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Original Article

Lobular distribution of enhanced expression levels of heat shock proteins using *in-situ* hybridization in the mouse liver treated with a single administration of CCl4

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Abstract: This study was conducted to visualize the lobular distribution of enhanced mRNA expression levels of heat shock proteins (HSPs) in liver samples from carbon tetra chloride (CCl4)-treated mice using *in-situ* hybridization (ISH). Male BALB/c mice given a single oral administration of CCl4 were euthanized 6 hours or 1 day after the administration (6 h or 1 day). Paraffin-embedded liver samples were obtained, ISH for HSPs was conducted, as well as hematoxylin-eosin staining and immunohistochemistry (IHC). At 6 h, centrilobular hepatocellular vacuolization was observed, and increased signals for *Hspa1a, Hspa1b*, and *Grp78*, which are HSPs, were noted in the centrilobular area using ISH. At 1 day, zonal hepatocellular necrosis was observed in the centrilobular area, but mRNA signal increases for HSPs were no longer observed there. Some discrepancies between ISH and IHC for HSPs were observed, and they might be partly caused by post-transcriptional gene regulation, including the ribosome quality control mechanisms. It is known that CCl4 damages centrilobular hepatocytes through metabolization by cytochrome P450, mainly located in the centrilobular region, and HSPs are induced under cellular stress. Therefore, our ISH results visualized increased mRNA expression levels of HSPs in the centrilobular hepatocytes of mice 6 hours after a single administration of CCl4 as a response to cellular stress, and it disappeared 1 day after the treatment when remarkable necrosis was observed there. (DOI: 10.1293/tox.2023-0053; J Toxicol Pathol 2024; 37: 29–37)

Key words: carbon tetra chloride, heat shock protein, *in-situ* hybridization, RNAscope, liver

Introduction

Drug-induced liver injury is one of the leading causes of termination of clinical drug development programs and withdrawal of approved drugs from the market[1–3]. Therefore, mechanistic analysis of hepatotoxicity has become important in toxicologic pathology[4–6]. The liver is the primary organ for metabolizing nutrients, drugs, and xenobiotics. Such metabolism is mainly conducted by a family of enzymes called cytochrome P-450 (CYPs), and many compounds are known to exhibit hepatotoxicity through metabolic activation[5, 6]. Carbon tetrachloride (CCl4) is an example of a hepatotoxicant that is converted into toxic metabolites by metabolic activation[5–8]. A prominent acute histopathological change caused by CCl4 is zonal hepatocellular necrosis in the centrilobular area, where expression levels of CYPs are known to be higher than those in the periportal region[9, 10]. CCl4 is converted into free radicals, such as trichloromethyl and trichloromethylperoxy radicals. They are detoxified by glutathione or glutathione S-transferase; however, the excessive generation of free radicals depletes the glutathione levels and causes oxidative stress, which results in hepatocellular damage[7, 8, 11, 12].

Heat shock proteins (HSPs) are crucial for the maintenance of cellular homeostasis and cellular survival under various stresses. The HSP70 family acts as a molecular chaperone, reducing denaturation and aggregation of intracellular proteins induced by cellular stress[11, 13, 14]. Therefore, the expression levels of HSPs are expected to increase in the centrilobular area of liver under cellular stress caused by CCl4 administration. It was reported that the expression levels of HSP 70 families were enhanced early after CCl4 administration using Northern blot analysis[12]; however, lobular distribution of the enhanced expression levels has not been visualized yet.

In this study, we conducted *in-situ* hybridization (ISH)

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of liver samples from CCl4-treated mice to visualize lobular distribution of the enhanced expression levels of HSP 70 families. As HSP 70 molecules, we selected Hspa1a, Hspa1b, and Grp78. Hspa1a and Hspa1b are expressed at low or undetectable levels in most unstressed normal cells, but it is prominently upregulated in stressed cells to guarantee cell survival through protein homeostasis[14]. Grp78 is localized on the membrane of the endoplasmic reticulum (ER), edits the folding and assembly, and avoids the transport of misfolded proteins (or subunits). The expression of Grp78 is increased in ER stress[15]. We used RNAscope as an ISH method. RNAscope is commercially available from Advanced Cell Diagnostics (ACD, Hayward, CA, USA)[16]. It is a novel RNA ISH technique that improves target signal amplification, reduces background using a unique double Z probe design, and allows for the visualization of targeted RNA using formalin-fixed paraffin-embedded (FFPE) samples[16–19]. We also conducted immunohistochemistry (IHC) for Hsp70 (corresponding to *Hspa1a* and *Hspa1b*) and Grp78 to evaluate the distribution of these proteins and compare them with the results of ISH. Additionally, we conducted blood chemistry to detect hepatotoxicity, including glutamate dehydrogenase (GLDH) and miR-122. Serum GLDH is reported to be a specific biomarker of liver injury[20]. mir-122 is a circulating microRNA, and it is enriched in the liver and recognized as a sensitive and informative biomarker for drug-induced liver injury[21].

