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ORIGINAL ARTICLE

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A novel evaluation system for whole-organ-engineered liver graft by ex vivo application to a highly reproducible hepatic failure rat model

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Abstract

In recent years, studies on liver graft construction using the decellularized liver as a template for transplantation therapy have attracted much attention. However, the therapeutic effect of constructed liver grafts in hepatic failure has not been evaluated. Therefore, we aimed to develop a novel evaluation system demonstrating the curative effect of a constructed liver graft in animals with hepatic failure. First, we developed a highly reproducible rat model of hepatic failure by combining 80% partial hepatectomy with warm ischemia. In this model, severity could be controlled by the warm ischemic period. We also constructed a liver graft by recellularization of decellularized liver, and confirmed the ammonia metabolic function in the graft in vitro as one of the most important functions for recovery from hepatic failure. The graft was then applied to our developed hepatic failure rat model using a blood extracorporeal circulation system. In this application, the graft metabolized the ammonia in the blood of animals with hepatic failure and was thus suggested to be effective for the treatment of hepatic failure. In summary, a novel evaluation system for whole-organ-engineered liver graft as a preliminary stage of transplantation was developed. This system was expected to provide much information about the curative effect of a constructed liver graft.

Keywords Decellularized liver · Recellularized liver · Hepatic failure model · Blood extracorporeal circulation · Liver tissue engineering

Introduction

Liver is the largest abdominal organ in our body that constitutes about 1/45 of body weight, and is known to possess various functions indispensable for maintaining homeostasis. Currently, liver transplantation alone is the gold standard treatment for end-stage fulminant hepatic failure (FHF). However, severe donor shortage is a great problem globally. In the United States alone, liver transplantation is required in approximately 14,000 patients per year, but only about 8000 liver transplantations are performed [1]. Therefore, an innovative approach for the construction of transplantable liver grafts is needed to combat donor shortage and to save FHF patients.

In 1993, Robert Samuel Langer and Joseph Philip Vacanti proposed the tissue engineering approach for construction of tissues by combining cells, scaffolds, and growth factors [2]. Accordingly, various studies for liver

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tissue construction are underway [3–5]. For example, in the transplantation of hepatocytes based on cell sheet engineering, the constructed tissue was maintained for a long time (> 200 days). However, even though cell sheets were layered, the thickness of the constructed tissue was limited to approximately 1 mm, possibly because of poor oxygen supply [3].

To construct a thick liver tissue or a liver graft for transplantation therapy, construction of blood vessels is an essential consideration because hepatocytes have a high oxygen consumption rate [6]. Therefore, construction of liver grafts using the decellularized liver (DC-liver) as a 3D scaffold with an intact fine vascular network has attracted much attention for liver tissue engineering in recent years.

Some groups have studied the construction of transplantable liver grafts by seeding cells into the DC-liver [7–10]. In our previous study, we successfully obtained a DC-liver with an intact fine vascular network and endothelialized the DC-liver using human umbilical vein endothelial cells [7]. We also constructed a recellularized liver (RC-liver) by seeding primary rat hepatocytes into the DC-liver, and confirmed albumin production in the RC-liver [8]. There are some reports of attempts to transplant RC-livers. Uygun et al. obtained the DC-liver by perfusing detergent, and constructed the RC-liver by seeding hepatocytes into the DC-liver via the portal vein. After that, left nephrectomy of the recipient rat was performed, and the portal vein and hepatic vein of the graft were connected to the renal artery and the renal vein of the recipient rat, respectively [9]. Bao et al. constructed the RC-liver using the heparinized DC-liver to prevent coagulation. Then, 90% partial hepatectomy of the recipient rat was performed and the portal vein and hepatic vein of the graft were connected to the recipient rat's inferior and superior portal vein incisions, respectively. At 72 h postoperation, the RC-liver sustained functional and viable hepatocytes [10]. As described above, it is expected that RC-livers be used as alternatives to donor livers.

