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Scanning Electron Microscopy Findings of Machine Perfused Liver Graft After Warm Ischemia Between Hypothermic and Rewarming Machine Perfusion in Pigs

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ABSTRACT

Background. The shortage of organ donors is a universal problem. Use of grafts from donors after cardiac death would greatly contribute to the expansion of the donor organ pool. The two major methods of preservation are cold storage and machine perfusion (MP) preservation, and each has its own advantages. Several studies have reported the relative merits of MP for the preservation for grafts from donors after cardiac death. In this study, we used scanning electron microscopy (SEM) to assess the damage to the liver between hypothermic and rewarming preservation conditions.

Methods. Porcine livers were perfused with a newly developed MP system. The livers were perfused for 4 hours with a modified University of Wisconsin solution–gluconate solution. In group 1, grafts were preserved with warm ischemic time for 60 minutes and hypothermic machine perfusion (HMP) for 4 hours. In group 2, grafts were preserved with warn ischemic time for 60 minutes and had rewarming up to 22°C by MP (RMP) for 4 hours.

Results. A significant enlargement of the mitochondria were observed in both the HMP and RMP groups under higher magnification, Additionally, vacuoles appeared occasionally in hepatocytes in the RMP for 4 hours group, but not in the HMP for 4 hours group.

Conclusions. An analysis by scanning electron microscope appears to be useful to evaluate the levels of damage of hepatocytes compared with transmission electron microscopy, and further study is needed to analyze the significance of the appearance of swelling of mitochondria and vacuolization during preservation.

THE SHORTAGE of organ donors is a universal problem. The use of grafts from donors after cardiac death would greatly contribute to the expansion of the donor organ pool. The development of machine perfusion preservation has improved the preservation condition of grafts [1]. Recently, the clinical application of a liver perfusion system has been developed, and we previously reported our original machine perfusion preservation system to control the perfusate temperatures from hypothermic to subnormothermic conditions [2]. We also previously showed that subnormothermic perfusion is superior to hypothermic perfusion in the preservation of liver with ischemic damage by analyzing the level of deviation enzymes [3]. However, the levels of damage of hepatocytes under each condition remain unclear. Nonetheless, many pathological analyses were performed because observation by optical microscopy

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only shows the condition of the liver at the tissue level. In this study, we assessed the ability of scanning electron microscopy (SEM) compared with transmission electron microscopy (TEM) to evaluate the damage of hepatocytes under each experimental condition.

MATERIALS AND METHODS

Animals

Domestic female cross-bred Large-Yorkshire, Landrace, and Duroc pigs (approximately 28 kg, 2 months to 3 months of age) were purchased from The Nakase livestock in Asahikawa.

Perfusion Preservation Machine

Pigs were anesthetized, and potassium chloride was administered to induce cardiac arrest (Fig 1). The grafts were procured after 1 hour of warm ischemia and perfused with the machine for 4 hours; the parameter was obtained at pre-perfusion, with machine perfusion at 0 hours, 2 hours, and 4 hours. We divided the pigs into two groups: Group 1 (n = 2) underwent hypothermic machine perfusion (HMP) for 4 hours. Group 2 (n = 4) received rewarming up to 22°C by machine perfusion (RMP) for 4 hour.

SEM

Tissue samples for the analysis by SEM were prepared according to the osmium maceration methods described previously [4]. Specifically, biopsy samples from the livers of experimental animals were cut into small pieces (1 mm × 1 mm × 10 mm) and immediately immersed in a fixative mixture of 0.5% paraformaldehyde and 0.5% glutaraldehyde in 0.1 mol/L phosphate buffer (PB, pH adjusted to 7.4) for 30 minutes at 4°C. After fixation, the tissue blocks were directly immersed in 1% osmium tetroxide (OsO_4) in 0.1 mol/L PB for 6 hours at 4°C. Then the samples were washed thoroughly with 0.1 mol/L PB three times, immersed in 25% and 50% dimethyl sulfoxide for 30 minutes each for cryoprotection, and frozen on a metal plate deeply chilled with liquid nitrogen. The frozen liver blocks were cracked into two pieces with a screwdriver and a hammer and transferred into 50% dimethyl sulfoxide for thawing. After freeze cracking, the samples were washed three times in 0.1 mol/L PB, and transferred to 0.1% OsO_4 diluted with 0.1 mol/L PB for 100 hours at 20°C under light for osmium maceration. The macerated tissues were immersed in 1% OsO_4 in 0.1 mol/L PB for 1 hour for further fixation. After rinsing with 0.1 mol/L PB, the samples were treated with 1% tannic acid in mol/L PB and then with 1% OsO_4 in 0.1 mol/L PB for conductive staining. Then, the conductive-stained samples were dehydrated in ascending concentrations of ethanol (70%, 80%, 90%, 95%, and 100%), transferred into isoamyl acetate and dried in a critical point dryer (HCP-2, Hitachi Koki Co., Ltd., Tokyo, Japan) using liquid CO_2. The dried samples were mounted onto an aluminum metal plate and coated with platinum-palladium in an ion sputtering device (E1010, Hitachi Koki Co., Ltd.). After the process described above, the specimens were analyzed on a field emission SEM (S-4100, Hitachi High- Technologies Corporation, Tokyo, Japan). This study was performed with the approval of our institutional ethics committee, and all the experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

