学位論文

Hypoxic-Ischemic Encephalopathy-Associated Liver Fatty Degeneration and the Effects of Therapeutic Hypothermia in Newborn Piglets

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Hypoxic-Ischemic Encephalopathy-Associated Liver Fatty Degeneration and the Effects of Therapeutic Hypothermia in Newborn Piglets

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Keywords
Hypoxic-ischemic encephalopathy · Hypoxia · Liver injury · Therapeutic hypothermia · Lipid droplets

Abstract
Background: Although liver can be injured under the hypoxic-ischemic encephalopathy (HIE) condition, there is currently no histopathological evidence. Therapeutic hypothermia is used to protect the brain; however, the therapeutic potential for concomitant liver injury is unknown. ***Objectives:*** This study aimed to histopathologically prove HIE-associated liver injury and to investigate the influence of therapeutic hypothermia in a newborn piglet HIE model. ***Methods:*** Eighteen newborn piglets were divided into 3 groups: control (n = 4), HIE (n = 8), and therapeutic hypothermia (n = 6) groups. The hypoxic insult was induced by decreasing the fraction of inspiratory oxygen from 21 to 2–4% over 40 min while monitoring cerebral blood volume and cerebral hemoglobin oxygen saturation. For therapeutic hypothermia, whole-body cooling at 33–34°C was administered for 24 h after the hypoxic insult. We hematologically and histopathologically investigated the liver injury in all groups. ***Results:*** Alanine transaminase and lactate dehydrogenase levels in the HIE group were significantly elevated compared with those in the control group. Micro-lipid droplet accumulation in the periportal zone, but not in the perivenous zone, was significantly greater in the HIE group than in the control group and significantly smaller in the therapeutic hypothermia group than in the HIE group. ***Conclusions:*** We demonstrated that micro-lipid droplet accumulation in the cytoplasm of hepatocytes in the periportal zone occurs under the HIE condition and that this accumulation is suppressed by therapeutic hypothermia.

Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) is one of the most serious conditions occurring in the perinatal period. HIE primarily results from systemic hypoxia due to respiratory or circulatory failure at birth such as during asphyxia [1].

In the past decade, many preclinical and clinical studies have investigated HIE etiology and evaluated potential

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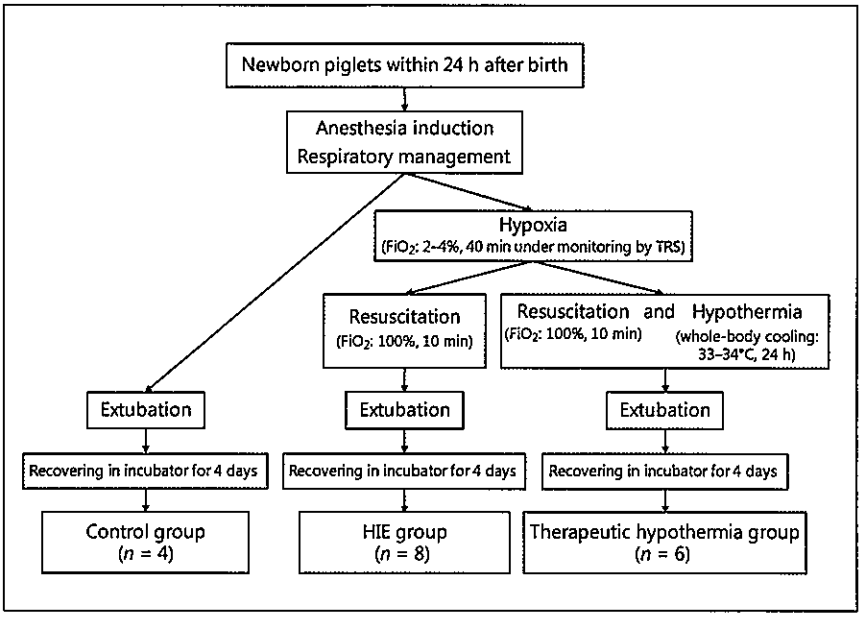

Fig. 1. Experimental protocol. TRS, time-resolved spectroscopy.

therapeutic interventions, mainly focusing on the brain. Recent studies indicate that HIE affects not only the brain itself but also other organs, including the liver, kidneys, heart, and lungs [2]. Multiple organ injury associated with HIE may be caused not only by direct hypoxia but also by vital reactions such as the diving reflex [2], ischemic-reperfusion injury [3], and the interactions of organ injury [4]. HIE-associated liver injury is involved in high mortality [5]. Although the pathogenetic mechanism underlying HIE-associated liver injury is similar to that of ischemic hepatitis [5], there is currently no histopathological evidence of HIE-associated liver injury.

