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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Noninvasive diagnostic method for idiopathic portal hypertension based on measurements of liver and spleen stiffness by ARFI elastography

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Abstract
Background Acoustic radiation force impulse (ARFI) elastography is an ultrasound technique that is capable of measuring tissue stiffness noninvasively. It is difficult to differentiate idiopathic portal hypertension (IPH) from liver cirrhosis (LC) or chronic hepatitis (CH), and liver biopsy is essential. We investigated whether it would be possible to noninvasively diagnose IPH by measuring the stiffness of the liver and spleen by ARFI.
Methods The subjects were 17 IPH patients, 25 LC patients, 20 CH patients, and 20 normal controls (NC). We measured liver stiffness, spleen stiffness, and the spleen/liver stiffness ratio, and plotted ROC curves.
Results The median value of liver stiffness in the IPH group was lower than that in the LC group ($p = 0.00077$) and about the same as in the CH group ($p = 0.79$). The median value of spleen stiffness was highest in the IPH group (IPH vs. LC group, $p = 0.003$; IPH vs. CH group, $p < 0.00001$). The spleen/liver stiffness ratio was lower in the LC group and in the CH group, and higher in the IPH group ($p < 0.001$, respectively). When an ROC curve of spleen/liver stiffness ratios was plotted to differentiate between the IPH group and the combined group of patients with other liver diseases (LC + CH group), when a cutoff value of 1.71 was used, the AUROC was 0.933 sensitivity 0.941, specificity 0.800, and accuracy 0.839.
Conclusion Measuring the spleen/liver stiffness ratio by ARFI made it possible to noninvasively, specifically, and accurately diagnose IPH.

Keywords ARFI · VTTQ · Esophageal varices · Spleen

List of abbreviations

ARFI	Acoustic radiation force impulse
IPH	Idiopathic portal hypertension
LC	Liver cirrhosis
CH	Chronic hepatitis
NC	Normal controls
ROC	Receiver operatorating characteristic curve
INCPH	Idiopathic non-cirrhotic portal hypertension
EVs	Esophageal varices
ROI	Region of interest
VTTQ	Virtual touch tissue quantification
Se	Sensitivity
Sp	Specificity
PPV	Positive predictive value
NPV	Negative predictive value
LR	Likelihood ratio

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HBV	Hepatitis B virus
HCV	Hepatitis C virus
NBNC	Non B non C hepatitis
PT-INR	Prothrombin time international normalized ratio
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
HCC	Hepatocellular caricinoma
PALS	Periarterial lymphoid sheath
LFs	Lymph follicles

Introduction

Idiopathic portal hypertension (IPH) is a disease in which occlusion and narrowing of peripheral portal veins in the liver leads to portal hypertension, and in western European countries it is classified as a type of idiopathic non-cirrhotic portal hypertension (INCPH). Its etiology is unknown, and further elucidation of the pathology is needed. Depending on its severity, IPH is manifested by esophagogastric varices, portal hypertensive gastropathy, ascites, hepatic encephalopathy, splenomegaly, anemia, liver function disorders, etc., but it does not lead to liver cirrhosis (LC), and it never develops as a complication of hepatocellular carcinoma [1]. Moreover, IPH has a favorable prognosis, and unmanageable bleeding of esophageal varices (EVs) is much less common than in LC [2]. By contrast, in addition to LC progressing to liver failure over a period of more than 10 years, hepatocellular carcinoma sometimes develops as another complication, and recurs. Thus, LC requires more frequent US examinations, CT examinations, blood examinations, and treatment than IPH does.

Liver biopsy examinations remain essential to the diagnosis of IPH. They are indispensable to rule out LC and chronic hepatitis (CH), because IPH is indistinguishable from cirrhosis on radiological examinations. Even in renowned hepatology centers, IPH patients are frequently misdiagnosed with LC. Krasinskas et al. [3] showed that the majority of IPH patients who underwent liver transplantation had a pretransplantation diagnosis of cirrhosis.

If a method that made it possible to diagnose IPH both noninvasively and accurately were available, it would be of inestimable value to patients, and as a means of reducing such misdiagnoses as well. Seijo et al. [4] reported that liver stiffness retrospectively measured by transient elastography in IPH patients was lower than that in LC patients. But, for the difference of liver stiffness between IPH patients and CH patients, the accuracy and the sensitivity were unclear. Meanwhile, the study by magnetic resonance elastography suggested that spleen stiffness in patients with LC was more closely correlated to portal hypertension compared with liver stiffness and was able to predict the presence of EVs [5]. We, therefore, used acoustic radiation force impulse (ARFI) elastography, which is capable of measuring tissue stiffness noninvasively by gauging the shear wave propagation velocity of ultrasound waves [6, 7], and investigated the possibility of specifically diagnosing IPH by measuring the stiffness of both the liver and the spleen. In addition, we elucidated how the pathology of IPH differs from that of LC and CH.

Subjects and methods

Subjects

There were 82 subjects, and they consisted of 17 IPH patients, 25 LC patients, 20 CH (eight F1 and 12 F2 fibrosis) patients, and 20 normal control (NC) subjects (Table 1). A histological diagnosis by biopsy was made in all of the cases of IPH, LC, and CH. In the LC group, the cases having ascites or hepatocellular carcinoma were excluded. Moreover, we chose the cases as all IPH patients and LC patients had the similar portal hypertension signs (i.e. having esophageal varices or previous history of treatment of esophageal varices). The protocol of this study was approved by the clinical research ethics committee of the Tokyo Medical University (Japan) and conformed to the principles of the Helsinki agreement. All subjects gave written informed consent before participating in the study.

ARFI elastography

ARFI measurements were performed by using the virtual touch tissue quantification (VTTQ) mode of a Siemens Acuson S2000™ ultrasound system, and we measured liver stiffness, spleen stiffness. The region of interest (ROI) was set at a depth of 2 cm beneath the body surface and 10 successful ARFI measurements (stiffness) were performed in the liver and in the spleen of each patient by an intercostal approach. The median values in each group were then calculated, and the results are expressed in meters/second (m/s). All ARFI examinations were performed by the independent blinded examiner, who had experience of over 10 years as a radiologist and was not informed of the patient characteristics. Since the ARFI measurements were independent and blind comparison with the gold standard (that is, liver biopsy) of diagnosis, this study can be said as a phase 3 design for a diagnostic method [8].

ARFI imaging technology involves the mechanical excitation of tissue by exposing an ROI chosen by the examiner to short-duration acoustic pulses (push pulses), which produce shear waves that generate localized, micron-scale displacements in the tissue as they propagate away from the ROI. Detection waves of lower intensity than the push pulse are simultaneously generated.