Materials and Methods

Animal experimental design

Six-week-old male BALB/cAnNCrlCrlj mice were obtained from Charles River, Japan (Atsugi, Japan). After a 2-week acclimation period, they were allocated to control or CCl4 treatment groups according to body weight. They were housed in plastic cages under controlled temperature and humidity with a 12-h light/dark cycle. The animals were allowed free access to normal chow (CE-2, CLEA Japan, Tokyo, Japan) and tap water. CCl4 (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) was dissolved in corn oil (FUJIFILM Wako Pure Chemical Corporation) and administered once by gavage (0.1 mL/kg). The dosing volume was set at 10 mL/kg. Control mice received corn oil. Six hours or 1 day after the administration (6 h or 1 day), animals were weighed, and blood samples were collected from the vena cava inferior under isoflurane anesthesia using a syringe treated with an anticoagulant (EDTA-2K). Then, animals were sacrificed through exsanguination from the abdominal aorta under isoflurane anesthesia. The liver was dissected and weighed. One transversal slice of the left lateral lobe of the liver was immersed in 10 vol% neutral buffered formalin for five days. We used 16 mice in total in the present study, and four animals were assigned to each of the sampling points (6 h and 1 day) for the CCl4-treated or the control group. This animal study was conducted under the approval of the Institutional Animal Care and Use Committee and Shonan Health Innovation Park.

Blood chemistry

Blood samples were centrifuged at 7,500 × *g* for 10 min at 4°C to obtain plasma. The values of plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), and GLDH were determined with an automated blood chemistry analyzer (LABOSPECT008, Hitachi High-Technologies Corporation, Tokyo, Japan). Total RNA, including small RNA, was extracted from the plasma using a miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Then, real-time quantitative PCR was performed for miR-122 measurement using the ID3EAL system (MiRXES) and ABI7900 (ThermoFisher Scientific, Waltham, MA, USA). Relative miRNA expression levels were calculated as follows according to previous literature[22]. The ΔCt value was represented as Ct (miR-122) – Ct (reference). The ΔΔCt value was calculated as ΔCt (CCl4 group) – ΔCt (control group). The fold miRNA expression was calculated as $2^{(-\Delta\Delta Ct)}$.

Histopathology

For histopathological observation, the fixed liver slices were embedded in paraffin, and FFPE blocks were prepared. Then, they were sectioned and stained with hematoxylin and eosin (H.E.) and examined using a light microscope. RNAscope ISH was performed using the FFPE blocks, and they were sectioned at a thickness of approximately 3 μm and mounted on a coated glass slide (Matsunami, Osaka, Japan; Cat No. CRE-04). Target probes specific to *Hspa1a* (RNAscope 2.5 LS Probe-Mm-Hspa1a, Cat No. 488358), *Hspa1b* (RNAscope 2.5 LS Probe-Mm-Hspa1b, Cat No. 478218), and *Grp78* (RNAscope 2.5 LS Probe-Mm-Hspa5, Cat No. 438838), a positive control probe to peptidylprolyl isomerase B (*PPIB*, RNAscope 2.5 LS Positive Control Probe-Mm-PPIB, Cat No. 313918), a negative control probe to dihydrodipicolinate reductase (*DapB*, RNAscope 2.5 LS Negative Control Probe-DapB, Cat No. 312038), and staining kits (RNAscope 2.5 LS Reagent Kit-BROWN, Cat No. 322100, BOND Polymer Refine Detection kit, Cat No. DS9800) were purchased (ACD or Leica Biosystems, Wetzlar, Germany). RNAscope ISH was conducted using an automated staining equipment (Leica BOND RX) according to the automated ISH protocol for RNAscope 2.5 LS Reagent Kit-Brown. The signals by ISH were scored for each slide for each target gene based on the ACD Score (RNAscope Reference Guide, ACD) using a light microscope. Based on results from the positive control probe, whose scores were 2 or 3 for all samples, and the negative control probe, whose scores were 0 for all samples (see results of histopathology), the quality of the FFPE samples was confirmed to be suitable for RNAscope ISH. For IHC, the same FFPE blocks were used, and primary antibodies for Hsp70 (Cat No. ab181606, Rabbit monoclonal [EPR16892] to Hsp70) and Grp78 (Cat No. ab21685, Rabbit polyclonal to GRP78 BiP) were purchased (Abcam, Cambridge, UK). According to the manufacturer's website, the antibody for Hsp70 reacts with Hspa1a and Hspa1b in mice. It was reported that *Hspa1a* and *Hspa1b* genes only differ in 8 bp, and they encode a similar protein with 99%

identity[14]. Probably due to that, we could not find a commercial antibody that reacts with Hspa1a but not Hspa1b, or vice versa. Therefore, we used the antibodies mentioned above for IHC and compared the result with ISH. The suitability of these commercial antibodies for IHC using FFPE blocks was expressed on the manufacturer's website. IHC staining was conducted according to the protocol using the automated staining equipment (Leica BOND RX) with heat treatment for antigen retrieval. The dilution of the primary antibody was set at 1:1000 for Hsp70 and Grp78.