Currently, one treatment for HIE is therapeutic hypothermia, the standard treatment in newborns to minimize brain damage [6]. This treatment is believed to improve HIE prognosis by suppressing cerebral metabolism and inflammation [7]. However, the potential of this treatment for HIE-associated liver injury is unknown.

Ischemic hepatitis is characterized by centrilobular liver cell necrosis or fatty degeneration [8]. Therapeutic hypothermia has a protective effect against acute liver failure [9]; thus, we hypothesized that HIE-associated liver injury may also induce histopathological liver changes and that these changes may be ameliorated by therapeutic hypothermia. We aimed to histopathologically demonstrate the presence of HIE-associated liver injury and to investigate the influence of therapeutic hypothermia using a newborn piglet HIE model.

Methods

Animal Model and Experimental Protocol

The animal preparation method for the HIE model was previously reported in detail [10]. Eighteen newborn piglets were obtained within 24 h of birth. Piglets were initially anesthetized with 1–2% isoflurane in air using a face mask. Each piglet was then intubated and mechanically ventilated with an infant ventilator. The umbilical vein and artery were cannulated with a neonatal umbilical catheter for drip infusion, blood pressure monitoring, and blood sampling. After cannulation, the piglets were anesthetized with fentanyl citrate at an initial dose of 10 μg/kg, followed by an infusion at 5 μg/kg/h. They were then paralyzed with pancuronium bromide at an initial dose of 100 μg/kg, followed by an infusion at 100 μg/kg/h. Maintenance solution (electrolytes plus 2.7% glucose, KN3B; Otsuka Pharmaceutical Co., Tokyo, Japan) was continuously infused at a rate of 4 mL/kg/h via the umbilical vein. Each piglet was placed under a radiant warmer to maintain the rectal temperature at 39 ± 0.5°C.

The piglets were classified into 3 groups. The control group ($n = 4$) was maintained under 21% fraction of inspiratory oxygen (FiO_2) for 24 h and extubated. The HIE group ($n = 8$) was stabilized for 120 min under 21% FiO_2 after the initial induction of anesthesia. Subsequently, they were exposed to a systemic hypoxic insult, induced by decreasing FiO_2 to 2–4% over 40 min, under monitoring by near-infrared time-resolved spectroscopy to measure cerebral blood volume and cerebral hemoglobin oxygen saturation. The hypoxic-ischemic insult was terminated by resuscitation with 100% oxygen for 10 min. Thereafter, the ventilator rate and FiO_2 were gradually reduced until the piglets began breathing spontaneously; they were extubated after resuscitation. During the experiment, a base excess less than −5.0 mM, caused by hypoxia, was corrected by sodium bicarbonate infusion to maintain a pH of 7.3–7.5. Phenobarbital was administered when they convulsed. Finally,

