

Results

Liver injury

The liver histopathology appeared to be almost normal in the sham-operated group (Fig. 1a). Warm ischemia of the liver and reperfusion (hepatic warm I/R) caused inflammatory cell infiltration, congestion, and vacuolization with condensed nucleus at R6 h (Fig. 1b), whereas these changes were attenuated in the NaHS-treated mice (Fig. 1c). The injury score was augmented by hepatic warm I/R at R6 h in the NaHS(-) group, whereas it was significantly reduced by NaHS treatment (Fig. 1d). Plasma alanine aminotransferase (ALT) activity at R6 h was augmented in the hepatic

warm I/R in NaHS(-) group, whereas it was significantly reduced by NaHS treatment (Fig. 1e).

Plasma cytokines and chemokines

Plasma levels of TNF- α (Fig. 2a), IL-6 (Fig. 2b), IL-1 β (Fig. 2c), IFN- γ (Fig. 2d), IL-23 (Fig. 2e), IL-17F (Fig. 2f) and CD40L (Fig. 2g) were significantly higher in the NaHS(-) group 3 h after reperfusion (R3 h) than the respective value in the sham group, whereas the augmentation was significantly suppressed in the NaHS(+) group. By 6 h after reperfusion (R6 h), these molecules, except for CD40L, had decreased in the NaHS(-) group, and were even lower in the NaHS(+) group. Inter-group comparison