Statistical analysis

The values on body weight, liver weight, and blood chemistry were tested by the F test for homogeneity of variance between the groups. When the variances were homogeneous, Student's t-test was used, and when the variances were heterogeneous, the Aspin & Welch t-test was performed to compare the mean in the CCl4 group with that in the control group at the same time point. The F test was conducted at the significance level of 0.20, and the other tests were conducted at the significance levels of 0.05 and 0.01. Analyses were performed using SAS version 9.3 (SAS Institute Inc.).

Results

Body and liver weights

Mean body weights in the CCl4 group were not significantly different from those of the control group at 6 h and 1 day. Mean liver weight in the CCl4 group at 6 h showed no significant difference, but the value at 1 day significantly increased compared with that in the control group (Table 1).

Blood chemistry

Plasma AST, ALT, and miR-122 values were significantly increased at 6 h, and plasma AST, ALT, and GLDH values were also significantly increased at 1 day compared with the corresponding values of the control group (Table 2). An increased tendency was also observed in plasma miR-122 value at 1 day.

Histopathology

H.E. sections revealed centrilobular hepatocellular vacuolization in the CCl4 group at 6 h and zonal hepatocellular necrosis, inflammatory cell infiltration, and hemorrhage in the centrilobular area at 1 day in the CCl4 group (Table 3, Fig. 1). ISH using RNAscope on the mouse liver sections from formalin-fixed tissue revealed increased signals for *Hspa1a*, *Hspa1b*, and *Grp78* in the centrilobular hepatocytes at 6 h compared with the control group. At 1 day, ISH signals of *Hspa1a* and *Hspa1b* were no longer detected in the centrilobular area, where remarkable hepatocellular necrosis was observed. Additionally, signals for *Grp78* in the periportal hepatocytes slightly increased compared with

Table 1. Body and Liver Weights

Sampling timing	Group	Body weight (g)	Liver weight (mg)
6 hours	Corn oil	26.2 ± 0.9	1,364.4 ± 39.4
	CCl4	26.2 ± 0.9	1,438.7 ± 66.6
1 day	Corn oil	25.6 ± 1.1	1,423.5 ± 102.4
	CCl4	25.3 ± 0.5	1,800.3 ± 57.5**

Values are means ± SDs from 4 animals for each point. Control mice received corn oil.
**: Significantly different from the control at p<0.01 (Student's t test).

Table 2. Blood Chemistry

Sampling timing	Group	AST (U/L)	ALT (U/L)	GLDH (U/L)	miR-122 (2^−ΔΔCt)
6 hours	Corn oil	59 ± 9	75 ± 11	80.3 ± 20.6	1.08 ± 0.51
	CCl4	126 ± 13**	349 ± 46&&	73.1 ± 15	15.25 ± 5.31&
1 day	Corn oil	62 ± 11	66 ± 12	95.7 ± 29.8	1.10 ± 0.55
	CCl4	12,539 ± 6,802&	25,500 ± 8,087&&	5,365 ± 864.5&&	29.91 ± 21.55

Values are means ± SDs from 4 animals for each point. Control mice received corn oil.
**: Significantly different from the control at p<0.01 (Student's t test).
&, &&: Significantly different from the control at p<0.05, 0.01, respectively (Aspin & Welch t-test).
AST: aspartate aminotransferase; ALT: alanine aminotransferase; GLDH: glutamate dehydrogenase.

Table 3. Histopathological Changes by H.E. Observation

	6 hours		1 day	
	Corn oil	CCl4	Corn oil	CCl4
Vacuolization, Hepatocyte, Centrilobular	-	++	-	-
Zonal necrosis, Hepatocyte, Centrilobular	-	-	-	+++
Infiltrate, Inflammatory cell, Centrilobular	-	-	-	+
Hemorrhage, Centrilobular	-	-	-	++

-: Not remarkable, +: Minimal, ++: Mild, +++: Moderate, ++++: Marked
One section for each 4 animals were evaluated for each point. Control mice received corn oil.