Table 1 Patients characteristics

	IPH	LC	CH	NC
Number of cases	17	25	20	20
Gender (M:F)	11:6	18:7	10:10	14:6
Ages (years old)[a]	40.0 (23–86)	71.0 (56–82)	66.5 (32–81)	38.0 (29–55)
Pathogenesis				
HBV		4	4	
HCV		14	8	
NBNC		7	8	
Platelet ($\times 10^4 \mu l$)[a]	8.6 (2.5–24.1)	9.8 (3.2–18.9)	15.3 (7.4–35.8)	21.2 (18.6–24.3)
PT-INR[a]	1.06 (1.00–1.34)	1.10 (0.95–1.25)	1.01 (0.97–1.18)	0.99 (0.94–1.06)
AST[a]	27.5 (11–47)	42.0 (17–136)	26.0 (15–83)	14.5 (11–19)
ALT[a]	17.5 (11–37)	29.0 (6–114)	43.1 (9–111)	8.5 (7–15)
Total bilirubin[a]	1.15 (0.81–2.31)	1.02 (0.60–2.04)	0.87 (0.25–1.39)	0.54 (0.39–0.78)
Albumin[a]	4.1 (2.9–4.5)	3.6 (2.4–4.5)	4.3 (3.5–4.6)	4.4 (4.0–4.9)
Esophageal varices				
Presence	12	18	0	0
Previous	6	7	0	0
Absence	0	0	20	20
Ascites				
Presence	0	0	0	0
Absence	18	25	20	20
HCC				
Presence	0	0	0	0
Absence	18	25	20	20

Data are numbers of patients

HBV hepatitis B virus, *HCV* hepatitis C virus, *NBNC* non B non C hepatitis, *PT-INR* prothrombin time international normalized ratio, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *HCC* hepatocellular caricinoma

[a] Data are median values with ranges in parentheses

As a result, we can estimate the propagation speed of share waves by measuring this detection waves and the stiffness of the region in the tissue can be expressed in meters/second (m/s). Generally, the stiffer the region in the tissue is, the greater the shear wave velocity is [9].

Evaluation and statistical analysis of liver and spleen stiffness

The statistical analysis was performed using SPSS software version 16.0 j (SPSS Inc. Chicago, IL, USA) and statistical computing software R (R 2.15.1 for Windows).

Multiple comparisons of the median values of liver stiffness and spleen stiffness in the four groups of subjects (IPH group, LC group, CH group, and NC group) were performed by the nonparametric Kruskal–Wallis test, and the Steel–Dwass test was used for multiple comparisons between groups. In addition, the spleen stiffness to liver stiffness ratio (spleen/liver stiffness ratio) was calculated, and the values obtained were tested in a similar manner. A p-value of less than 0.05 was used as the criterion for significance in all of the tests.

The diagnostic performance of ARFI elastography in the liver and spleen was assessed by plotting receiver operating characteristic (ROC) curves that were designed to differentiate between the IPH group and the LC group, between the IPH group and the CH group, between the IPH group and the NC group, and between the IPH group and the group with other liver diseases (CH group + LC group). An ROC curve is a plot of sensitivity versus 1-specificity for all possible cut-off values. The most commonly used index of accuracy is the area under the ROC curve (AUROC), with values indicating higher diagnostic accuracy as they approach 1. Optimal cut-off values were chosen to maximize the sum of sensitivity (Se) and specificity (Sp). Se, Sp, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR), negative LR and accuracy were calculated according to standard methods.

Spleen size and stiffness

Spleen size was calculated in the form of a cross sectional spleen area in the ultrasound images by using the formula:

the cross section area (square cm) = longest diameter (in cm) × shortest diameter (in cm), and a diagnosis of splenomegaly was made if the area was 40 cm^2 or more [10–12]. Each group was investigated to determine whether there was a correlation between the spleen size values and spleen stiffness by calculating Spearman rank correlation coefficients, and when rs was greater than $r0.01$, a p-value of less than 0.01 was considered to indicated a significant difference.

Results

Comparison of stiffness in the subject groups

1. First, we measured liver stiffness by ARFI elastography. The median values were (m/s): IPH group 1.56 (0.98–2.37), LC group 2.44 (1.08–3.83), CH group 1.81 (1.03–2.36), NC group 1.13 (0.86–1.53) (Fig. 1). The results showed that liver stiffness in the IPH group was higher than in the NC group (IPH group vs. NC group, $p = 0.0022$), lower than in the LC group (IPH group vs. LC group, $p = 0.00077$), and about the same in the CH group (IPH group vs. CH group, $p = 0.79$).
2. Next, we measured the median values for spleen stiffness. The results were (m/s): IPH group 3.88 (2.69–4.79), LC group 3.18 (2.06–4.52), CH group 2.27 (1.89–2.77), and NC group 1.99 (1.10–2.55), and the median value was highest in the IPH group (IPH group vs. LC group, $p = 0.003$; IPH group vs. CH group, $p < 0.00001$).
3. We then calculated the spleen/liver stiffness ratios, and the median values were: IPH group 2.47 (1.71–3.34), LC group 1.3 (0.78–2.64), CH group 1.32 (0.86–2.15), and NC group 1.78 (0.72–2.30). The measurements in the CH group and the LC group were lower than the measurements in the NC group (CH group vs. NC group, $p = 0.016$; LC group vs. NC group, $p = 0.024$). However, in contrast to the above, the measurements in the IPH group were higher than the measurements in the NC group (IPH group vs. NC group, $p = 0.00063$). Thus, whereas the spleen/liver stiffness ratio decreased with the progression of the pathology from CH to LC, it increased instead in the IPH group (IPH group vs. CH group, $p = 0.000014$; IPH group vs. LC group, $p < 0.000036$). Based on the above findings, it was concluded that IPH is a disease in which the spleen becomes markedly stiffer than the liver.

Sensitivity and specificity obtained in the ROC curves

We plotted ROC curves for liver stiffness, spleen stiffness, and the spleen stiffness/liver stiffness ratio.

First, we plotted an ROC curve to differentiate between the IPH group and the LC group (Fig. 2.). When we set the spleen/liver stiffness ratio cut-off value at 1.53, AUROC was 0.920, sensitivity 1.000 (0.857–1.000, 95 % confidence interval), specificity 0.720 (0.623–0.720), PPV 0.708 (0.607–0.708), NPV 1.000 (0.865–1.000), and accuracy 0.833 (0.718–0.833), showing that the spleen/liver stiffness ratio was useful for differentiating between the IPH group and the LC group.

Next, we plotted an ROC curve in order to differentiate between the IPH group and the CH group. When we set the cut-off value for spleen stiffness at 2.69 m/s, AUROC was 0.997, sensitivity 1.000 (0.895–1.000), specificity 0.950

