for anesthesia management, and it is possible that long-term use of isoflurane caused a decrease in blood pressure [16]. As no other significant changes in vital signs or platelet count were observed (Tables 2 and 3), we concluded that we were able to perform CRRT without placing significant burden on our experimental animals.

In this study, pigs were made to develop renal disorders similar to those seen in ICU patients. However,
patients undergoing CRRT in the ICU may have increased secretion of inflammatory substances such as cytokines due to infectious diseases and highly invasive surgery [17]. Therefore, it is necessary to further investigate how to induce an inflammatory condition in the future.

5 | CONCLUSION

We used a porcine model designed to resemble the renal impairment of ICU patients to whom CRRT is given to construct an AKI-CRRT model wherein animals were given 24 h of CHDF-type CRRT circulation. This study demonstrates that the use of our model can enable blood biochemical, hematological, and histopathological pre-clinical evaluation of the effects of under-development CRRT devices on living organisms under conditions similar to those encountered in an actual clinical setting. We expect this model to contribute to the launching of products whose real-world clinical performance deviates very little from results obtained during R&D testing. In the future, we will employ existing and under-development products to confirm that this model correlates well with actual clinical conditions.

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CONFLICT OF INTEREST

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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