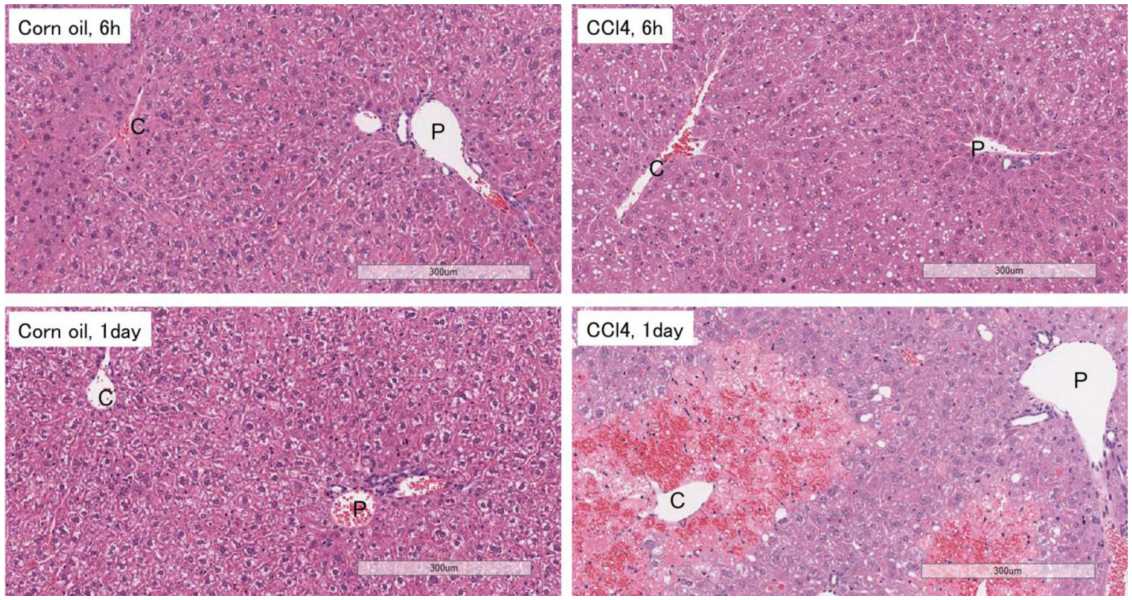

Fig. 1. H.E. sections of the liver. CCl4 or corn oil was orally administered to BALB/cAnNCrlCrlj mice. Then mice were sacrificed under isoflurane anesthesia, and the liver was dissected 6 hours or 1 day after the administration. Control mice received corn oil. C: centrilobular; P: periportal, Bars=300 µm.

Table 4. Scores of *In-Situ* Hybridization (ISH)

	6 hours		1 day	
	Corn oil	CCl4	Corn oil	CCl4
Hspa1a				
Centrilobular hepatocyte	0	3	0	NA
Periportal hepatocyte	0	0	0	0
Hspa1b				
Centrilobular hepatocyte	0	3	0	NA
Periportal hepatocyte	0	0	0	0
Grp78				
Centrilobular hepatocyte	2	4	2	NA
Periportal hepatocyte	2	2	2	3
PPIB				
Centrilobular hepatocyte	2	2	2	NA
Periportal hepatocyte	2	2	2	3
DapB				
Centrilobular hepatocyte	0	0	0	NA
Periportal hepatocyte	0	0	0	0

Scores were evaluated as 0–4 according to ACD Score (RNAscope Reference Guide, ACD).
PPIB: positive control probe; *DabB*: negative control probe.
NA: No signals were observed in the area where remarkable necrosis was observed.
One section for each 4 animals were evaluated for each point. Control mice received corn oil.

the control group, and the signals for the positive control probe (*PPIB*) were also enhanced in the periportal area (Table 4, Figs. 2–5). IHC at 6 h showed increased Hsp70 signals in the centrilobular area. For Grp78, only a few sporadic positive cells were observed in the transition zone, but no significant change was observed in the centrilobular or periportal area compared with the control group at 6 h. At 1 day, increased Hsp70 and Grp78 signals in the periportal hepatocytes were observed using IHC compared with the control group (Table 5, Figs. 6, 7).

Discussion

At 6 h, centrilobular hepatocellular vacuolization before necrosis was observed using H.E. with increased blood chemical parameters, including miR-122. ISH revealed increased signals for *Hspa1a*, *Hspa1b*, and *Grp78* in the centrilobular hepatocytes. CCl4 is known to damage centrilobular hepatocytes by cellular stress due to metabolization by cytochrome P450, mainly located in the centrilobular region[5–12]. HSPs are crucial for maintaining cellular homeostasis and are induced under cellular stress[11, 13, 14]. Furthermore, Song *et al*. reported that remarkable centrilobular hepatocellular necrosis was observed 24 hours after CCl4 treatment only in *Hsp70* knock-out mice but not in wild-type mice[11]. Although the mouse strain (C57BL/6), dosing route (intraperitoneal injection), and dose (0.3 mL/kg) of the experimental design of Song *et al.* were different from our experiment, we supposed that HSPs have a protective effect on hepatocytes against CCl4 treatment, and HSPs gene expression will be enhanced in the centrilobular hepatocytes after CCl4 administration. As far as we know, our result is the first data that visualizes the lobular distribution of enhanced expression of HSPs in the liver after treatment of CCl4. Increased Hsp70 protein signals in the centrilobular area were detected using IHC at 6 h, corresponding to the result of ISH. However, Grp78 protein signals in the centrilobular area were not increased using IHC at 6 h. It was