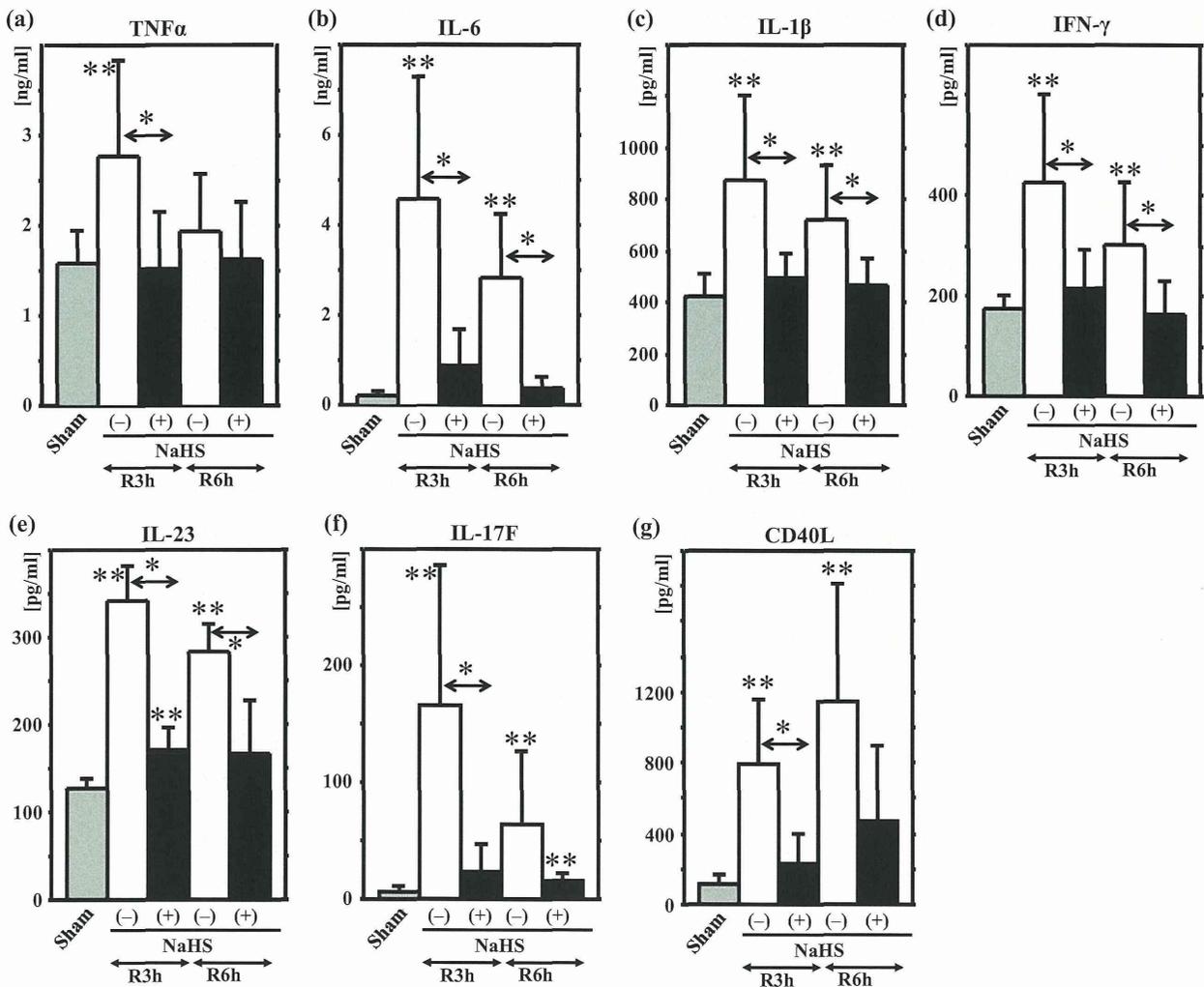


Fig. 2 Sodium hydrogen sulfide (NaHS) inhibits the expression of inflammatory cytokines and chemokines. Mice were subjected to partial warm ischemia for 75 min and subsequent reperfusion (I/R). The plasma concentrations of inflammatory cytokines and chemokines at

3 and 6 h after reperfusion were measured by an ELISA-based assay. **a** TNF- α , **b** IL-6, **c** IL-1 β , **d** IFN- γ , **e** IL-23, **f** IL-17F, and **g** soluble CD40 ligand. Results are expressed as the mean \pm SD. * P < 0.05, NaHS (-) vs. NaHS (+). ** P < 0.05 vs. Sham

revealed significant decreases in IL-6, IL-1 β , IFN- γ , and IL-23, although the decreases in TNF- α and IL17F were not significant. It noteworthy that CD40L continued to rise from R3 h to R6 h in both groups, but that NaHS treatment tended to decrease its value ($P = 0.065$).

Pro-survival signals

Pro-survival signals at R6 h were evaluated by western blots of phosphorylated PDK-1 (p-PDK1-Ser²⁴¹), Akt

(p-Akt-Ser⁴⁷³), mTOR (p-mTOR-Ser²⁴⁴⁸), and p70S6k (p-p70S6 K-Ser³⁷¹). Phosphorylated PDK-1 was significantly attenuated by hepatic warm I/R, whereas the reduction was significantly less pronounced in the NaHS treatment groups (Fig. 3a). Phosphorylated Akt tended to decrease only in the NaHS(-) group, whereas in the NaHS(+) group, it was significantly higher than in the other groups (Fig. 3b). Phosphorylated mTOR and phosphorylated p70S6k were almost unchanged by hepatic warm I/R in the NaHS(-) group, whereas they were