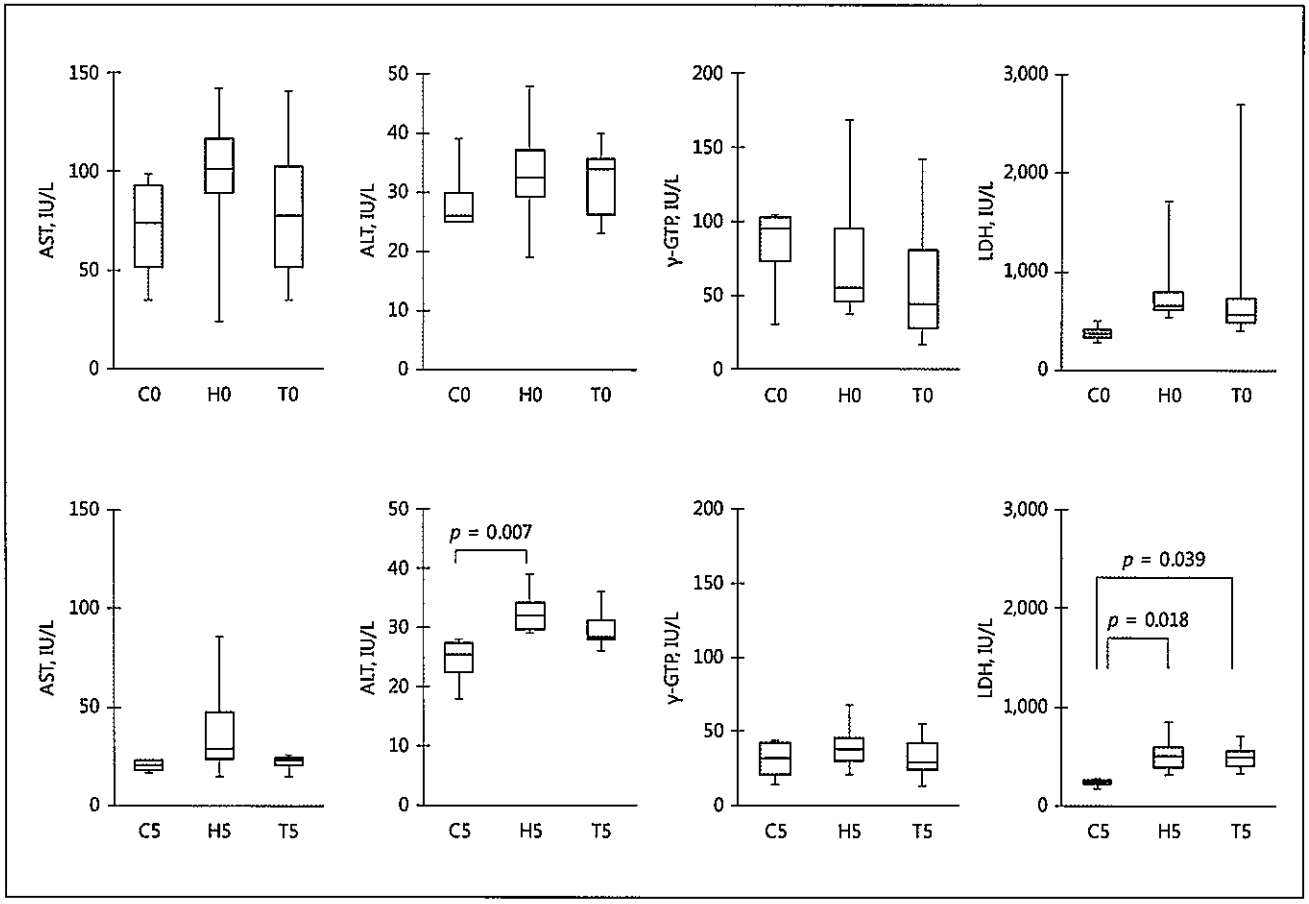

Fig. 2. Blood data on the 5th day after birth and at anesthesia induction. C, control group; H, HIE group; T, therapeutic hypothermia group. Median serum levels of AST, ALT, γ-GTP, and LDH in the HIE group were higher than those in the control group on the 5th day after birth (C5, H5, T5). In particular, ALT ($p = 0.007$) and LDH ($p = 0.018$) were significantly elevated in the HIE group compared with the control group. There were no differences in these levels among the 3 groups at anesthesia induction (C0, H0, T0).

the therapeutic hypothermia group ($n = 6$) was exposed to a systemic hypoxic insult in the same way as the HIE group, followed by therapeutic hypothermia, consisting of whole-body cooling to 33–34°C for 24 h during the resuscitation period. After extubation, all piglets were placed in an incubator and perorally fed with synthetic milk for 4 days (Fig. 1).

Specimen Sampling

Four days after extubation, all piglets were sacrificed under deep anesthesia by isoflurane inhalation. Blood samples were collected. The systemic organs, including the liver, were perfused with phosphate-buffered paraformaldehyde and immersed in buffered formalin. After fixing, liver tissue samples were collected from the outermost part of the right lobe of the liver. Blood samples were also collected at initial anesthesia induction.