As mentioned above, RC-liver grafts were transplanted to healthy or partially hepatectomized animals [9–11]. However, due to blood leakage from the graft and thrombus in the graft, in vivo evaluations have not been realized completely [12, 13]. Moreover, RC-livers were not evaluated with hepatic failure (HF) animals. To obtain information about the therapeutic efficacy of the graft and facilitate optimization of the graft, an ex vivo evaluation system with HF animals is indispensable. Therefore, we aimed to develop an ex vivo application system with HF animals for RC-livers and we evaluated our RC-liver in this system.

In this study, we first developed an HF rat model without a circulatory disorder by combining partial hepatectomy and warm ischemia. Subsequently, ammonia metabolism of the constructed liver graft was evaluated in vitro and then we applied the graft to the developed HF rat model using the blood extracorporeal circulation system (BECS) as an ex vivo evaluation.

Materials and methods

Creation of HF rat models

In this study, 8-week-old male SD rats (Japan SLC, Shizuoka, Japan), whose weights were approximately 300 g, were used. Each rat was anesthetized with isoflurane (Pfizer Japan, Tokyo, Japan), and the left lobe, the middle lobe, the right lobe, and the caudate lobe of the liver were ligated and excised. Subsequently, the weight of each liver lobe was measured. Other 8-week-old male SD rats were anesthetized and warm ischemia was performed for 30–60 min by clamping the proper hepatic artery and the right portal vein connected to the right lobe using bulldog clamps (Fig. 1a). After warm ischemia, the left lobe, the middle lobe, and the caudate lobe were ligated and excised (Fig. 1b). The abdomen of the rat was then sutured and closed. Blood of the treated rat was collected from the tail vein or the femoral vein at the required time. Follow-up observation was performed for 2 weeks postoperatively, and survival rate was evaluated. Additionally, the 30, 45, and 60 min ischemia models were killed at 6 h after the end of ischemia. Prothrombin time of the collected blood was measured using Thrombocheck PT (Sysmex, Hyogo, Japan). The liver of the HF rat model was harvested at 6 h after the end of ischemia and evaluated by hematoxylin and eosin staining (H&E staining). Necrotic area of the harvested liver was automatically extracted from the H&E staining image using the analysis software BZ-X Analyzer (KEYENCE, Osaka, Japan). In 30-min ischemia models, the ammonia concentration in blood with time was evaluated using Pocket Chem BA (Arkray, Kyoto, Japan). The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in blood with time were evaluated using GPT-JS (Denka Seiken, Tokyo, Japan) and GOT-JS (Denka Seiken).

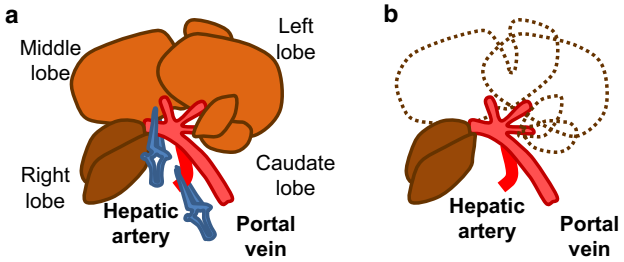

Fig. 1 The image of the hepatic failure procedure in rats. **a** Warm ischemia was performed by clamping the proper hepatic artery and the right portal vein via the right lobe. **b** Other liver lobes except the right lobe were resected after the end of warm ischemia