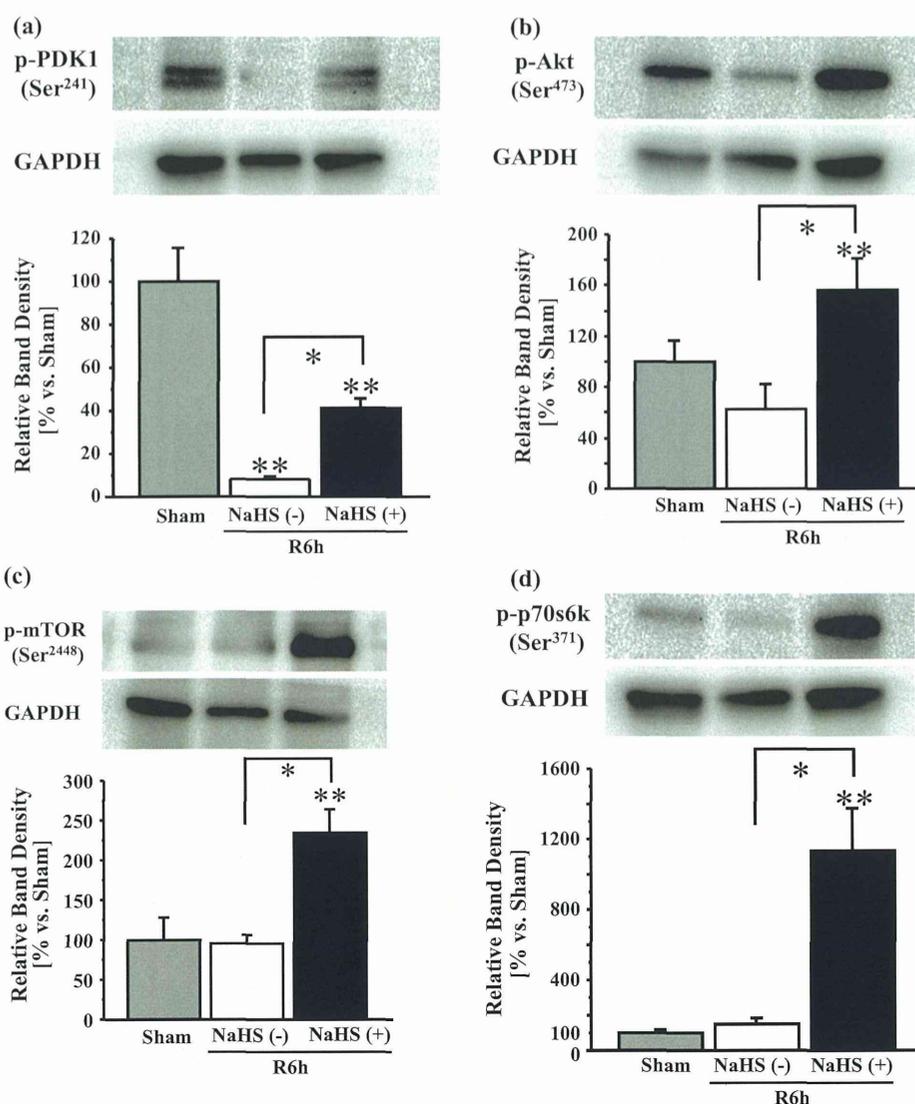


Fig. 3 Sodium hydrogen sulfide (NaHS) activates survival signals. Mice were subjected to partial warm ischemia for 75 min and subsequent reperfusion (I/R) for 6 h. Cytosolic protein in the ischemic lobe was applied to the western blot (*top*), and the relative intensity (*bottom*) is shown. *Panels a–d* show the results for **a** phosphorylated PDK-1 (Ser²⁴¹), **b** phosphorylated Akt (Ser⁴⁷³), **c** phosphorylated

mTOR (Ser²⁴⁴⁸), and **d** phosphorylated p70s6k (Ser³⁷¹). Relative quantitation of each sample was performed, using GAPDH as an internal control. Each normalized value was further normalized by the mean value in the sham-operated group, and expressed as the mean \pm SD. * $P < 0.05$, NaHS (-) vs. NaHS (+). ** $P < 0.05$ vs. Sham