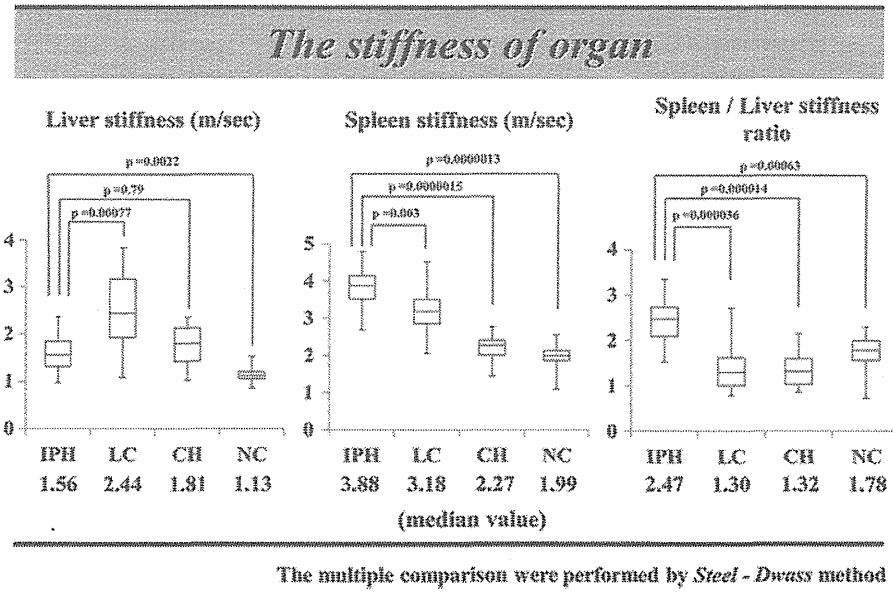
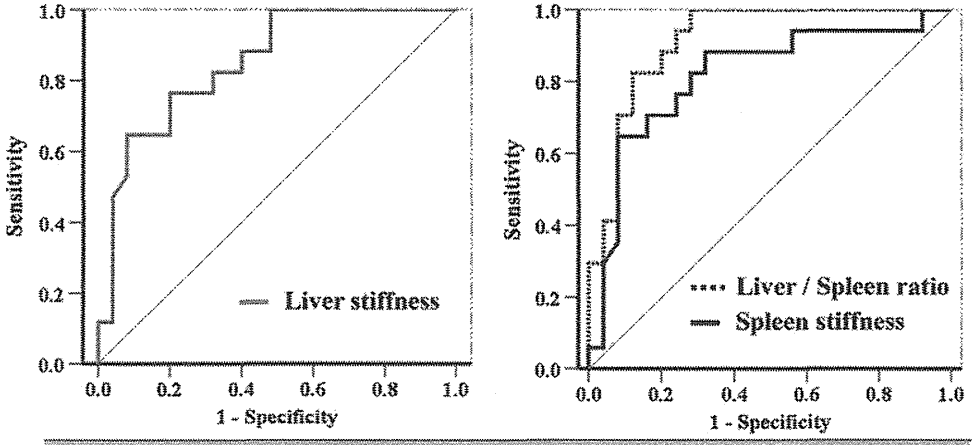


Fig. 1 The stiffness of liver and spleen and the ratio. *Box plots* of the liver stiffness values, spleen stiffness values, and spleen/liver stiffness ratios of the IPH group, the liver cirrhosis group, the hepatitis group, and the normal controls group. The *top* and the *bottom* of each box are the first quartile and third quartile, respectively, and thus the *height of the box* represents the interquartile range within which 50 % of the values were located. The *line through the middle of each box* represents the median value. The *bar* shows the minimum and maximum values (range)



	Liver stiffness	SPL stiffness	SPL / Liver ratio
AUROC	0.85	0.82	0.92
Cut off value	<1.65	>3.86	>1.53
Se	0.65 (0.48-0.73)	0.65 (0.48-0.73)	1.00 (0.86-1.00)
Sp	0.92 (0.81-0.98)	0.92 (0.81-0.98)	0.72 (0.62-0.72)
LR+	8.09 (2.52-30.0)	8.13 (2.52-30.0)	3.57 (2.27-3.57)
LR-	0.38 (0.28-0.64)	0.38 (0.28-0.64)	0.00 (0.00-0.23)
PPV	0.85 (0.63-0.95)	0.85 (0.63-0.95)	0.71 (0.61-0.71)
NPV	0.79 (0.70-0.84)	0.79 (0.70-0.84)	1.00 (0.87-1.00)
Accuracy	0.81 (0.68-0.88)	0.81 (0.68-0.88)	0.83 (0.72-0.83)

Fig. 2 The measurement of ARFI elastography for diagnosis of IPH compared with liver cirrhosis ROC curves for liver stiffness, spleen stiffness, and the spleen/liver stiffness ratio are represented between the IPH group and the liver cirrhosis (LC) group. The values of ARFI elastography in IPH group are shown as positive for calculation. The *values in parentheses* indicate the 95 % confidence intervals. *AUROC* area under the curve of ROC, *Se* sensitivity, *Sp* specificity, *PPV* positive predictive value, *NPV* negative predictive value, *LR+* likelihood ratio of a positive test, *LR−* likelihood ratio of a negative test

(0.850–0.950), PPV0.944 (0.833–0.944), NPV 1.000 (0.895–1.000), and accuracy 0.973 (0.865–0.973), and it was concluded that spleen stiffness measurements are important for differentiating between IPH and LC.

We then plotted an ROC curve in order to differentiate between the IPH group and the NC group. When we set the cut-off value for spleen stiffness at 2.69 m/s, AUROC was 1.000, sensitivity 1.000 (0.898–1.000), specificity 1.000 (0.913–1.000), PPV 1.000 (0.898–1.000), NPV 1.000 (0.913–1.000) and accuracy 1.000 (0.906–1.000), and it was concluded that spleen stiffness measurements are important.

We also plotted an ROC curve in regard to differentiation between the IPH group and the group with liver diseases (CH group and LC group combined). When we set the spleen/liver stiffness ratio cut-off value at 1.71, AUROC was 0.933, sensitivity 0.941 (0.768–0.989), specificity 0.800 (0.735–0.818), PPV 0.640 (0.522–0.673), NPV 0.973 (0.894–0.995) and accuracy 0.839 (0.744–0.865).

Based on the above findings, it was concluded that measuring both spleen stiffness and the spleen/liver stiffness ratio is critically important to specifically diagnosing IPH and making the differential diagnosis from other liver diseases, including CH and LC.

Moreover, we prospectively validated the usefulness of these cut-off values of ARFI elastography to another four new patients with similar characteristics being suspected as IPH or LC before definitive diagnosis. As a result, the spleen/liver stiffness ratios were 2.86, 2.07, 1.23 and 0.99. These results indicated two patients were IPH and another two patients were LC by the cut-off value (less than or more than 1.53) in this study (Fig. 2). After this study, the accuracy of this result was confirmed by liver tissue biopsy, and this cut-off value was demonstrated to be useful.

Correlation between spleen size and spleen stiffness

The spleen size was largest in the IPH group (102.5 cm^2, median value), and the IPH group was followed by the LC group (44.0 cm^2), the CH group (28.9 cm^2) and the NC group (22.4 cm^2). Significant differences were found in the multiple comparisons by the Steel–Dwass method (IPH group vs. LC group, $p = 0.0000037$; IPH group vs. CH group, $p = 0.0000013$; IPH group vs. NC group, $p = 0.0000013$), but no correlations were found between spleen size and spleen stiffness in each of the groups (rs = −0.081, 0.091, 0.300, 0.071, respectively, $p = 0.742$, 0.656, 0.190, 0.757) (Fig. 3.). Thus, it was concluded that spleen size was not the cause of the increase in spleen stiffness in IPH.