Construction of a graft in vitro

6-week-old male Wistar rats (Kyudo, Saga, Japan) were anesthetized with isoflurane and laparotomy was performed. Then, the middle lobe, the left lobe, and the caudate lobe were ligated and excised. The blood in the right lobe was removed by perfusion of blood-removing buffer (Supplementary Table S1) from the portal vein, and the right lobe was then excised from the rat. The right lobe was decellularized using a protocol based on our previous study [8]. Briefly, the liver was decellularized by perfusing Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (CMF-PBS) containing 4% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) via the portal vein for 2 h. After decellularization, the DC-liver was washed with CMF-PBS to remove the detergent. Primary rat hepatocytes for recellularization of the DC-liver were obtained from 6- to 8-week-old male Wistar rats using a two-step collagenase perfusion method [14]. As reported previously, 1×10^7 hepatocytes suspended in 1 ml collagen sol (Nitta Gelatin, Osaka, Japan) were injected into the DC-liver from surroundings using a syringe with a 26 G needle (Terumo, Tokyo, Japan) and the DC-liver was then incubated at 37 °C for 30 min to facilitate gelation of the injected collagen sol; thus, we obtained the RC-liver [8]. As control, 1 ml collagen sol was injected into another DC-liver and the DC-liver was also incubated in the same manner. As a positive control, the right lobe of the native liver was used immediately after excision from the rat. On the other hand, the organ culture system comprising a chamber, polyvinyl chloride tubes (Kawasumi Laboratories, Tokyo, Japan), and a peristaltic pump (Gilson, Middleton, WI, USA) was constructed to evaluate the ammonia metabolism function of the graft (Supplementary Figure S1). Thus, 20 ml perfusate consisting of 2 ml rat blood for sufficient oxygen supply and 18 ml MEM medium was prepared. Then, 200 µl of 100 mM ammonium chloride aqueous solution was added to the perfusate and the final concentration of ammonium chloride in the perfusate was 1 mM [15]. The graft was placed in the chamber and circulation was started at a flow rate of 0.4 ml/min. Circulation was performed for 1 h and ammonia concentration in the perfusate was analyzed using the Ammonia Test Wako (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Evaluation of grafts by BECS

The BECS circuit consisted of a chamber, a peristaltic pump (Gilson), polyvinyl chloride tubes (Kawasumi Laboratories), air vents, a water bath, and a heat exchanger (Supplementary Figure S2). The volume of the circuit was about 5 ml. These circuits were sterilized and then primed with saline containing 100 U/ml heparin sodium (Mochida Pharmaceutical, Tokyo, Japan) before blood extracorporeal circulation. Subsequently, the RC-liver was constructed by seeding 2×10^7 primary rat hepatocytes into the DC-liver, in the same manner as described above. Moreover, the DC-liver and native liver were also prepared. These liver grafts were placed in the chamber and connected to the circuit. The 45-min ischemia HF rat model could not endure blood extracorporeal circulation for 1 h without grafts in a previous study because of the physical stress of blood extracorporeal circulation. Therefore, in this study, the 30-min ischemia HF rat model was used in the following experiments. Then, 24 G cannulas (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) were inserted into the left carotid artery and the right jugular vein of the HF rat model. The HF rat model was administered 30 units of heparin via the right jugular vein before the start of circulation. These inserted cannulas were connected to the blood extracorporeal circulation circuit at 1 h after the end of ischemia. Blood extracorporeal circulation was then carried out for 1 h at a flow rate of 0.4 ml/min. We took a blood sample at the start of circulation, the end of circulation, and at 4 h after the end of circulation. Then, ammonia, lactate, lactate dehydrogenase (LDH), ALT, and AST in the blood sample were analyzed. Lactate and LDH were analyzed using determiner LA (Kyowa Medex, Tokyo, Japan) and LD-JS (Denka Seiken). During circulation, 1 ml of 10 U/ml heparinized saline was administered every 30 min. The treated rat was killed at 4 h after the end of blood extracorporeal circulation, and the right lobe of the rat was harvested. The graft and the liver of the applied rat were evaluated by H&E staining.

Ethics and statistics

Institutional guidelines for the care and use of laboratory animals were observed. The study has been approved by the Ethics Committee for Animal Experiments of Kyushu University on animal research, and all protocols have been found acceptable (Approval number: A27-326-0, A29-413-0).

Significant difference tests for the results were performed using Tukey's test or Dunnett's test with Ekuseru-Toukei 2010 (Social Survey Research Information, Tokyo, Japan). P value < 0.05 was considered statistically significant.

Results

Creation of an HF rat model

The weight of each lobe against the whole liver was measured (Supplementary Table S2, $n = 3$). These results confirmed that approximately 20% of the whole liver remained in this HF rat model after resection of all the lobes except the right lobe.

We thus tried to construct an HF rat model. The survival ratio for the 30-min, 45-min, 50-min, and 60-min ischemia models was 100% ($n = 3$), 60% ($n = 5$), 40% ($n = 5$), and 0% ($n = 3$), respectively (Fig. 2a).