Blood Data Analysis

Serum levels of aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyltranspeptidase (γ-GTP), and lactate dehydrogenase (LDH) were measured. The values were compared among the control ($n = 4$), HIE ($n = 8$), and therapeutic hypothermia ($n = 6$) groups using 1-way ANOVA, followed by a post hoc Tukey test. $p < 0.05$ was considered statistically significant.

Histopathological Analysis

Liver specimens were embedded in paraffin, and 4-μm sections were stained using hematoxylin and eosin (H&E) to investigate hepatocyte necrosis and invasion of inflammatory cells. Three slides per animal were examined to investigate histological findings. Other paraffin sections were also processed for periodic acid-Schiff (PAS) staining. To estimate whether apoptosis was induced in hepatocytes, immunohistochemistry using the rabbit monoclonal antibody for apoptosis protease-activating factor 1 (APAF-1; Abcam, Cambridge, UK) and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining were examined according to their protocols. Additionally, frozen sections were embedded in OCT (optimal cutting temperature) com-

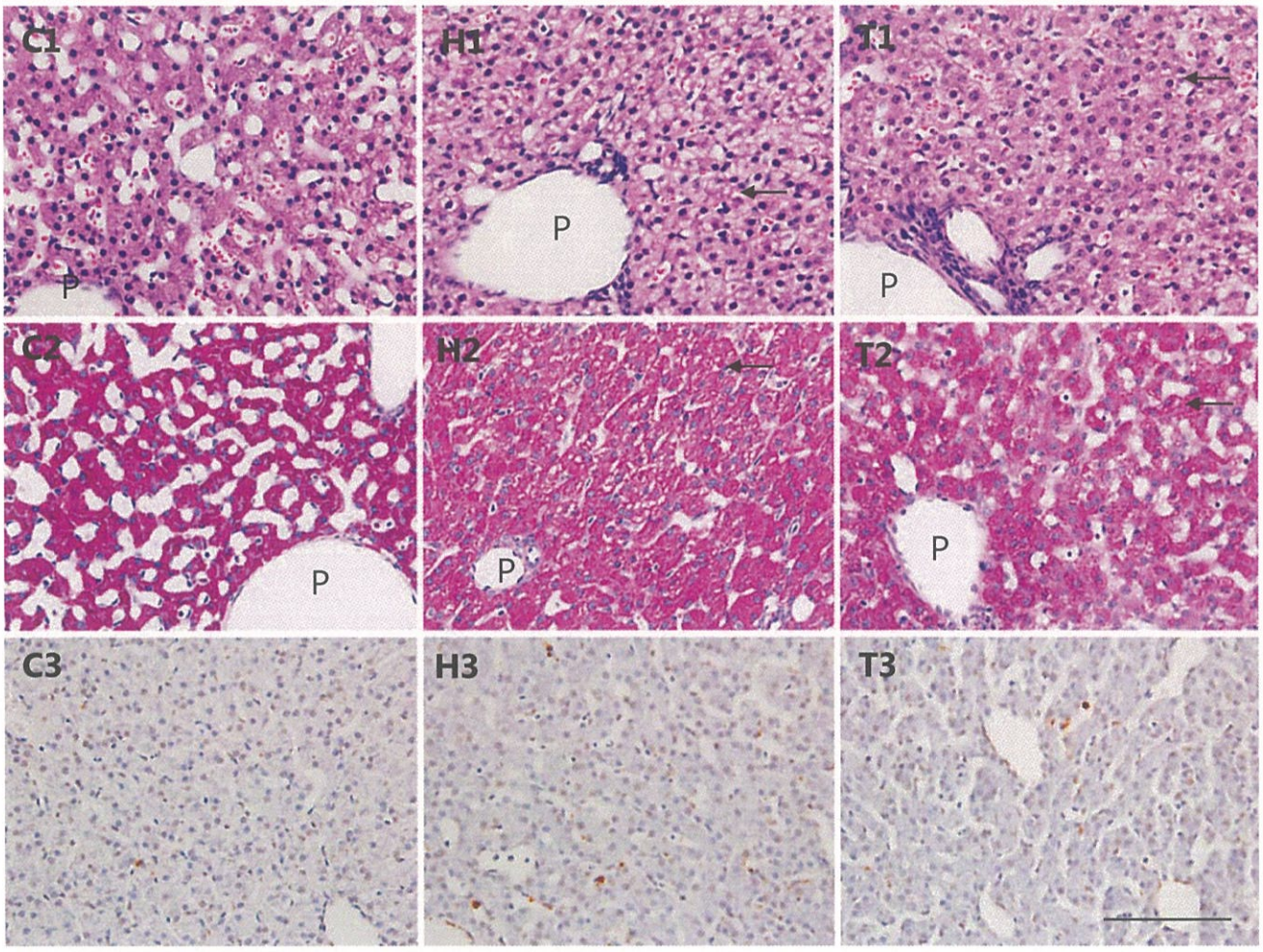

Fig. 3. H&E staining of the 3 groups. C: H&E (C1), PAS (C2), and TUNEL (C3) staining of the control group; H: H&E (H1), PAS (H2), and TUNEL (H3) staining of the HIE group; T: H&E (T1), PAS (T2), and TUNEL (T3) staining of the therapeutic hypothermia group. P, portal vein. Vacuolations (H1, H2: arrows) were more frequently observed in the cytoplasm of hepatocytes surrounding the portal vein in the HIE group than in the control group. Vacuolations (T1, T2: arrows) were occasionally observed in the cytoplasm of hepatocytes surrounding the portal vein in the therapeutic hypothermia group. A few TUNEL-positive cells, which were supposed to be blood cells, were observed in the 3 groups, while TUNEL-positive hepatocytes were not clear. Scale bar, 100 μm.

pound, and 10-μm frozen sections were stained with oil red O to investigate lipid accumulation. To estimate the hepatic zone and the amount of lipid accumulation objectively, 1 slide stained with oil red O from each piglet was used to capture 10 random images of the surrounding portal vein and central vein of the liver tissue. We measured the surface area of lipid droplets, which were stained red by oil red O, using image processing software (ImageJ; US National Institutes of Health [11]). The mean surface area was calculated for each piglet. The mean surface areas of red in the control ($n = 4$), HIE ($n = 8$), and therapeutic hypothermia ($n = 6$) groups were compared using the Kruskal-Wallis test, followed by a post hoc Steel-Dwass test. $p < 0.05$ was considered statistically significant.

Results

In blood data analysis, the median serum levels of AST, ALT, γ-GTP, and LDH in the HIE group were higher than those in the control group at 4 days after extubation. In particular, ALT ($p = 0.007$) and LDH ($p = 0.018$) levels were significantly elevated in the HIE group compared with those in the control group. The median serum levels in the therapeutic hypothermia group were lower than those in the HIE group, although the difference was not statistically significant. On the other hand, there were no

differences in these levels among the 3 groups at initial anesthesia induction (Fig. 2).

On H&E staining, obvious necrotic changes in hepatocytes or invasion of inflammatory cells were not seen in any of the groups. However, higher amounts of vacuolations were mainly observed in the cytoplasm of hepatocytes surrounding the portal vein in the HIE group compared with those in the control group. A few vacuolations were also observed in the cytoplasm of hepatocytes surrounding the portal vein in the therapeutic hypothermia group (Fig. 3: C1, H1, and T1). Because the identity of the vacuolations was not clear, PAS, TUNEL, APAF-1, and oil red O staining were performed. Hepatocytes in the HIE group were more weakly stained with PAS than those in the control group, while hepatocytes in the therapeutic hypothermia group were partially weakly stained with PAS (Fig. 3: C2, H2, and T2). A few vacuoles were observed in the cytoplasm of hepatocytes in the HIE group and in the therapeutic hypothermia group, also with PAS staining. As vacuoles showing no staining for PAS were observed in the HIE and the therapeutic hypothermia groups, the vacuoles were supposed to be lipid accumulation but not glycogen. TUNEL staining revealed that apoptotic or necrotic death of hepatocytes was not clear in the 3 groups (Fig. 3: C3, H3, and T3). In addition, immunoreactivity of APAF-1 was mainly observed in mononuclear cells and Kupffer cells commonly in the 3 groups, and not clear in hepatocytes (data not shown).