RESULTS

After hematoxylin and eosin staining and analysis under an optical microscope, the structure of the hepatic lobules were well-preserved in both the HMP and RMP groups.

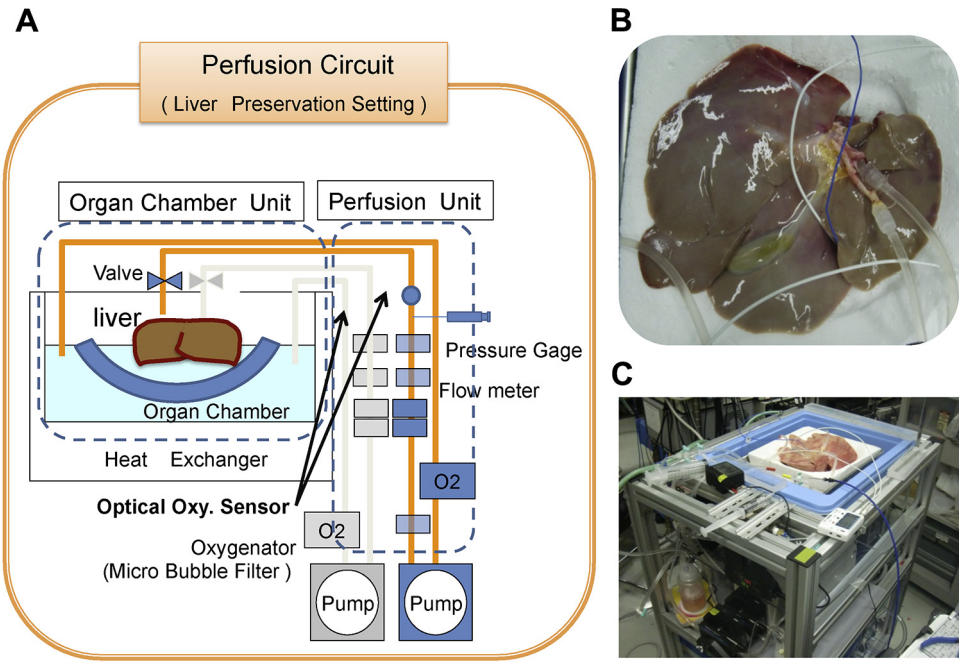

Fig 1. The liver biopsy of preperfusion was obtained after cardiac arrest was induced with injection of potassium chloride. Liver grafts were left for 60 minutes under warm ischemic conditions and perfused with Euro Collins solution; the tissues of liver biopsy specimens were sampled at 0 hours and 4 hours in each group. (**A**) Diagrammatic machine perfusion system. (**B**) Porcine liver processed with the routes. (**C**) Porcine liver managed in the machine perfusion.

Occasional microvascular steatosis as well as dilatation of the sinusoidal and the space of Disse were observed in both groups (Fig 2). Under SEM and TEM, in addition to hepatocytes and nucleus, the mitochondria and Golgi apparatus were well-observed. The mitochondria and Golgi apparatus were more clearly observed on SEM, compared to TEM (Fig 3A and B). Significant enlargements of the mitochondria were observed in both the HMP and RMP groups under higher magnification. Additionally, vacuoles appeared occasionally in the hepatocytes in the RMP for 4 hours group, but not in the HMP for 4 hours group (Fig 3C and D).