We also performed histological analysis and determined the necrotic areas. In Fig. 2b, typical necrotic parts of the each HF rat model's injured liver were shown. In the liver of the 30-min ischemia model, a very small necrotic area was observed (Fig. 2b, i) and the calculated necrotic area was approximately 1.11 ± 0.39% ($n = 2$). In contrast, 8.37 ± 5.20% ($n = 3$) necrotic area was observed in the liver of the 45-min ischemia model. Finally, 20.89 ± 2.43% ($n = 2$) necrotic area was observed in the liver of the 60-min ischemia model and the microstructure in the liver was found to be collapsed (Fig. 2b, iii).

Prothrombin time was also analyzed. Prothrombin times of 30-, 45-, and 60-min ischemia models at 6 h after the end of warm ischemia were approximately 18 ± 2.1 s ($n = 2$), 20 ± 2.5 s ($n = 3$), and 28 ± 2.1 s ($n = 2$), respectively (Fig. 2c). These results indicated that the necrotic area

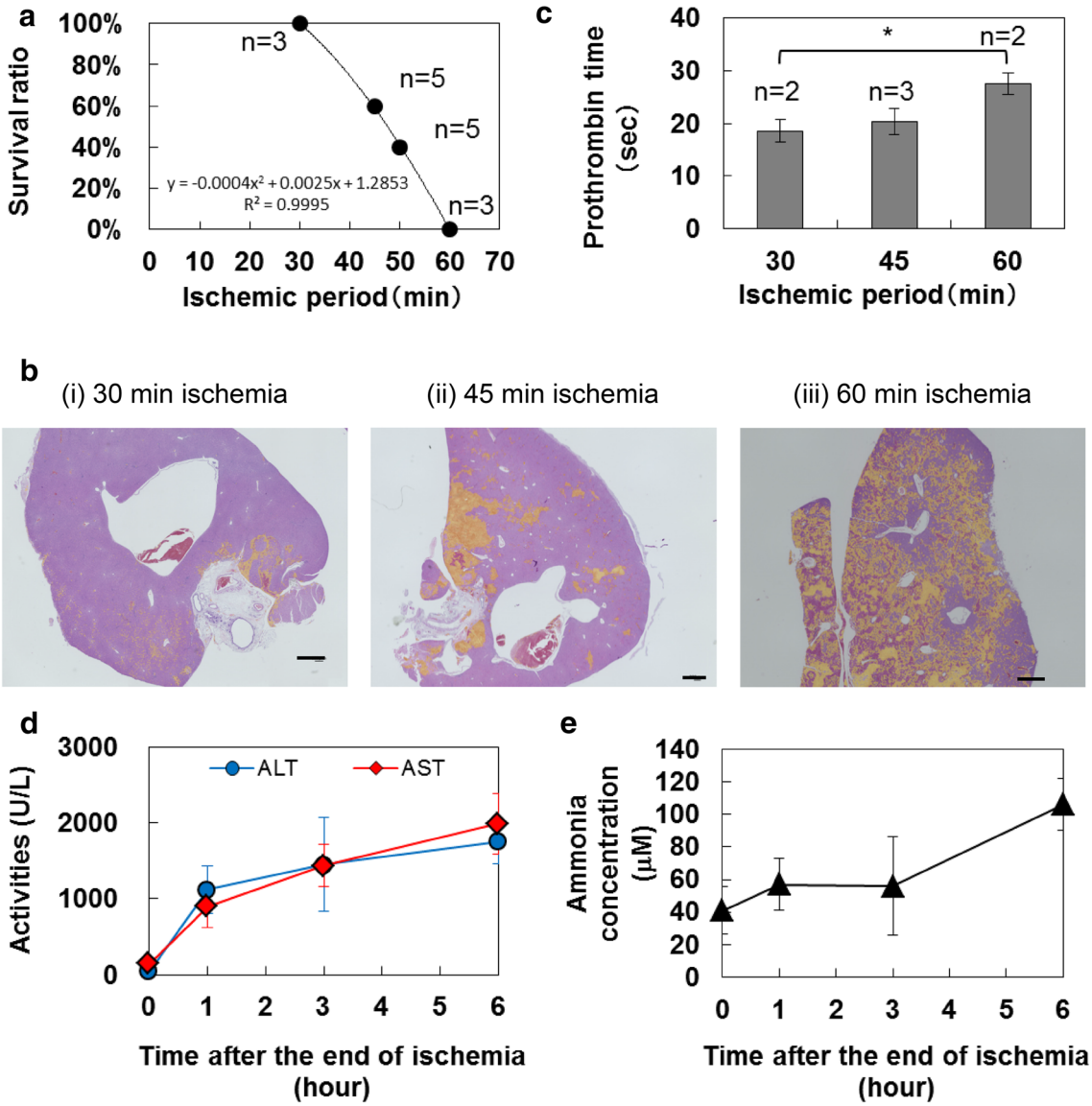

Fig. 2 **a** The relation between the warm ischemic period and survival ratio in the developed HF rat model (n indicates the number of rats used in each condition). **b** H&E staining of the liver in the HF rat model. (i) 30-min ischemia (ii) 45-min ischemia, and (iii) 60-min ischemia. Bars: 1 mm. Necrotic area is shown in yellow. **c** Prothrombin time of 30-, 45-, and 60-min ischemia rat models at 6 h after the end of warm ischemia (n indicates the number of rats used in each condition). Significant differences were analyzed using Tukey's test. *$p < 0.05$. **d** ALT and AST activities in blood of 30-min ischemia rat model ($n = 3$). The value at 0 h indicates ALT and AST activities of the rat before HF operation. Blue circle: ALT, red diamond: AST. **e** Ammonia concentration in the blood of 30-min ischemia rat model ($n = 3$). The value at 0 h indicates the ammonia concentration before the HF operation

increased with the prolongation of ischemic period, and consequently, the survival ratio and coagulation activity were decreased. In summary, the severity of the HF rat model can be controlled by changing the ischemic period.