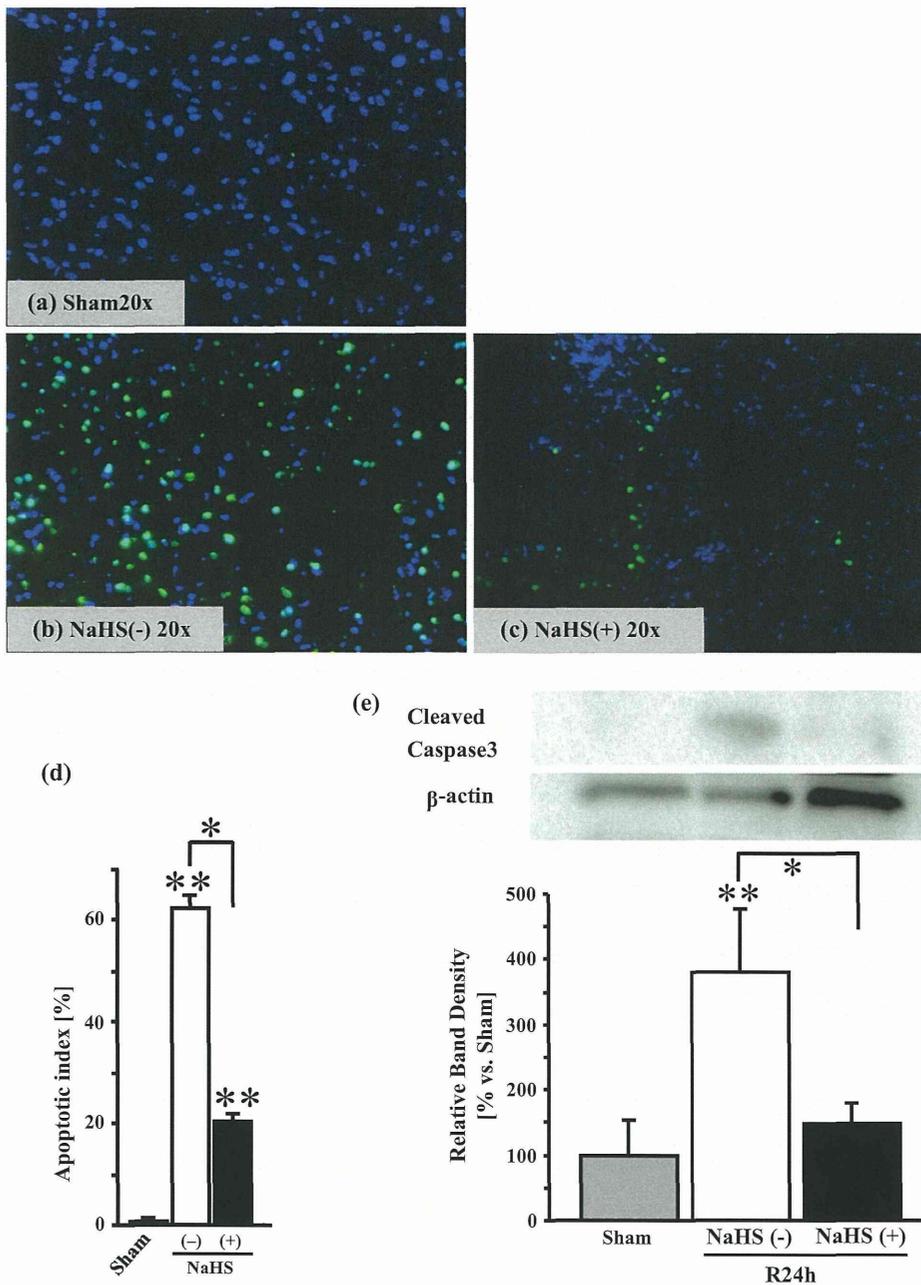
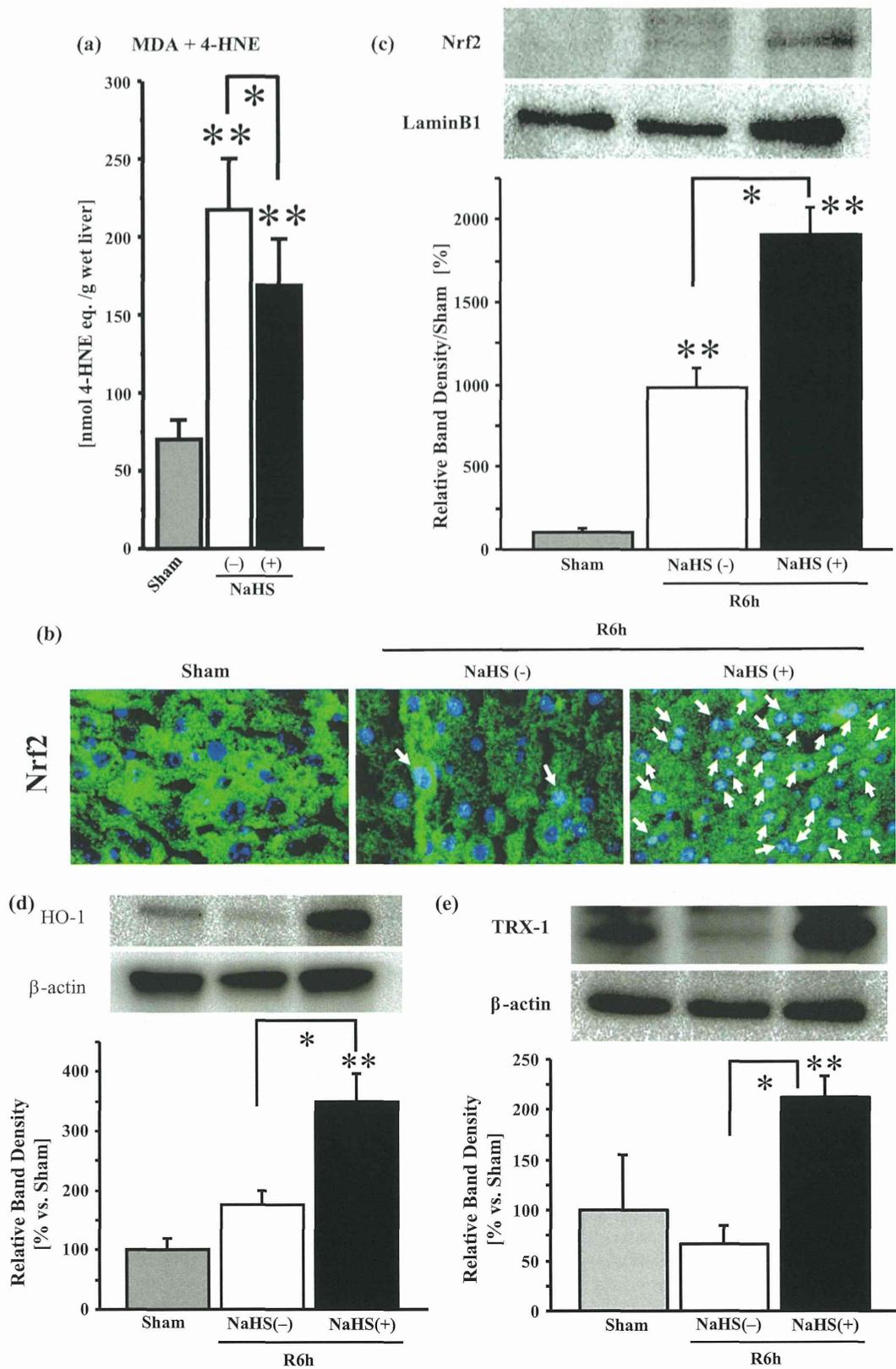