DISCUSSION

The pathological changes after preservation are associated with the immediate post-transplantation graft function. Broken hepatocyte cords, hepatocyte edema and vacuolization, sinusoidal congestion, and necrosis are well-defined pathological characteristics of preservation injury. SEM observation could support the findings of light microscopy. Furthermore, the analysis of osmium macerated tissue samples by SEM can clearly show the internal structures of intracellular organelles compared to TEM. This may be due to the differences in electron movement; during TEM, a beam of electrons is passed through a thin specimen, whereas during SEM a narrow beam of electrons is scanned back and forth across the surface of a specimen. In SEM, backscattered or secondary electrons are viewed; whereas in TEM, the images are dependent on the electron beam passing through the sample onto the phosphor screen or film camera. SEM is typically used for surface analyses and TEM for analyzing sections. This study could not clearly show differences in the hepatic damage between the HMP and RMP groups using optical microscopy, TEM, or SEM, although our previous study comparing laboratory data results related to hepatic damage, including effluent enzymes of aspartate transaminase, lactate dehydrogenase, and hyaluronic acid, showed significantly better results in the RMP group than the HMP group. This study showed the significance of the enlargement of the mitochondria and vacuolization in hepatocytes, which correlated with the laboratory results, due to swelling of the hepatocyte with damage. Preservation after warm ischemia will induce liver mitochondrial and cellular damage, which subsequently induce damage to the mitochondrial respiratory chain, leading to mitochondrial swelling. The consequences are the loss of adenosine triphosphate, the initiation of apoptosis through

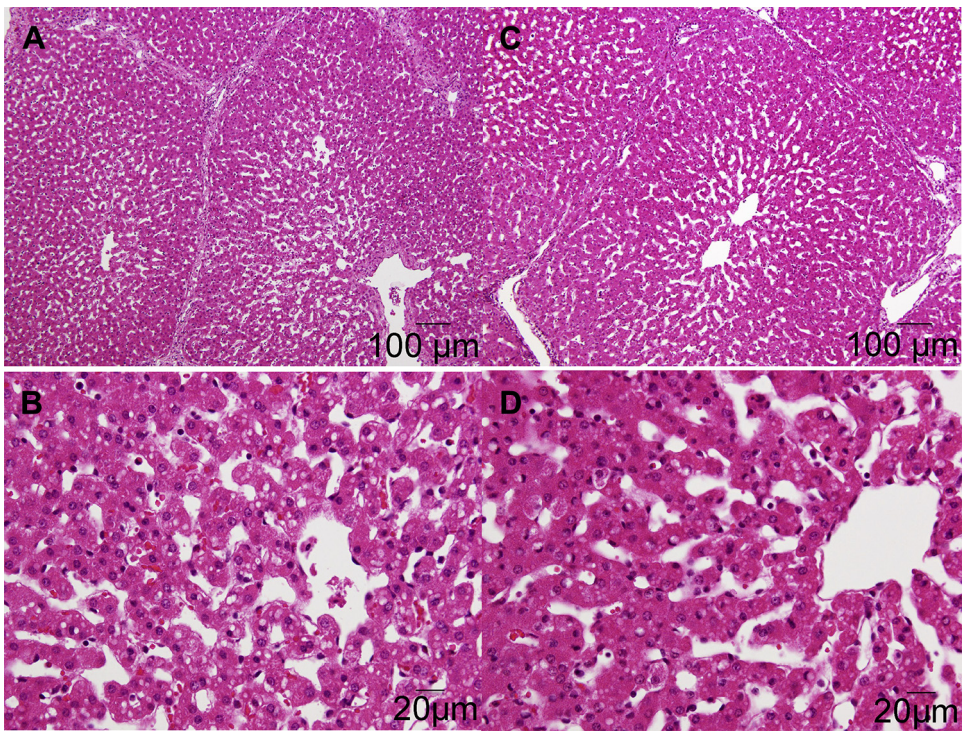

Fig 2. Comparisons between hypothermic machine perfusion (HMP) and rewarming machine perfusion (RMP) tissue characteristics of hepatic parenchyma at 4 hours after perfusion by HMP and 4 hours after perfusion by RMP. (**A**) HMP 4 hours after perfusion (hematoxylin and eosin stain [HE], lower magnification). (**B**) HMP 4 hours after perfusion (HE, higher magnification). (**C**) RMP 4 hours after perfusion (HE, lower magnification). (**D**) RMP 4 hours after perfusion (HE, higher magnification). With HE stain under the optical microscope, the structure of hepatic lobules were well-preserved and occasional microvascular steatosis as well as dilatation of the sinusoidal and the Disse's space were observed in both groups.