Next, the blood components of 30-min ischemia models were analyzed with time ($n = 3$). As indexes of hepatic injury, both ALT and AST activities increased with time after the end of warm ischemia and these values were significantly higher than those before the HF treatment (Fig. 2d). The ammonia concentration was also analyzed as an index of metabolic function of the liver in the HF rat model ($n = 3$). The ammonia concentration increased with time and reached 100 μM at 6 h after the end of warm ischemia (Fig. 2e). This value has been reported to cause encephalopathy [16]. Remarkably, individual differences in the blood components of treated rats were very small. The values of ALT, AST, and ammonia at 6 h after the end of warm ischemia were 1759 ± 294 U/l, 1988 ± 395 U/l, and 106 ± 16 mM, respectively. This suggested that the HF rat model could be induced in a reproducible manner in just 6 h after the end of ischemia.

These results indicated that we succeeded in developing the HF rat model with good reproducibility. The severity of the developed HF rat model could be controlled by the applied warm ischemic period.

Ammonia metabolism of grafts in vitro

We focused on the right hepatic lobe as our target for liver construction because the volume of right lobe was approximately 20%, which is sufficient for survival [17, 18]. Therefore, in this research, the right lobe was used for constructing the DC-liver and RC-liver.

Ammonia metabolism of the constructed graft was evaluated in vitro. In conditions where the DC-liver was applied, the ammonia concentration in the perfusate was hardly decreased ($n = 2$). On the other hand, in each condition where the native liver ($n = 2$) and RC-liver ($n = 2$) were applied, the ammonia concentration in the perfusate was decreased. It was thus confirmed that the average ammonia concentration was 0.897 ± 0.095 mM under application of the DC-liver. In contrast, under application of the native liver and the RC-liver, the average ammonia concentrations were 0.284 ± 0.099 mM and 0.445 ± 0.071 mM, respectively (Fig. 3a). Thus, ammonia was metabolized by the native liver and the RC-liver in vitro.