Discussion

Even in renowned hepatology centers, IPH patients are frequently misdiagnosed with LC, because their characteristics and findings of radiological examinations are very similar with those of LC patients. IPH is a disease that is characterized by splenomegaly and portal hypertension. Occlusion of peripheral portal veins in the liver and

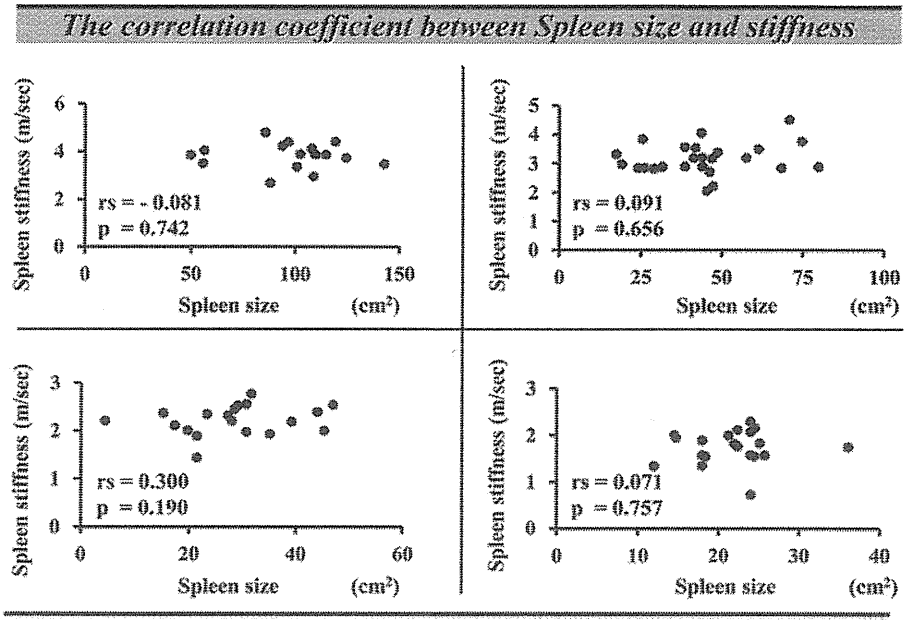


Fig. 3 The correlation coefficient between spleen size and stiffness. This figure shows the coefficients for the correlations (rs values) between spleen size (cross sectional spleen area) and spleen stiffness in each of the groups. Significant correlation was not found between spleen size and spleen stiffness in any of the groups. A p value of less than 0.05 was considered to indicate a statistically significant difference

increased splenic blood flow as a result of splenomegaly has been reported to be the causes that give rise to portal hypertension. However, these causes have never been completely explained. Since some cases are complicated by autoimmune diseases, some reports have pointed to immunologic involvement [13, 14]. The presence of fibrotic portal tracts and thin fibrous septa in the absence of cirrhosis are pathological criteria for making a diagnosis of IPH [15, 16]. Although the precise pathogenic mechanisms causing spleen enlargement are still poorly elucidated, it was clear that there were some architectural alterations, including pulp hyperplasia, congestion from increased blood flow and fibrosis in the spleen of portal hypertensive patients [17–19]. In the previous report, it was demonstrated that spleen stiffness increased with higher degrees of hepatic fibrosis and was unrelated to age, sex or body mass index [5].

Histologically, the spleen is usually divided into white pulp, red pulp, and a marginal zone. As the splenic artery repeatedly divides, it eventually branches into trabecular arteries and penicillar arteries, and they penetrate the white pulp. A periarterial lymphoid sheath (PALS) and lymph follicles (LFs) are present around them, and lymphocytes migrate from within the arteries and are responsible for immune functions. The terminal arterial branches end in an open manner in the splenic cords. After the blood, i.e., both the blood cells and the plasma, flows out into the reticular fibers of the splenic cords, it slips through the slits between the endothelial cells (rod shaped cells) that form the venous sinuses, and enters the sinus lumen. From the sinus lumen the blood passes through the veins of the pulp, which join to form the splenic vein, and it reaches the portal vein [20].

In 1924, the splenomegaly in IPH (Banti's syndrome) was found to be attributable to an increase in venous sinuses in the red pulp [21]. In addition, the lymph follicles and the reticular fibers and collagen fibers around the splenic cords grow thicker in a diffuse manner, and narrowing of cord width and narrowing of the sinus lumens is seen [22]. Moreover, characteristically, the rod shaped cells become deformed, fall into disarray, and diversify, and the slits between the rod shaped cells enlarge [23, 24]. As a result, the basal lamina of the sinuses also thickens [25]. Therefore, this splenic tissue hyperplasia characterized by fibrogenesis enlargement and passive spleen congestion are likely to cause the increase of spleen tissue stiffness. The increased spleen stiffness in IPH group in this study may be a manifestation of splenic fibrosis and congestion. In the livers of the IPH group, the degree of stiffness was about the same level as that in chronic hepatitis, suggesting that a little fibrosis occurred just in the parts of liver limited to the periphery of portal vein [1]. The previous study by transient elastography also reported that liver stiffness in IPH patients is significantly lower than that in LC patients [4]. Thus, IPH appeared to be a disease that exhibits a relatively soft liver and hard spleen, and the result was that the spleen/liver stiffness ratio increased.

On the other hand, the spleen is also known to undergo fibrosis in LC, primarily in the red pulp. The appearance of venous sinus hyperplasia is not so marked as in IPH, and the rod shaped cells in the venous sinuses do not diversify as much [23–25]. In this study, the IPH patients' characteristics (liver functions, esophageal varices and findings of radiological examinations) were similar with those of LC patients. But, on the ultrasonographic result, the size of

spleens in the LC group was not as large as those in the IPH group, and the spleen stiffness of ARFI elastography in the LC group was not as hard as in the IPH group. Therefore, there was the possibility that the degree of splenic congestion and fibrosis in the LC group were smaller than that in the IPH group. In contrast, the liver stiffness in the LC group was much more marked than in the IPH group, and the spleen/liver stiffness ratio was lower. This is the difference between IPH and LC.

No reports of previous pathological studies of the spleen have elucidated the difference between the severity of the fibrosis and congestion in IPH and LC disease. Sato et al. [26] investigated differences in the numbers of Gamna-Gandy nodules, which develop in the lymphocyte sheaths around arteries and the splenic trabeculae, in IPH and LC. Gamna-Gandy nodules are lesions that are often seen in the spleen in IPH and LC, and they are formed as a result of bleeding, fibrosis, and calcareous deposits after an increase in red pulp pressure due to venous congestion [27]. The nodules also seem to be involved in increases in spleen stiffness, but no pathological differences between IPH and LC have ever been reported. In that regard, the ARFI elastography used in the present study made it possible to express spleen stiffness in vivo in the form of numerical values, and it is now possible to elucidate pathology that had been impossible to elucidate by pathological studies outside the body.

There were some reports in which spleen stiffness was studied by ultrasonographic elastography [28–34]. Elastographic methods are divided into two major categories, which are named strain elastography and are named shear wave elastography. The former method (e.g. real-time tissue elastography) is calculated by the strain, which is induced by passive power of the compression or the cardiac beat. The latter method (e.g. Transient elastography or ARFI elastography) uses shear wave. Transient elastography is done by using a vibrator probe which transmits low frequency vibrations to the tissue, inducing an elastic shear wave, and the stiffness is showed as Young's modulus. On the other hand, in ARFI elastography, these shear waves are induced by acoustic push pulse from the probe, and the speed of shear wave is calculated by detection pulse. As the result we can know the stiffness of the tissue as the spread speed of shear wave.

The weakness point in real-time tissue elastography is that the elasticity cannot be expressed as the absolute value, and the elastic ratio among two regions of interest can be only calculated. Namely, the acquired value (=ratio) is lacking objectivity. Meanwhile, the weakness point in transient elastography is that the system does not have B-mode, and this measurement should be performed by A-mode or M-mode, which demand higher skill to examiners. Therefore, we used ARFI elastography in this study in order to overcome these problems.