On oil red O staining, the control group showed almost no staining. However, the HIE group was densely stained – in particular, the cytoplasm of hepatocytes surrounding the portal vein. There was smaller lipid droplet accumulation in the cytoplasm of hepatocytes surrounding the portal vein in the therapeutic hypothermia group compared with that in the HIE group. Minimal staining was seen in the cytoplasm of hepatocytes surrounding the central vein in all groups (Fig. 4: C1, H1, and T1).

On high-power field examination, lipid droplets were observed as micro-lipid droplets, which were accumulated in the periportal zone, particularly in the HIE group (Fig. 4: C2, H2, and T2).

Image processing examination (Fig. 4: C3, H3, and T3) revealed that the digitized red area in the periportal zone in the HIE group was significantly larger than that in the control group ($p = 0.018$). The digitized red area in the periportal zone of the therapeutic hypothermia group was significantly smaller than that in the HIE group ($p = 0.037$). There was no significant difference in the periportal zone between the control and therapeutic hypothermia groups. There was also no significant difference in the perivenous zone among the 3 groups (Fig. 5).

Discussion

HIE is a much-studied process in neonatal care and has been extensively investigated using rodents. However, to understand the etiological mechanisms of human HIE, rodent models are not adequate because of differences in developmental events. Conversely, newborn piglets show comparable developmental levels with human neonates [12] and are widely used as an animal model for examining the etiology of various human perinatal diseases, including HIE.

We have developed a hypoxic-ischemic piglet model and reported clinical and pathological findings [13, 14]. The hypoxic-ischemic insult induced an increased cerebral blood volume, longer low amplitude-integrated electroencephalography, and severe brain injury in gray and white matter, including neuronal cell death. Clinical data on heart rates, mean arterial blood pressure, arterial pH, $PaCO_2$, PaO_2, base excess, lactate concentration, cerebral hemoglobin oxygen saturation, and cerebral blood volume were shown in the papers [13, 14].

To date, only 2 reports describing the histopathology of multiple organ injury with HIE in newborn piglets have been published [15, 16]. However, 1 model [15] is of selective cerebral ischemia by the ligation of the carotid arteries, not systemic hypoxia, while the other [16] does not refer to the effect of high concentrations of oxygen during resuscitation. Accordingly, findings on vital reactions such as the diving reflex, ischemia-reperfusion injury, and the interaction of organ injury after hypoxic insult and resuscitation have not been clarified.

Overall, 85% of severe HIE neonatal patients have concurrent liver injury [2] represented by elevated serum levels of AST, ALT, γ-GTP, and LDH. Similar to other studies [2, 5], our results demonstrated significantly elevated levels of ALT and LDH in the HIE group compared with those in the control group. Dispersion in the blood data at initial anesthesia induction is considered to be due to the stress associated with delivery.

Although blood analyses during acute HIE have been reported [2, 5], there are no reports on the histopathology of HIE-associated liver injury. Ischemic hepatitis is characterized by hepatocyte necrosis, particularly in the perivenous zone, which is vulnerable to ischemic damage due to low oxygen [17]. However, neither noticeable necrotic nor apoptotic changes in hepatocytes were observed in our study, suggesting that the hypoxic level of our HIE model may have been too mild to induce liver necrosis or apoptosis.