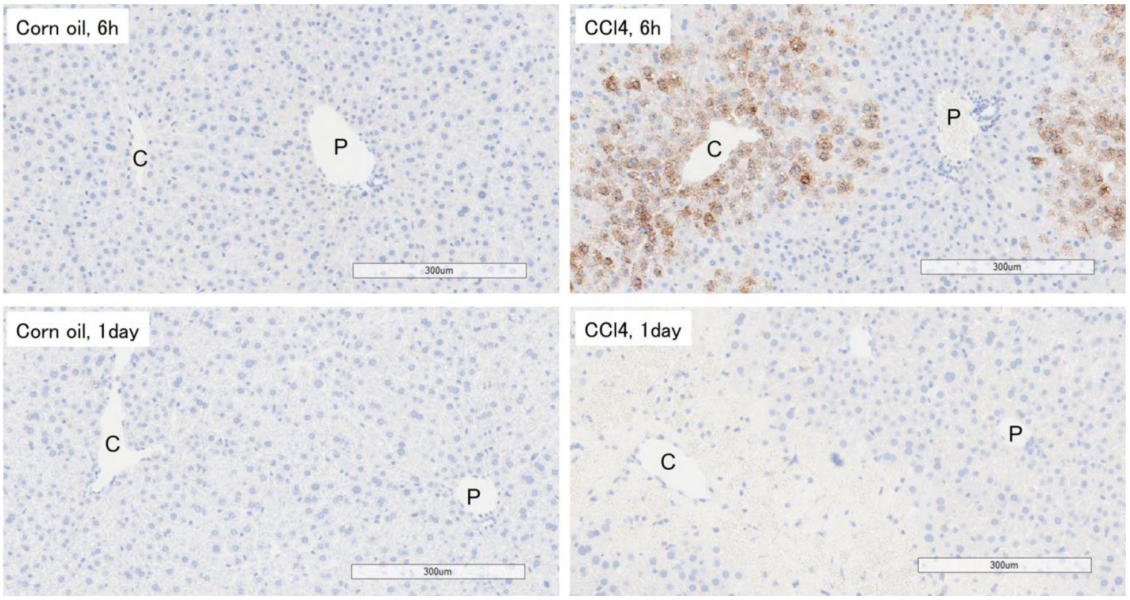

Fig. 2. ISH for *Hspa1a* in the liver. Control mice received corn oil. C: centrilobular; P: periportal, Bars=300 µm.

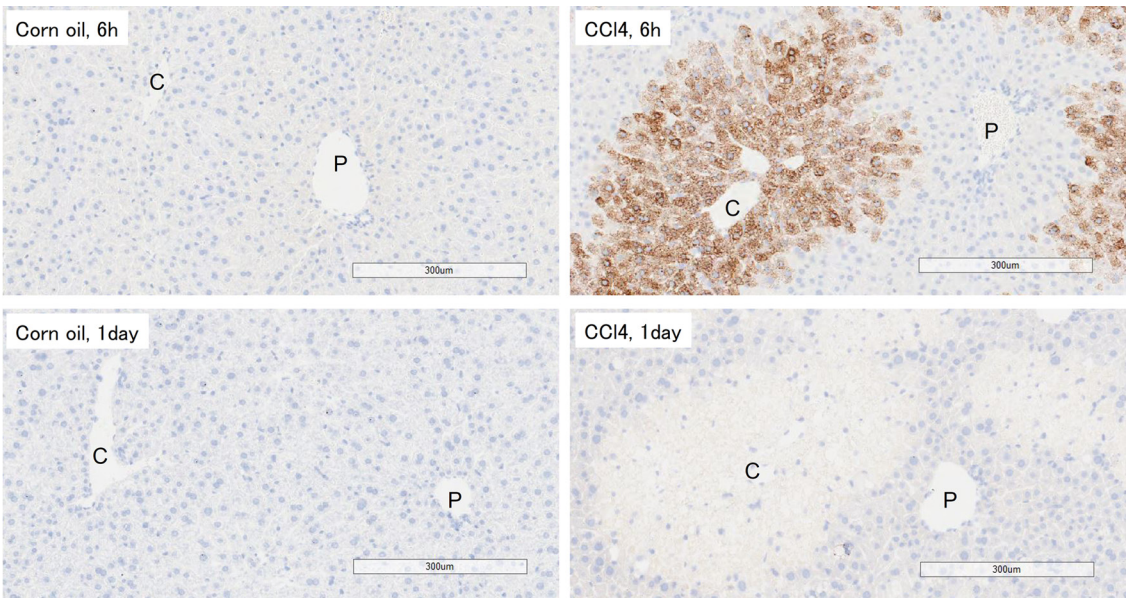

Fig. 3. ISH for *Hspa1b* in the liver. Control mice received corn oil. C: centrilobular; P: periportal, Bars=300 µm.

reported that because Grp78 is known to be an ER stress sensor, its expression is tightly controlled at a post-transcriptional level[23]. Therefore, increased levels of *Grp78* mRNA induced by CCl4 might not increase the protein levels in relation to its gene regulation mechanism. Cheng *et al.* reported that when ER stress was induced in HeLa cells by dithiothreitol treatment, *GRP78* mRNA concentration showed an early and transient increase, whereas protein concentration gradually increased[24]. Therefore, it is possible that an early and transient increase in mRNA was observed, but a delayed and gradual protein increase was not detected at 6 h in the present study. Another conceivable mechanism is that synthesized Grp78 protein was rapidly degraded, not accumulated in the hepatocellular cytoplasm, and could not be detected using IHC.