Stefanescu et al. reported increased spleen stiffness in a group of LC patients who had EVs, and that measuring spleen stiffness is useful for differentiating between the presence and absence of EVs [30–34]. There is a strong possibility that increased spleen stiffness is in some way related to whether EVs develop. We investigated the relationship between spleen size and spleen stiffness in IPH group, LC group, CH group, and NC group, but no correlations were found between them. The mechanism of the development of splenomegaly in LC is poorly elucidated, and it is thought to be caused by an increase in splenic blood flow associated with portal hypertension, angiogenesis enlargement, and hyperactivation of the splenic lymphoid compartment [35, 36]. However, in the present study, there is no correlation between splenomegaly and spleen stiffness. Therefore, the cause of the increase in spleen stiffness in IPH and LC cannot be explained by just splenic congestion due to portal hypertension. In other words, there is a strong possibility that portal hypertension due to narrowing of portal vein branches in the liver is not the only cause of IPH, and specific increase of spleen stiffness is the material primary factor. The previous report suggested that the primary increase in splenic blood flow causes a secondary increase in portal vein pressure [37]. Whether the liver or the spleen is the cause of IPH has been debated a great deal, and the results of our study may provide a clue to resolving the issue. But, for acquiring more accurate diagnostic performance, we need to plan a multicenter clinical study and enroll a large number of consecutive patients with similar characteristics, suspected LC or IPH, before performing liver biopsy, since the population of our pilot study is relatively small.

Up until now, even when we discovered cases in which we suspected IPH, liver tissue biopsy was indispensable to differentiating IPH from other liver diseases. However, in the present study we demonstrated that it is possible to specifically and noninvasively diagnose IPH by measuring spleen stiffness and the spleen/liver stiffness ratio by performing ARFI elastography. We hope that this will be helpful in making the diagnosis of IPH in the future.

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Conflict of interest None of the authors has any conflicts of interest.

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Increased oxidative stress may lead to impaired adaptive cytoprotection in the gastric mucosa of portal hypertensive rat

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Key words
adaptive cytoprotection, anti-oxidants, nitric oxide, oxidative stress, portal hypertension.

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Abstract

Background and Aims: In the gastric mucosa of portal hypertensive rats, adaptive cytoprotection against ethanol-induced damage is impaired. The aim of this study was to determine relation between impaired adaptive cytoprotection and oxidative stress.

Methods: Portal hypertension was produced in male Sprague-Dawley rats by inducing staged portal vein occlusion. Oxidative stress levels were evaluated by measuring malondialdehyde and nitrotyrosine levels in the rat gastric mucosa with or without 10% ethanol pretreatment. Inhibition of oxidative stress by an anti-oxidant agent was estimated, and glutathione levels were also measured. Adaptive cytoprotection to 70% ethanol treatment was evaluated by measuring the gastric mucosal injury index in the presence or absence of the anti-oxidant.

Results: The portal hypertensive gastric mucosa pretreated with 10% ethanol had significantly higher oxidative stress levels than the mucosa not pretreated with 10% ethanol. However, the sham-operated gastric mucosa pretreated with 10% ethanol had significantly lower oxidative stress levels than the mucosa not pretreated with 10% ethanol. Pretreatment with 10% ethanol increased glutathione levels in the sham-operated but not in the portal hypertensive gastric mucosa. Administration of the anti-oxidant agent prior to 10% ethanol pretreatment significantly reduced oxidative stress levels, increased glutathione levels, and decreased the injury index in response to 70% ethanol in the portal hypertensive gastric mucosa.

Conclusion: Increased oxidative stress may lead to impaired adaptive cytoprotection in the gastric mucosa of portal hypertensive rats, probably through damage to the system of endogenous anti-oxidant production.

Introduction

Portal hypertensive (PHT) gastropathy is frequently observed in patients with portal hypertension.[1,2] Previous studies have revealed morphological and functional abnormalities in PHT gastric mucosa. These abnormalities occur because of an increase in the susceptibility of the gastric mucosa to damage by various noxious factors.[3,4] Impairment in the mucosal defense mechanism is thought to be the main cause of this increased susceptibility to mucosal damage. In addition, the mucosal hypoxia, which is produced by gastric mucosal microvessels and the shunting of submucosal blood flow, has been implicated in the increased susceptibility of the gastric mucosa to damage.[3,5] Nitric oxide (NO) plays an important role in gastric mucosal circulation. In the PHT gastric mucosa, excess of NO is produced, which then reacts with superoxide (O_2^-) to produce peroxinitrite ($ONOO^-$). Subsequently, lipid peroxidation is mediated by superoxide.[6,7] In the PHT gastric mucosa, oxidative stress induced by lipid peroxidation and peroxynitrite is increased. Malondialdehyde (MDA) is a lipid peroxidation product, and nitrotyrosine is one of the proteins formed by peroxynitrite. These substances are widely used as markers of oxidative and nitrosative stress in many situations.[8,9]

Adaptive cytoprotection of the gastric mucosa is an autodefense mechanism in which a prior mild irritant prevents gastric mucosal damage by irritants such as alcohol and aspirin.[10] Recent reports have shown that the mechanism of adaptive cytoprotection in the gastric mucosa may be related to natural anti-oxidants such as glutathione and superoxide dismutase (SOD).[11,12] We previously showed that adaptive cytoprotection is impaired in the PHT gastric mucosa and that this impairment may be caused by increased NO production.[13] Recently, we found that excessive NO production mediated by overexpression of heat shock protein 90 may cause impairment of adaptive cytoprotection in the PHT gastric mucosa.[14] Therefore, we have suggested that the phenomenon

might be related to oxidative stress.[14] In the present study, we measured oxidative stress and glutathione levels in the PHT gastric mucosa after mild exposure to ethanol in order to investigate the relationship between adaptive cytoprotection and oxidative stress. In addition, the effects of an anti-oxidant on adaptive cytoprotection were evaluated.

Methods

Animals. Ninety-six male Sprague-Dawley rats (age, 6 weeks; weight 200–250 g; Kyudo, Fukuoka, Japan) were used. The rats were fed a standard laboratory diet and water, and kept in a room under a 12-h light : dark cycle. Portal hypertension was produced by inducing staged portal vein occlusion, as described previously.[15] Operation was performed under 4% sevoflurane (Maruishi Pharmaceutical Co., Ltd, Osaka, Japan). The portal vein was isolated, and stenosis was created by a single ligature of 4-0 silk placed around the combined portal vein and a 21-G blunt-tipped needle. The needle was removed from the ligature. In addition, 2-0 silk was placed as a loose ligation, and both ends were passed through the abdominal wall. Three days after surgery, both ends of ligature were pulled simultaneously. Sham-operated (SO) controls underwent similar procedures without portal vein occlusion. The experiments were performed 2 weeks after the initial surgery. Before the experiment, the animals were placed in individual cages with wide wire mesh floors for 24 h. During this period, the animals were not given any food but were allowed free access to tap water, which was removed 1 h prior to the experiment. This study was approved by the Animal Committee of Oita University and conformed to the Guidelines for Animal Experimentation of Oita University.