Ex vivo evaluation of grafts by BECS

First, grafts that were applied to the BECS were evaluated by H&E staining after blood extracorporeal circulation. Intact cells with cytoplasm were not observed in the DC-liver, and areas that seemed to be injected with collagen gels were observed (Supplementary Figure S3 i, white arrows). In addition, primary rat hepatocytes were confirmed in the RC-liver, and the hepatocytes (Supplementary Figure S3 ii, black arrows) were surrounded with what seemed to be collagen gel (Supplementary Figure S3 ii, white arrow). These results indicated that recellularization of the DC-liver was achieved successfully. Moreover, erythrocytes were found uniformly in all the grafts. This suggested that blood was flowing throughout the grafts (Supplementary Figure S3 i, ii, iii).

On the other hand, in histological evaluation of the liver in the HF rat model, there was almost no difference in the necrotic area (Supplementary Figure S4 i, ii, iii). The ALT and AST activities increased over time under all conditions (Fig. 3b, c). However, though there was no significant difference, the trend of these activities in the rats with the native liver and the RC-liver application was lower than that in the rat with the DC-liver application. The LDH activity as an index of hepatic injury kept increasing after the application of the DC-liver. In contrast, it decreased after application of the native liver and the RC-liver (Fig. 3d). The tendency of ALT and AST was slightly different with that of LDH. This might be due to the shorter half-life of LDH compared to ALT and AST. From these results, it was expected that the native liver and the RC-liver helped the recovery of the HF rat models.

Lactate concentration in the blood of the HF rat model showed similar profile under all conditions (Fig. 3e). However, it showed lower value with the application of the native liver at 2 h from the end of ischemia (the end of blood extracorporeal circulation). This result indicated that the native liver metabolized the lactate in the blood. Ammonia concentration in the blood of the HF rat model was approximately 100 μM at 1 h from the end of ischemia (the start of the circulation) in all conditions (Fig. 3f). At the end of blood extracorporeal circulation, the ammonia concentration under the application of native liver (145 ± 40 μM, $p = 0.035$) and RC-liver (114 ± 46 μM, $p = 0.019$) was significantly lower than that under the application of DC-liver (326 ± 134 μM). This result suggested that the native liver and the RC-liver metabolized the ammonia in the blood of the HF rat models. Moreover, at 6 h after the end of ischemia, ammonia concentrations in the blood of HF rats treated with the native liver, the RC-liver, and the DC-liver were 52 ± 15 μM, 70 ± 12 μM, and 135 ± 60 μM, respectively. Ammonia concentration in the blood of HF rats treated with the native liver and the RC-liver were lower than that in rats treated with DC-liver (native liver: $p = 0.027$, RC-liver: $p = 0.061$). Based on the obtained results, recovery of the HF rat model was expected by application of the native liver and the RC-liver with BECS. The therapeutic effect of the constructed grafts can thus be evaluated using BECS with our HF rat model.