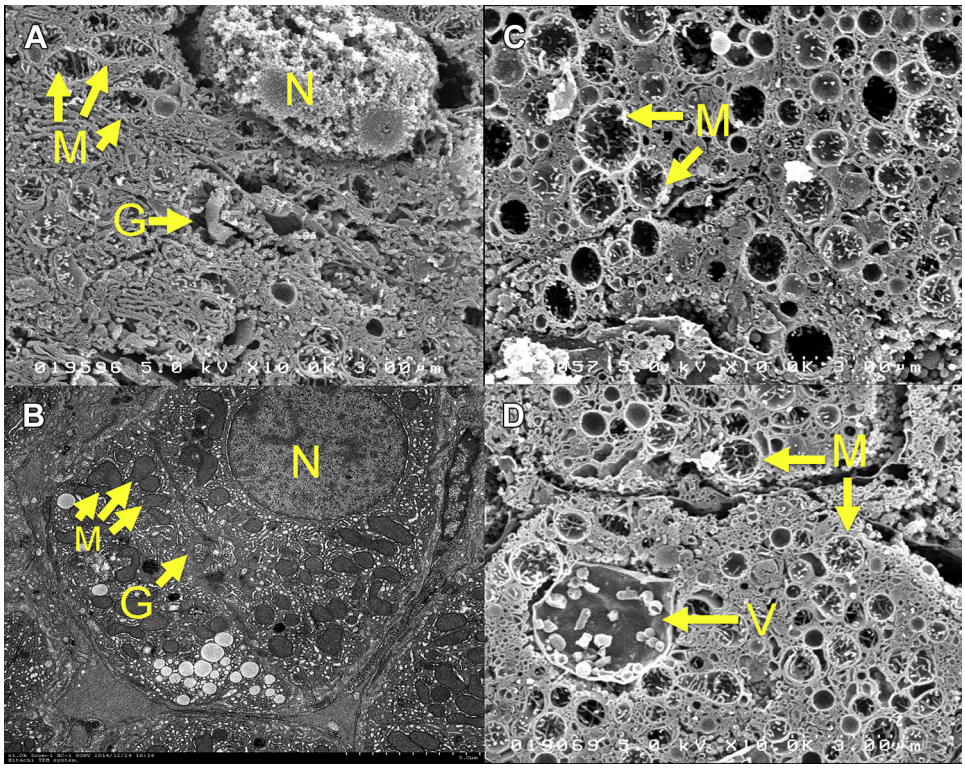

Fig 3. Ultrastructural characteristics in liver tissue of control and experimental groups (hypothermic machine perfusion and rewarming machine perfusion). (**A**) Scanning electron microscopic (SEM) image of control. (**B**) Transmission electron microscopic (TEM) image of control. (**C**) SEM image of hypothermic machine perfusion at 4 hours. (**D**) SEM image of rewarming machine perfusion at 4 hours. In SEM and TEM images, the mitochondria and Golgi apparatus were well-observed as well as hepatocytes and nucleus. The mitochondria and Golgi apparatus were more clearly observed in SEM (**A**) than TEM (**B**). Significant enlargement of the mitochondria was observed in both groups, and the vacuoles appeared occasionally at the hepatocyte in the rewarming machine perfusion at 4 hours group, but not in the hypothermic machine perfusion at 4 hours group. Abbreviations: N, nucleus; M, mitochondria; G, Golgi apparatus; V, vacuole.

cytochrome C release, and the activation of caspases [5,6]. Importantly, organelle injury under electron microscopy may be used as a predictor of the post-transplantation liver function.

CONCLUSIONS

In this study, our findings indicated that the analysis by SEM was useful to evaluate the levels of damage of the hepatocytes, and the SEM findings could support the findings of light microscopy; however, the influence of hepatocyte damage remained unclear. Further studies analyzing the relationships between the appearance and changes of organelles during preservation, particularly swelling of the mitochondria and vacuolization, by SEM are necessary.

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