Assays. The levels of tissue MDA, nitrotyrosine, and glutathione were measured. Frozen tissue samples of the gastric mucosa were homogenized using a tissue homogenizer (Dremel, Racine, WI, USA) and were then centrifuged at $10\,000 \times g$ for 10 min at 4°C. Protein content was determined using a Quick Start Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) and a Quick Start bovine serum albumin standard set (Bio-Rad Laboratories). MDA levels were measured using a commercial kit containing the chromogenic reagent, N-methyl-2-phenlindole (Percipio Bioscience, Inc., Burlingame, CA, USA); the levels were measured based on the reaction of the chromogenic reagent with MDA. Nitrotyrosine levels were measured using a commercial kit for 3-nitrotyrosine (Northwest Life Science Specialities LLC, Vancouver, WA, USA). Glutathione levels were measured using a commercial kit for glutathione (Northwest Life Science Specialities LLC). Absorbance was detected using an enzyme-linked immunosorbent assay reader (Bio-Rad Laboratories) at 586 nm for MDA, 450 nm for nitrotyrosine, and 405 nm for glutathione.

Adaptive cytoprotection. Twelve PHT and 12 SO rats were administered 10% ethanol or distilled water (10 mL/kg) by oral gavage. One hour later, the rats were sacrificed. The stomach was removed rapidly and opened along the greater curvature. The gastric mucosa was washed in phosphate-buffered saline, as described previously.[13] In addition, six other PHT and SO rats were administered in an anti-oxidant agent, dihydrolipoyl histidinate zinc complex (DHLHZn, 10 mg/kg), which is a derivative of alpha-lipoic acid, as reported previously,[16] by oral gavage in order to evaluate the contribution of oxidative stress to adaptive cytoprotection of the gastric mucosa. One hour later, 10% ethanol was administered to these rats, and the gastric mucosa was removed as described earlier. The levels of MDA, nitrotyrosine, and glutathione were compared between the groups.

Injury index. Rats were administered 70% ethanol (10 mL/kg) 3 h after 10% ethanol or distilled water (10 mL/kg). A gastric mucosal injury study was conducted, as we previously described.[13,14] One hour after the exposure to 70% ethanol, rats were killed, and the gastric mucosal surface was examined visually and photographed using a digital camera (FinePix S7000, Fujifilm Corporation, Tokyo, Japan). Macroscopic measurements were performed using Scion Image software (National Institutes of Health, Bethesda, MD, USA). The total areas of gross hemorrhagic and necrotic lesions were measured, and the injury index was expressed as the percentage of the injured area to the total glandular mucosal area.

Statistical analysis. All data are expressed as mean ± standard deviation. All data were evaluated using Mann–Whitney *U*-test or Kruskal–Wallis test with multiple comparisons. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS II (SPSS, Inc., Chicago, IL, USA).

Results

MDA and nitrotyrosine levels in the gastric mucosa. MDA levels in the PHT gastric mucosa were significantly higher than those in the SO controls ($P < 0.01$, Fig. 1a). Nitrotyrosine levels in the PHT gastric mucosa were also significantly higher than those in the SO gastric mucosa ($P < 0.05$, Fig. 1b).

Changes in the levels of MDA, nitrotyrosine andglutathione after pretreatment with 10% ethanol. MDA levels in the SO gastric mucosa pretreated with 10% ethanol were significantly lower than those not pretreated with 10% ethanol ($P < 0.01$, Fig. 2a). For the two groups pretreated with 10% ethanol, no significant differences were observed between those with and without prior DHLHZn administration. MDA levels in the PHT gastric mucosa pretreated with 10% ethanol were significantly higher than those in the mucosa not pretreated with 10% ethanol ($P < 0.05$, Fig. 2b). However, the prior administration of DHLHZn significantly decreased the MDA levels in the PHT gastric mucosa pretreated with 10% ethanol ($P < 0.01$, Fig. 2b).

Nitrotyrosine levels in the SO gastric mucosa pretreated with 10% ethanol were significantly lower than those in the mucosa not pretreated with 10% ethanol ($P < 0.05$, Fig. 3a). For the two groups pretreated with 10% ethanol, no significant difference was

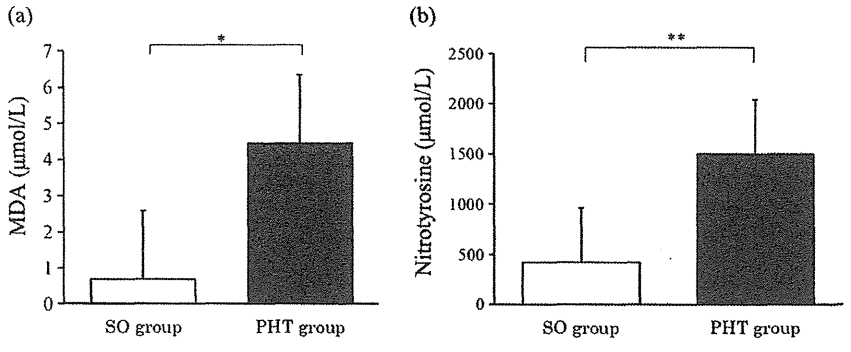

Figure 1 Malondialdehyde (MDA) and nitrotyrosine levels in gastric mucosa of sham-operated (SO) and portal hypertensive (PHT) rats. (a) MDA levels in the PHT group were significantly higher than those in the SO group. (b) Nitrotyrosine levels in the PHT group were significantly higher than those in the SO group. $*P < 0.01$, $**P < 0.05$. Each group consisted of 12 animals.

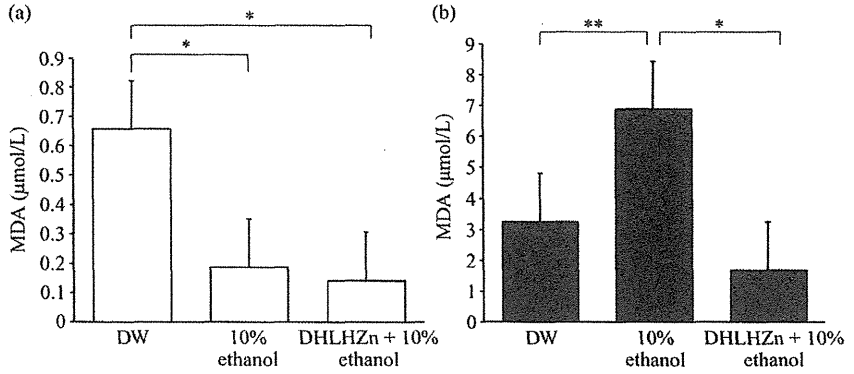

Figure 2 Malondialdehyde (MDA) levels in the gastric mucosa of sham-operated (SO) and portal hypertensive (PHT) rats pretreated with 10% ethanol and with prior administration of the dihydrolipoyl histidinate zinc complex (DHLHZn). (a) MDA levels in the SO group not pretreated with 10% ethanol were significantly higher than those in the SO group pretreated with 10% ethanol and those in the SO group pretreated with 10% ethanol after DHLHZn administration. (b) MDA levels in the PHT group pretreated with 10% ethanol were significantly higher than those in the PHT group not pretreated with 10% ethanol and those in the PHT group pretreated with 10% ethanol after DHLHZn administration. $*P < 0.01$, $**P < 0.05$. Each group consisted of 6 animals. DHLHZn, dihydrolipoyl histidinate zinc complex; DW, distilled water. (□) SO group; (■) PHT group.