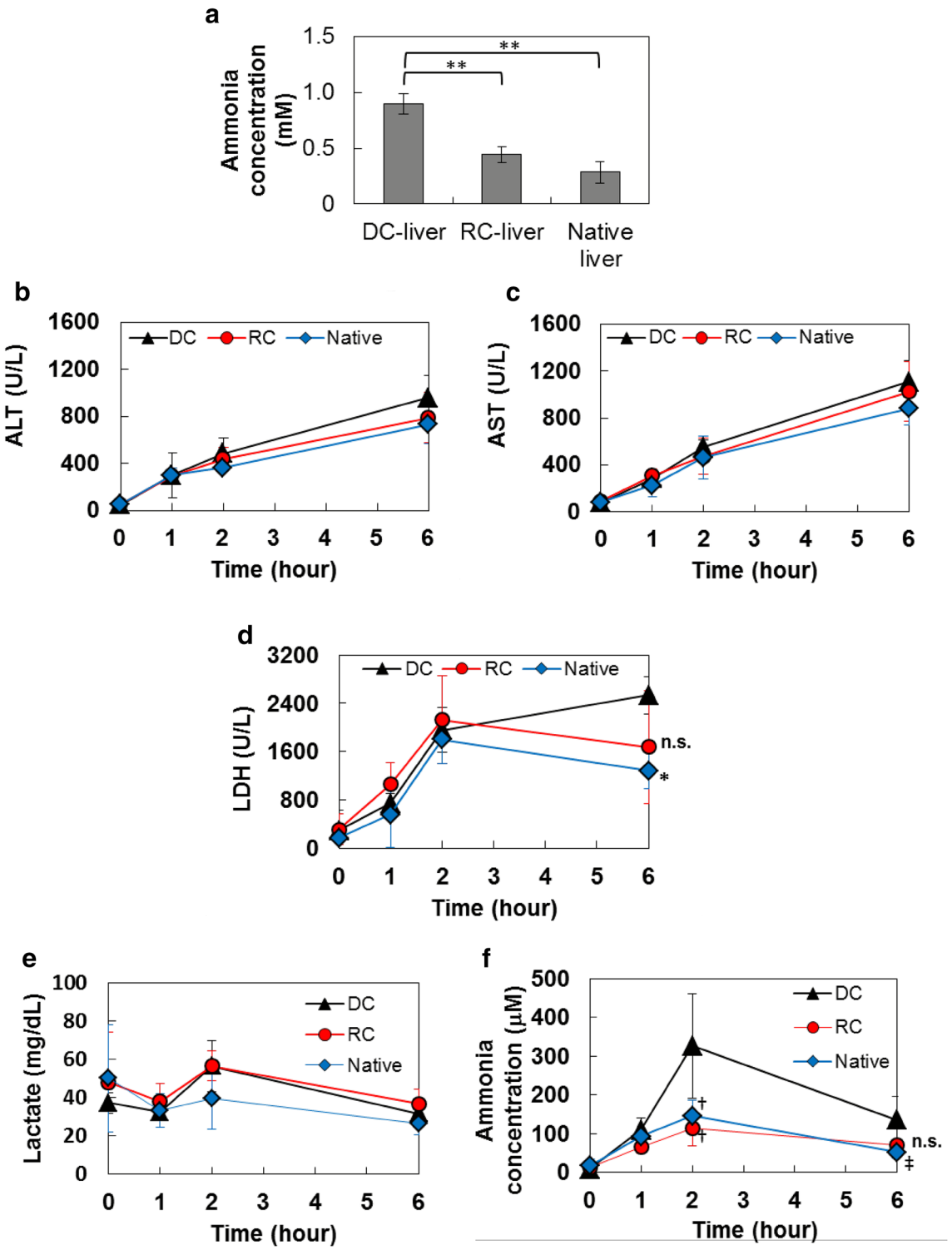

Fig. 3 **a** Ammonia concentration in the perfusate after circulation culture ($n = 2$). Significant differences were analyzed by Dunnett's test with DC-liver as the control group. $^{**}p < 0.01$. **b–d** The value of ALT, AST and LDH activity in the blood of the 30-min ischemia rat models after the end of ischemia. The value at 0 h indicates each activity in the blood of the rat before HF operation ($n = 3$). Black triangle: DC-liver, red circle: RC-liver, blue diamond: native liver. Significant differences were analyzed by Dunnett's test with DC-liver as the control group. RC-liver and native liver were compared with DC-liver. $^{*}p < 0.05$ *ns* not significant. **e**, **f** Lactate and ammonia concentration in the blood of 30-min ischemia rat models after the end of warm ischemia. The value at 0 h indicates each concentration in the blood of rats before HF operation ($n = 3$). Black triangle: DC-liver, red circle: RC-liver, blue diamond: Native liver. Significant differences were analyzed by Dunnett's test with DC-liver as the control group. RC-liver and native liver were compared with DC-liver at 2 h ($^{\dagger}p < 0.05$) and 6 h ($^{\ddagger}p < 0.05$) *ns* not significant

Discussion

In the present study, we developed the highly reproducible HF rat model in which the severity of HF could be controlled. After confirming the ammonia metabolism of the RC-liver in vitro, the RC-liver was applied to the HF rat model by BECS. Results showed that the RC-liver metabolized ammonia in blood of the HF rat model, and the therapeutic efficacy of the RC-liver was expected.