At 1 day in the centrilobular area, zonal hepatocellular necrosis, inflammatory cell infiltration, and hemorrhage were observed using H.E. Additionally, the increased liver weight and blood chemical parameters were observed simultaneously. ISH signals for *Hspa1a* and *Hspa1b* were no longer detected in the centrilobular area, where remarkable hepatocellular necrosis was observed. Based on these

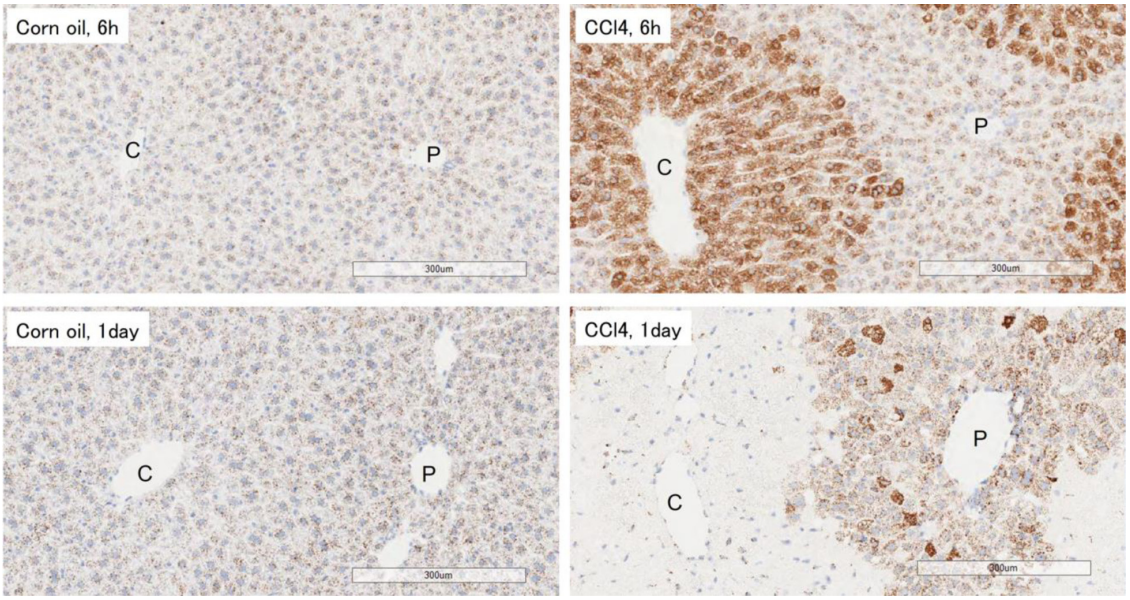

Fig. 4. ISH for *Grp78* in the liver. Control mice received corn oil. C: centrilobular; P: periportal, Bars=300 µm.

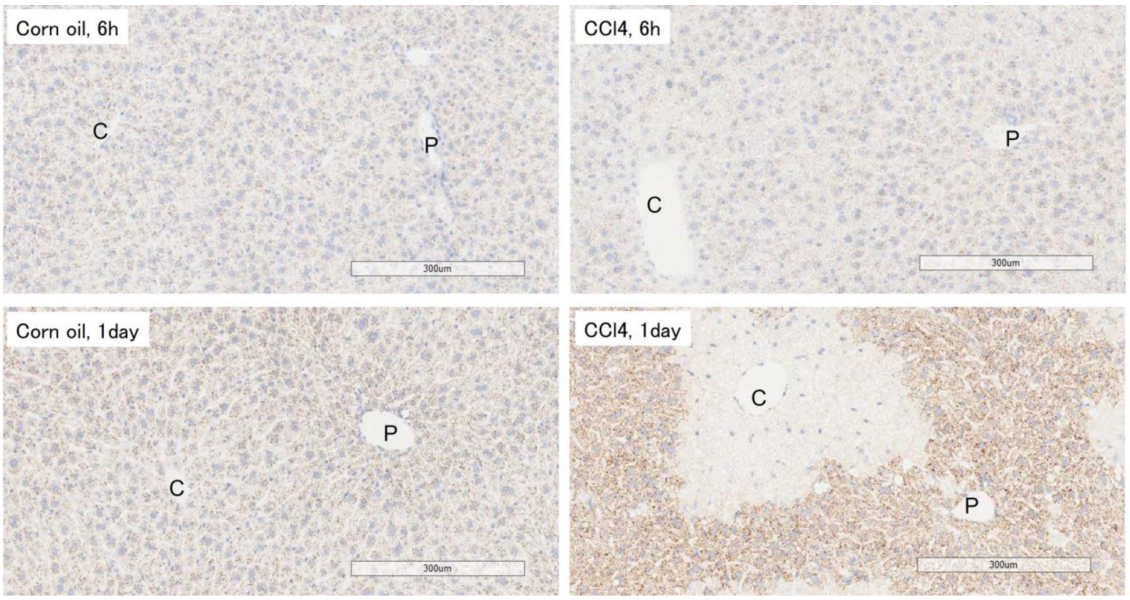

Fig. 5. ISH for *PPIB* in the liver. Control mice received corn oil. C: centrilobular; P: periportal, Bars=300 µm.

Table 5. Scores of Immunohistochemistry (IHC)

		6 hours		1 day	
		Corn oil	CCl4	Corn oil	CCl4
HSP70					
	Centrilobular hepatocyte	1	4	1	NE
	Periportal hepatocyte	0	0	0	2
Grp78					
	Centrilobular hepatocyte	1	1	1	NE
	Periportal hepatocyte	1	1	1	2

Scores were evaluated as 0–4. NE: Not evaluated due to necrosis.
Four animals were evaluated for each point. Control mice received corn oil.