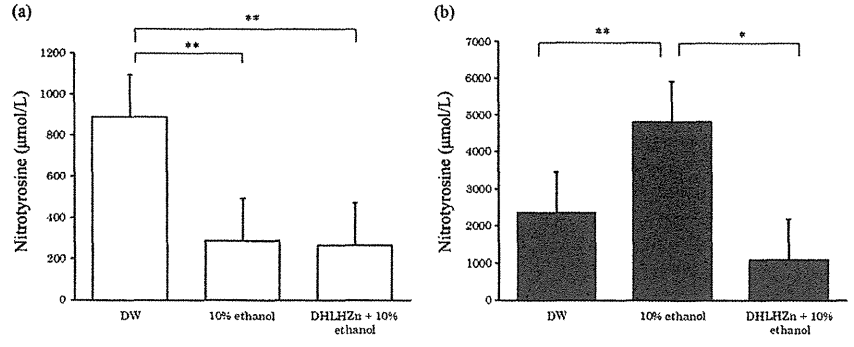

Figure 3 Nitrotyrosine levels in the gastric mucosa of sham-operated (SO) and portal hypertensive (PHT) rats pretreated with 10% ethanol and with prior dihydrolipoyl histidinate zinc complex (DHLHZn) administration. (a) Nitrotyrosine levels in the SO group not pretreated with 10% ethanol were significantly higher than those in the SO group pretreated with 10% ethanol and those in the SO group pretreated with 10% ethanol after DHLHZn administration. (b) Nitrotyrosine levels in the PHT group pretreated with 10% ethanol was significantly higher than those in the PHT group not pretreated with 10% ethanol and those in the PHT group pretreated with 10% ethanol after DHLHZn administration. $*P < 0.01$, $**P < 0.05$. Each group consisted of six animals. DW, distilled water. (□) SO group; (■) PHT group.

observed between those with prior DHLHZn administration and those without prior DHLHZn administration. Nitrotyrosine levels in the PHT gastric mucosa pretreated with 10% ethanol were significantly higher than those without pretreatment of 10% ethanol ($P < 0.05$, Fig. 3b). However, the prior administration of DHLHZn significantly decreased the nitrotyrosine levels in the PHT gastric mucosa pretreated with 10% ethanol ($P < 0.01$, Fig. 3b).

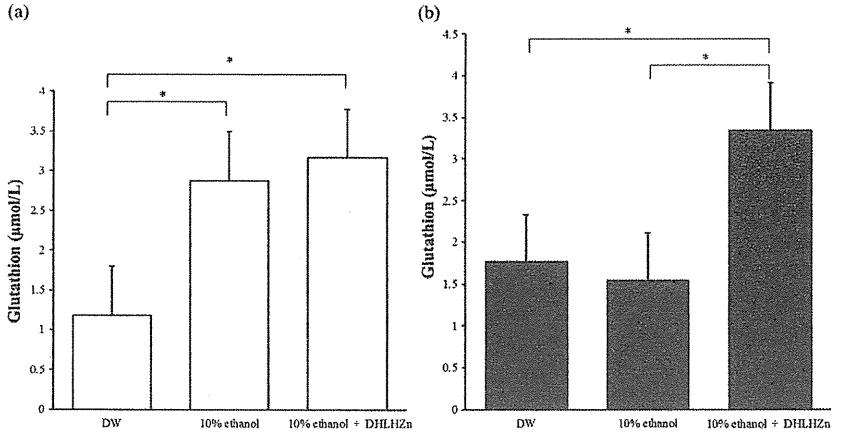

Figure 4 Glutathione levels in the gastric mucosa of sham-operated (SO) and portal hypertensive (PHT) rats pretreated with 10% ethanol and with prior dihydrolipoyl histidinate zinc complex (DHLHZn) administration. (a) Glutathione levels in the SO group not pretreated with 10% ethanol were significantly lower than those in the SO group pretreated with 10% ethanol and those in the SO group pretreated with 10% ethanol after DHLHZn administration. (b) Glutathione levels in the PHT group pretreated with 10% ethanol after DHLHZn administration were significantly higher than those in the PHT group pretreated with 10% ethanol and those in PHT group not pretreated with 10% ethanol. $*P < 0.01$, $**P < 0.05$. Each group consisted of six animals. DW, distilled water. (□) SO group; (■) PHT group.

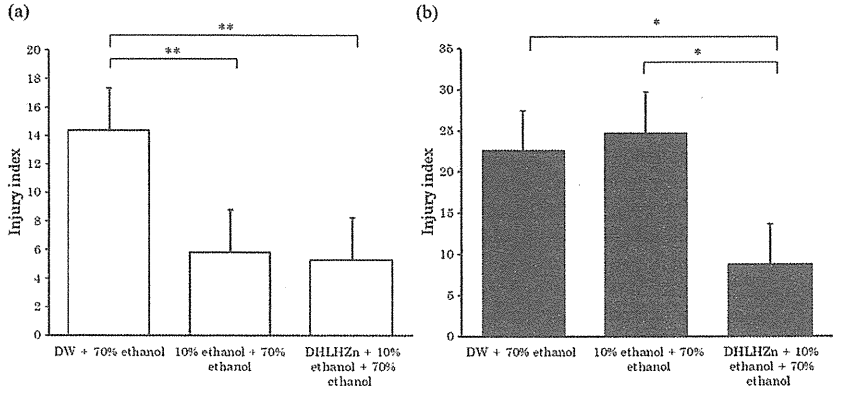

Figure 5 Injury index in the gastric mucosa of sham-operated (SO) and portal hypertensive (PHT) rats after treatment with 70% ethanol. (a) The injury index in the SO group not pretreated with 10% ethanol was significantly higher than those in the SO group pretreated with 10% ethanol and those in the SO group pretreated with 10% ethanol after dihydrolipoyl histidinate zinc complex (DHLHZn) administration. (b) The injury index in the PHT group pretreated with 10% ethanol after DHLHZn administration was significantly lower than those in the PHT group pretreated with 10% ethanol and those in the PHT group not pretreated with 10% ethanol. $*P < 0.01$, $**P < 0.05$. Each group consisted of six animals. DW, distilled water. (□) SO group; (■) PHT group.

Glutathione levels in the SO gastric mucosa pretreated with 10% ethanol were significantly higher than those in the mucosa not pretreated with 10% ethanol ($P < 0.01$, Fig. 4a). For the two groups pretreated with 10% ethanol, no significant difference was observed between those with prior DHLHZn administration compared with those without prior DHLHZn administration. In the PHT gastric mucosa, no significant difference was observed between the two groups pretreated or not pretreated with 10% ethanol (Fig. 4b). However, the prior administration of DHLHZn significantly increased the glutathione levels in the PHT gastric mucosa pretreated with 10% ethanol ($P < 0.01$, Fig. 4b).

Injury index and effects of inhibition of oxidative stress on adaptive cytoprotection. The injury index in the SO group not pretreated with 10% ethanol was significantly higher than that in the group pretreated with 10% ethanol and in the group pretreated with 10% ethanol after the prior DHLHZn administration ($P < 0.05$ for both, Fig. 5a). The injury index in the PHT group pretreated with 10% ethanol after DHLHZn administration was significantly lower than that in the group pretreated with 10% ethanol and in the group not pretreated with 10% ethanol ($P < 0.01$ for both, Fig. 5b).