An HF animal model induced by drug administration is poor in reproducibility because the sensitivity of the animal to drugs can easily change with the individual [19]. Therefore, for correct determination of the therapeutic effect, a surgically induced HF animal model seemed to be better than a drug-administered HF animal model. Some HF animal models developed by surgical treatment have been reported previously. Eguchi et al. reported a rat model wherein the middle lobe and the left lobe were excised and the right lobe ligated. This rat model was a very severe model with a residual liver of approximately 6% and it was impossible to control the severity of HF [20]. Yong et al. also reported that the severity of HF could be controlled by changing the site of hepatectomy [18]. However, some patterns of the residual liver are present upon partial hepatectomy, due to which the severity cannot be controlled gradually. On the other hand, Ijima et al. reported a rat model capable of finely controlling severity by performing 74% hepatectomy and warm ischemia to the remaining liver by clamping the portal vein and the hepatic artery [21]. However, this method can potentially cause circulatory disorders during warm ischemia, and these disorders affect the liver as well as other organs.

In this study, we developed an HF rat model combining warm ischemia of the right lobe alone and resection of the liver lobes except the right lobe at the end of warm ischemia. This method ensures blood flow in hepatic lobes except the right lobe from the portal vein to the inferior vena cava during warm ischemia and prevents circulatory disorders in the treated rat. With prolongation of the ischemic period, hepatic injury of the HF rat model became more severe and liver-specific function was decreased, consequently leading to a decline in the survival ratio of the HF rat model. Furthermore, the ALT and AST activities and ammonia concentration in the blood of the 30-min ischemia rat model was increased with minimal individual differences (Fig. 2d, e). Some researchers have stated that reproducibility is one of the most important elements for an HF animal model [19, 22, 23]. From the above, this HF rat model was considered suitable for evaluating the effectiveness of a graft on HF.

On the other hand, we produced the RC-liver by recellularization of the DC-liver with primary rat hepatocytes based on our previous report [10] and evaluated its ammonia metabolism in vitro. Based on the result, the RC-liver metabolized ammonia, and the metabolic rate per cell was calculated to be about 50 pmol/cell/day (Fig. 3a). This metabolic rate was suggested to be high compared to those in previous reports on the ammonia metabolic ability of 2D-cultured primary rat hepatocytes [24]. This result suggested that each of the primary rat hepatocytes in the RC-liver was working well.

During blood extracorporeal circulation, in the application of the native liver and RC-liver, the ammonia concentration in blood at the end of blood extracorporeal circulation was significantly lower than that in the application of the DC-liver (Fig. 3f). In other words, ammonia in the blood of the HF rat model was metabolized by the RC-liver. In the report of the hybrid artificial liver support system, the survival time of a hepatic failure animal was extended by the application of the support system, and the increase of blood ammonia concentration in the hepatic failure animal was completely suppressed [25]. Therefore, it is very valuable that the same tendency in blood ammonia concentration was observed in this study. Moreover, the LDH activity in blood of the HF rat model decreased after the application of the RC-liver (Fig. 3e). From these results, the RC-liver was expected to be effective for HF. The efficacy of RC-livers will have to be evaluated in detail by measuring the other parameters in the future. On the other hand, the number of hepatocytes in the RC-liver in this study was 1% of the whole liver. Therefore, it is necessary to increase the cell density to satisfy the clinically significant liver mass (for example, 20% liver).

Till date, there is no index which guarantees recovery from hepatic failure. Therefore, a constructed liver graft has to be applied to hepatic failure animals to prove its therapeutic effect. However, the constructed liver grafts using DC-liver have usually been evaluated in vitro. In this regard, we developed the evaluation system which was composed of the novel HF rat model and BECS. This system enables ex vivo evaluation of the therapeutic effect of the whole-organ-engineered liver graft.

Conclusion

We succeeded in developing a novel HF rat model with controllable severity and high reproducibility. We also developed an ex vivo application system for grafts by the BECS. Additionally, the effectiveness of RC-liver to the HF rat model was expected. In summary, we succeeded in developing an ex vivo evaluation system capable of evaluating the performance of grafts in HF.

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