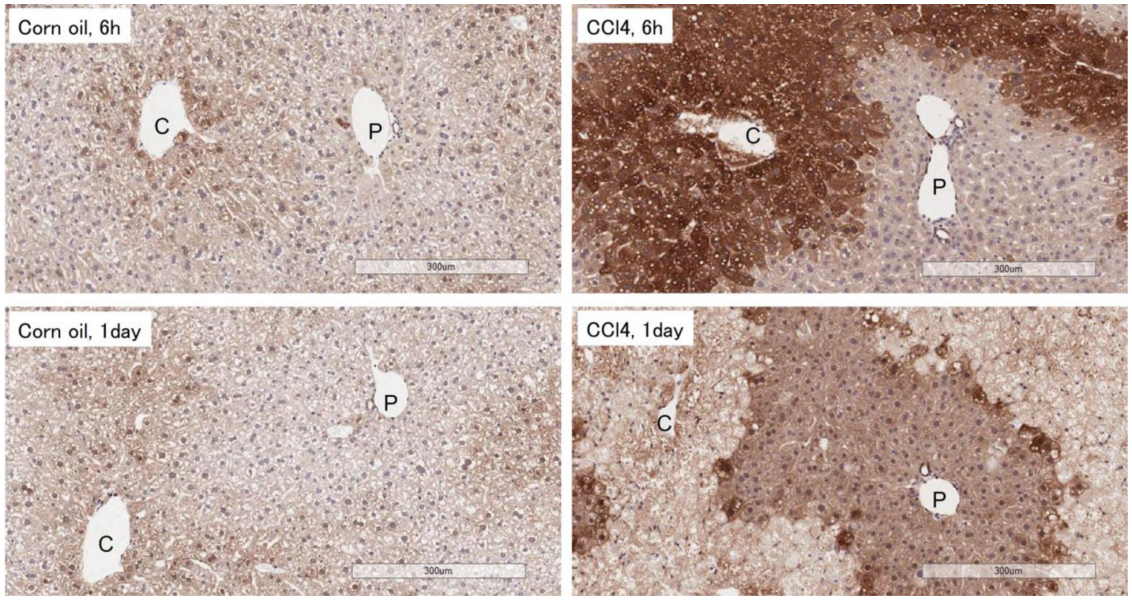

Fig. 6. IHC for Hsp70 in the liver. Control mice received corn oil. C: centrilobular; P: periportal, Bars=300 μm.

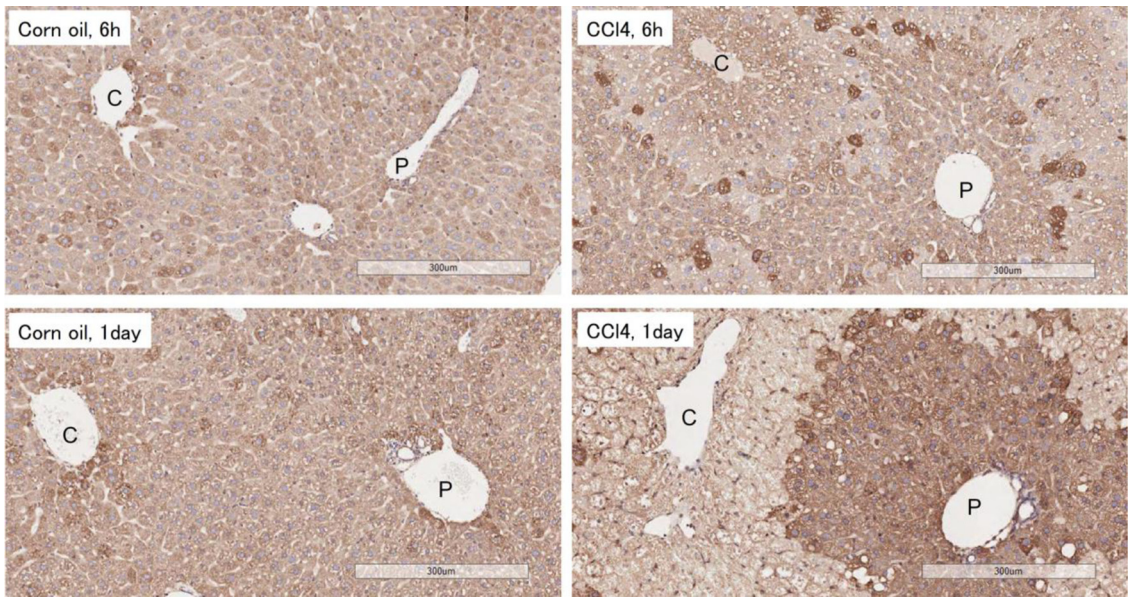

Fig. 7. IHC for Grp78 in the liver. Control mice received corn oil. C: centrilobular; P: periportal, Bars=300 μm.

findings, we concluded that enhanced mRNA expression of HSPs in the centrilobular hepatocytes at 6 h disappeared 1 day after the administration when remarkable necrosis was observed. Schiaffonati *et al.* detected increases of *Hsp70* and *Grp 78* mRNA expression in rat liver using Northern blot analysis 3 and 5 h after CCl4 administration, and the increase of *Hsp70* disappeared 24 h after the treatment[12]. Their results in rats were consistent with our results in mice using ISH.