Discussion

Several studies have described the pathogenesis of PHT gastropathy. Ohta *et al.* suggested that increased portal pressure may cause congestion of the upper stomach resulting in tissue damage and that these changes can activate the tumor necrosis factor alpha (TNF-α).[6] TNF-α activation results in the overexpression of endothelial NO synthase, which then produces an excess of NO. Overproduction of NO produces peroxinitrite by reacting with superoxide, and lipid peroxidation is produced by superoxide.[6,7] Further evidence of the pathogenesis involved in the PHT gastric mucosa was reported by Kawanaka *et al.*[17] Mitogen-activated protein (MAP) kinase activation in the PHT gastric mucosa is impaired by oxidative stress. This impairment is introduced by MAP kinase phosphatase-1 (MKP-1) overexpression and is improved by the inhibition of MKP-1.[7] In this study, MDA and nitrotyrosine levels in the PHT gastric mucosa were increased compared with those in the SO controls. Therefore, our data were comparable with their results.

The phenomenon by which mild irritants prevent gastric necrosis caused by various noxious factors is called adaptive cytoprotection.[11] Initially, this mechanism was thought to be mediated by endogenous prostaglandins.[11,18] However, another study reported that adaptive cytoprotection also occurs when prostaglandin generation is depressed.[19] Afterwards, it has been shown that epidermal growth factor and transforming growth factor α (TGF-α) prevent gastric mucosal damage induced by noxious factors[20,21] and that TGF-α is associated with adaptation of the gastric mucosa to the damaging effects of aspirin in vivo.[22] Recently, several studies have suggested that endogenous anti-oxidants are associated with adaptive cytoprotection.[11,12,23] SOD activity is increased in the gastric mucosa of rat after pretreatment of mild irritants.[11] In addition, glutathione levels increased in the H9c2cells after pretreatment with a lower H_2O_2 concentration.[23] Exposure of bovine aortic endothelial and smooth muscle cells to NO induces a prolonged increase in glutathione level beginning at 8–9 h.[24] Therefore, an overexpression of endogenous anti-oxidants such as glutathione because of exposure to mild irritants may be a mechanism underlying adaptive cytoprotection.

The levels of endogenous anti-oxidants, such as glutathione and SOD, have been reported to be decreased in the PHT gastric mucosa or in liver tissue.[25,26] Administration of vitamin E, which is a radical scavenger, could increase the levels of anti-oxidant defense enzymes, such as SOD and glutathione peroxidase.[26] In the present study, oxidative stress levels in the SO gastric mucosa pretreated with 10% ethanol were significantly decreased compared with those in the mucosa not pretreated with 10% ethanol, while glutathione levels in the SO gastric mucosa pretreated with 10% ethanol was significantly increased compared with those in the mucosa not pretreated with 10% ethanol. Pretreatment with mild irritants may lead to the production of glutathione and the attenuation of oxidative stress as a result. Oxidative stress levels in the PHT gastric mucosa pretreated with 10% ethanol were significantly increased compared with those in the mucosa not pretreated with 10% ethanol. The intrinsic increase in oxidative stress in the PHT gastric mucosa may impair the mechanisms of adaptive cytoprotection probably via production of endogenous anti-oxidants. The prior administration of an anti-oxidant would reverse the condition of the mucosa and retain adaptive cytoprotection.

Oxidative stress induced by lipid peroxidation and peroxynitrite can cause susceptibility to ethanol injury and impairment of the healing process in the PHT gastric mucosa. Therefore, anti-oxidant drugs may be effective against PHT gastropathy. Treatment with rebamipide, which is a gastroprotective drug that is effective as a radical scavenger, was suggested to contribute to the reduction of oxidative stress.[7] Other studies have reported that administration of vitamin E and glutamine reduced oxidative stress in the PHT gastric mucosa.[17,27] In addition, zinc and zinc complex have also been reported to decrease gastric mucosal damage because of their anti-oxidant effects.[28–30] Furthermore, the zinc complex stimulates cell proliferation and attenuates the cytokine-mediated oxidative damage to the gastric mucosa.[31] In the present study, we used DHLHZn as an anti-oxidant agent. DHLHZn, which is a derivative of alpha lipoic acid, is composed of dihydrolipoyl histidinate and zinc.[16] It has strong anti-oxidant activity and can attenuate oxidative stress.[16] Therefore, DHLHZn may also be effective against PHT gastropathy.

In conclusion, the present study demonstrated that increased oxidative stress is associated with impaired adaptive cytoprotection in the gastric mucosa of PHT rats. The impaired adaptive cytoprotection is probably related to damage to the mechanisms of endogenous anti-oxidant production. Anti-oxidative can reverse the condition of the PHT gastric mucosa.

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Brief Communication

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Use of per Rectal Portal Scintigraphy to Detect Portal Hypertension in Sinusoidal Obstructive Syndrome following Unrelated Cord Blood Transplantation

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Hepatic sinusoidal obstructive syndrome (SOS) is an important complication of allogeneic hematopoietic cell transplantation (HCT). SOS is a syndrome characterized by ascites, fluid retention, weight gain, painful hepatomegaly and jaundice. The diagnosis of this syndrome is usually based on clinical symptoms, according to the Seattle or Baltimore criteria. The reported incidence of SOS is 0–62.3% [1]. Risk factors for SOS identified in previous studies include older age, prior liver disease, elevated aspartate aminotransferase and serum ferritin levels before conditioning, the intensity of the conditioning regimen, HLA-mismatched or unrelated HCT [2, 3] and concomitant use of drugs such as acyclovir and amphotericin. Histological examination of the liver is required for definitive diagnosis; however, a percutaneous biopsy is often difficult due to large amounts of ascites, very low peripheral platelet counts and/or the presence of disordered coagulation. Although transjugular liver biopsy has been recommended in the past, biopsy specimens obtained by transjugular liver biopsy are sometimes too small to allow adequate histological analysis.

Elevation of the serum bilirubin level is a sensitive marker for SOS, but not specific, because hyperbilirubinemia often appears after HCT, due to various conditions such as graft versus host disease (GVHD), engraftment syndrome, infection and drug-related toxicity. Use of just two of the Seattle criteria to diagnose SOS is insufficient, with a diagnostic accuracy of only 42% [4].

Measurement of the hepatic venous pressure gradient as an index of portal pressure has been reported to discriminate between SOS and other liver complications in HCT recipients [5]. Per rectal portal scintigraphy is a unique, noninvasive method for evaluation of portal pressure. However, there have been no reports of the utility of this test in the diagnosis of SOS.

In April 2010, during his first complete remission following induction chemotherapy, a 55-year-old male with acute myeloid leukemia received a cord blood transplant, mismatched at two HLA loci. The conditioning regimen comprised fludarabine (180 mg/m^2), busulfan (12.8 mg/kg) and total body irradiation (4 Gy). Tacrolimus and short-term methotrexate were used for GVHD prophylaxis and ursodeoxycholic acid was used for SOS prophylaxis. Risk factors that raised concerns about SOS included his HBV carrier status, an elevated aspartate aminotransferase and a raised serum ferritin prior to conditioning.

On day 12, he developed hyperbilirubinemia (over 2 mg/dl) and tenderness in the right upper quadrant, fulfilling two Seattle criteria. Despite treatment that included management of water and sodium balance, his body weight continued to increase, attaining a 4% gain from baseline on day 17. On day 19, acute cutaneous GVHD

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