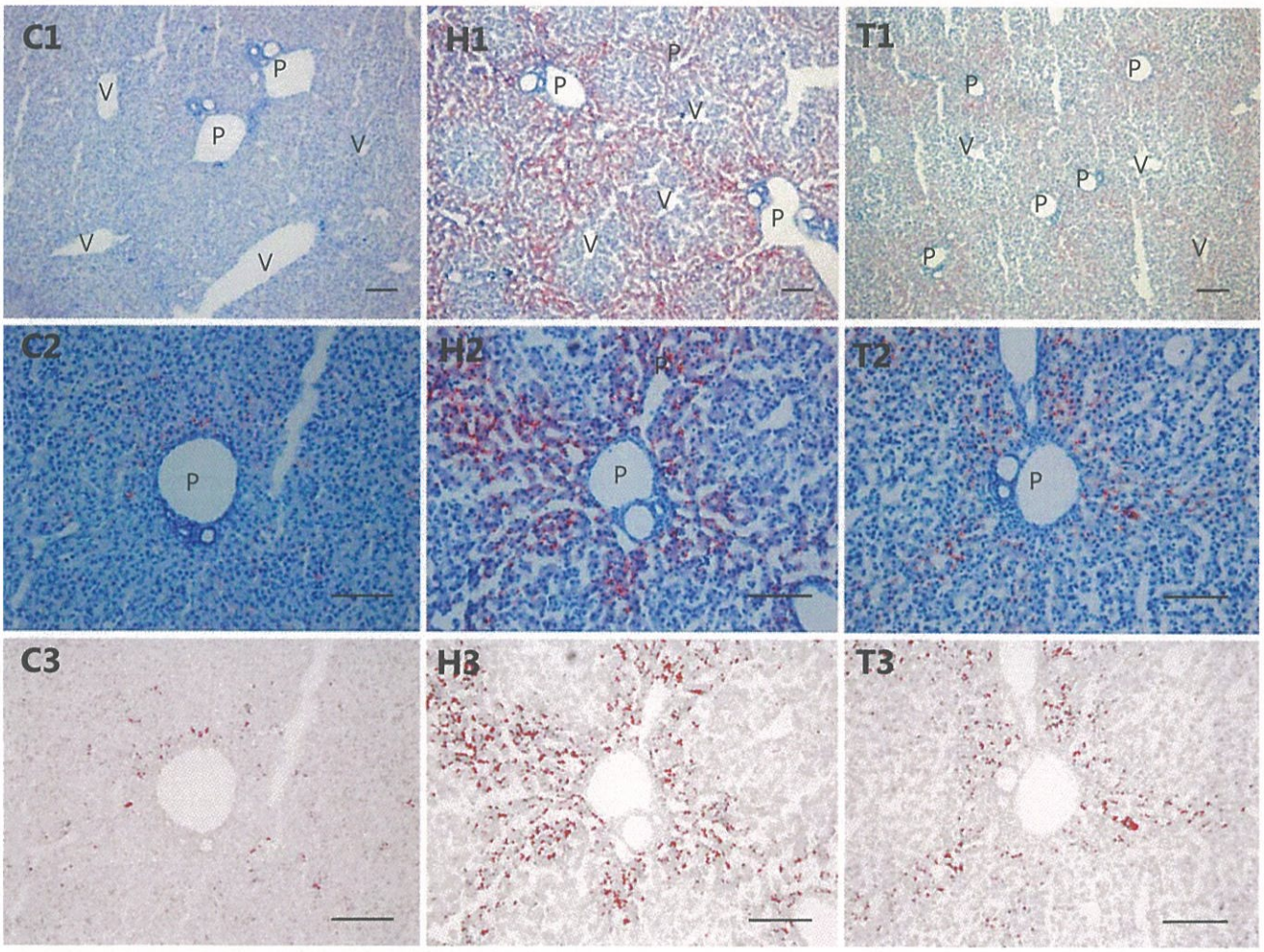

Fig. 4. Oil red O staining and image processing. C1, H1, and T1: oil red O staining of the control, HIE, and therapeutic hypothermia groups, respectively; C2, H2, and T2: portal vein surroundings in the high-power field of the control, HIE, and therapeutic hypothermia groups; C3, H3, and T3: image processing using ImageJ of C2, H2, and T2 for digitization of the red-stained areas of the control, HIE, and therapeutic hypothermia groups. P, portal vein; V, central vein. C1 and C2 depict the control group, which was minimally stained. H1 is a typical picture of oil red O staining in the HIE group. Hepatocytes surrounding the portal vein were strongly stained. Conversely, hepatocytes surrounding the central vein were minimally stained. H2 is a picture of the portal vein surroundings. Micro-lipid droplets were accumulated in the cytoplasm of hepatocytes mainly in the periportal zone. T1 and T2 depict the therapeutic hypothermia group, which has a reduced number of micro-lipid droplets compared with the HIE group. Scale bars, 100 μm.