At 1 day in the periportal area, the ISH signal for the positive control probe (*PPIB*) was enhanced in the periportal area. We used *PPIB* as a positive control probe according to the manufacturer's guide (the RNAscope Reference Guide, ACD), where *PPIB* (cyclophilin B) is recommended for use as a positive control for most tissues, including the mouse liver because it is expressed at a sufficiently low level to provide a rigorous control for sample quality and technical performance. Wang *et al.* reported increased expression of cyclophilin B in CCl4 treated-fibrosis model rats[25]. Therefore, the enhanced *PPIB* signal for ISH might be induced by the administration of CCl4. However, the increased expression of cyclophilin B in the report of Wang *et al.* was

observed after 6 weeks of administration accompanied by liver fibrosis. Because fibrosis was not observed in our single administration experiment, the detailed mechanism of this phenomenon remained unclear.

Signals of ISH for *Grp78* in the periportal area of the CCl4 group were slightly increased at 1 day compared with the control group. Additionally, increased signals of IHC for Grp78 were observed in the periportal area of the CCl4 group at 1 day. Schiaffonati *et al.* reported the increase of *Grp78* mRNA expression in rat liver detected using Northern blot analysis remained unchanged at 48 and 72 h after the administration of CCl4[12]. Their data did not contradict our results. Histopathological abnormality was not observed in the periportal hepatocytes, and they seemed to remain intact at 1 day in the present study. Therefore, it was supposed that cellular stress in the periportal hepatocytes might increase due to increased load to maintain liver function after rapid necrosis of centrilobular hepatocytes, and consequently, increased *Grp78* mRNA expression might be observed as a cellular stress response in the periportal area. However, as mentioned above, the positive control probe (PPIB) signal was also enhanced in the periportal area on 1 day by an uncertain mechanism. Therefore, we could not exclude the possibility that multiple unspecified genes were enhanced and *Grp78* expression was increased due to a nonspecific cause that is not directly related to the cell stress response, which is the original function of Grp78. The biological meaning of the phenomenon remained uncertain.

Signals of IHC for Hsp70 in the periportal area of the CCl4 group was increased at 1 day compared with the control group. However, no corresponding signals of ISH were detected, i.e., ISH signals for *Hspa1a* and *Hspa1b* were not detected in the periportal area at 6 h and 1 day. Alagar Boopathy *et al.* described that mRNA translation of HSP70 is suppressed by ribosome quality control (RQC) mechanisms[26]. Therefore, it is possible that the changes over time in the expression levels of mRNA and the protein levels did not match in the present study via post-transcriptional gene regulation, including the RQC mechanisms. In other words, mRNA for HSP70 might peak transiently in the periportal area between 6 h and 1 day, then disappear by 1 day, and only an increase in protein might be detected at 1 day. To clarify this point, additional experiments with sampling between 6 h and 1 day are considered necessary.

Comparing ISH and IHC in general, the advantage of ISH in terms of specificity was pointed out in several articles[16, 18, 27, 28]. Because we could not prepare a positive control sample in the present study in which the expression of HSPs was enhanced, we had to admit the specificity of IHC was not completely confirmed. However, we used commercial antibodies suitable for IHC using FFPE blocks; therefore, we assumed that our IHC results were reliable. We considered that the discrepancies between ISH and IHC might partly be caused by the post-transcriptional gene regulation, including the RQC mechanisms mentioned above.

In the present study, we conducted blood chemistry tests, including GLDH and miR-122, for detecting hepatotoxicity. Increased AST, ALT, and miR-122 plasma levels were observed at 6 h, but GLDH did not change significantly. GLDH is reported to be a liver-specific biomarker of hepatocellular injury, especially when muscle lesions are present[20]. Liver enzymes, such as ALT, in the blood are recognized as "leakage" enzymes that escape from the hepatocytes due to altered permeability of the hepatocellular membrane[5, 29, 30]. Like ALT, the GLDH enzyme leaks from the damaged hepatocyte into the circulation when hepatocellular membrane integrity is lost[20]. Therefore, the reason why a change in GLDH was not detected at 6 h remained unclear. mir-122 is also a sensitive biomarker, and elevation of serum miR-122 was reported to be observed before serum ALT elevation in drug-induced liver injury[31]. It was reported that HSP inductions are observed in intact cells under stress[13, 14, 32]. Therefore, the enhanced expression levels of HSPs and the elevation of plasma levels of mir-122 might be observed before changes in other toxicologic parameters, such as "leakage" enzymes, when the permeability of the hepatocellular membrane remains intact. Further studies are required to clarify this point in which hepatocytes are exposed to lower cell stress compared with the present study.

Our ISH results showed increased mRNA expression levels of HSPs in the centrilobular hepatocytes of mice 6 hours after a single administration of CCl4 due to cellular stress, and it disappeared 1 day after the administration when remarkable necrosis was observed there.

Disclosure of Potential Conflicts of Interest: The authors declare no conflict of interest.

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