Micro-lipid droplet accumulation was observed in the periportal zone of the HIE group. Recent studies have shown that lipid droplets accumulate in the cytoplasm of hepatocytes under hypoxic conditions and suggest acute liver injury [18]. Lipid droplets can be macro- or micro-droplets. Macro-lipid droplets are the most common form and are typically associated with alcohol abuse, diabetes, and obesity, while micro-lipid droplets are induced in acute fatty liver of pregnancy or Reye's syndrome [19, 20]. Accordingly, it is likely that the micro-lipid droplets observed in the present study were induced during an acute phase of liver injury. Although the zonal heterogeneity (periportal vs. perivenous) of lipid accumulation remains to be clarified, micro-lipid droplet accumulation in the periportal zone may be considered as a first stage of HIE-associated liver injury.

Therapeutic hypothermia is standard treatment for newborns with moderate-to-severe HIE and can be

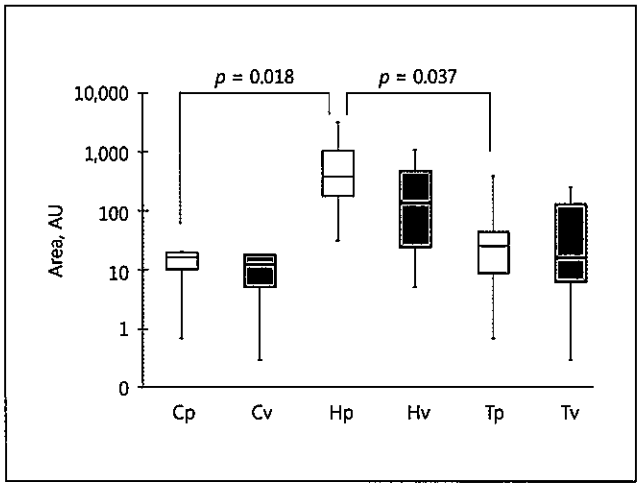

Fig. 5. Accumulation of lipid droplets in each zone in the 3 groups. C, control group; H, HIE group; T, therapeutic hypothermia group; p, periportal zone; v, perivenous zone. The digitized red area in the periportal zone in the HIE group was significantly larger than that of the control group ($p = 0.018$). The digitized red area in the periportal zone of the therapeutic hypothermia group was significantly smaller than that of the HIE group ($p = 0.037$). There was no significant difference in the periportal zone between the control and therapeutic hypothermia groups. There was also no significant difference in the area of the perivenous zone among the 3 groups.

achieved through either selective head cooling or whole-body cooling [21]. We adopted whole-body cooling for the direct cooling effect on the liver. Our results indicate that whole-body cooling suppressed micro-lipid accumulation induced by hypoxic insult, reducing HIE-associated liver injury. The therapeutic effect on HIE-associated liver injury may be mediated by metabolic inhibition, suppression of cytokines, or reduction of reactive oxygen species. Further investigation on the concrete effects of therapeutic hypothermia on HIE-associated liver injury using our experimental animal model is necessary.

Our study has some limitations. First, the study was performed on piglets, but not on humans. Second, because the piglet models were born normally before HIE induction, our model is not strictly HIE due to birth-related asphyxia. Micro-lipid droplet accumulation could have been influenced by drugs or cannulation of the umbilical vein in this study.

In conclusion, our study demonstrates that micro-lipid droplet accumulation in the cytoplasm of hepatocytes in the periportal zone occur under the HIE condition, and this accumulation is suppressed by therapeutic hypothermia. These results should contribute to the understanding of the pathology and treatment of HIE-associated liver injury.

Acknowledgment

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Statement of Ethics

This study was carried out in compliance with the guidelines for experimental use and care of laboratory animals set forth by the European Communities Council Directive of November 24, 1986 (86/609/EEC), and the Kagawa University Animal Ethics Committee.

Disclosure Statement

All authors confirm that they have no financial relationships or potential conflicts of interest to disclose.

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