Fig. 4 Sodium hydrogen sulfide (NaHS) attenuates apoptosis of hepatocytes. Mice were subjected to partial warm ischemia for 75 min and subsequent reperfusion (I/R) for 24 h. Liver sections were stained by the fluorescent TUNEL method and nuclear counterstaining with DAPI. Representative photographs (20 \times magnification) are shown. **a** Sham operation. **b** NaHS (-): I/R with vehicle treatment. **c** NaHS (+): I/R with NaHS (1.0 mg/kg) administration before reperfusion. **d** The apoptotic index was calculated as the number of TUNEL-

positive cells divided by the number of DAPI-positive cells. Data are expressed as the mean \pm SD. **e** Cytosolic protein of the ischemic lobe at R6 h was applied to a standard western blot. Representative western blots detected by cleaved caspase-3 antibody (*top*) and the ratio of the relative intensity, cleaved caspase-3/ β -actin (*bottom*), are shown. Each normalized value was further normalized by the mean value in the sham group, and expressed as the mean \pm SD. * P < 0.05, NaHS (-) vs. NaHS (+). ** P < 0.05 vs. Sham



◀ **Fig. 5** Sodium hydrogen sulfide (NaHS) reduces oxidative stress. Mice were subjected to partial warm ischemia for 75 min and subsequent reperfusion (I/R) for 6 h. **a** Lipid peroxidation was assessed by MDA + 4-HNE. **b** Representative photographs (40× magnification) are shown. Immunohistochemistry of the liver showed that Nrf2 (Green) was ubiquitous in the cytosol but not in the nucleus in the sham-operated group. In the NaHS (-) group, only faint staining of Nrf2 in the nucleus was seen, whereas in the NaHS (+) group, almost all cells showed Nrf2-positive (pale blue; Arrow). **c** Western blot of nuclear proteins with regard to Nrf2. **d** Western blots of cytosolic proteins were evaluated for the HO-1 protein, and **e** thioredoxin-1 (TRX-1). Representative western blots (top) and the relative ratio (target protein/ β -actin) (bottom) are shown. Each normalized value was further normalized by the mean value in the sham-operated group. Data are expressed as the mean \pm SD. * $P < 0.05$, NaHS (-) vs. NaHS (+). ** $P < 0.05$ vs. Sham

significantly higher in the NaHS treatment group than in the other groups (Fig. 3c, d).

Apoptosis

TUNEL staining at R24 h showed almost no positive cells in the sham group (Fig. 4a, d). TUNEL-positive cells were augmented by hepatic warm I/R in the NaHS(-) group at R24 h (Fig. 4b, d), whereas they were significantly reduced by NaHS treatment (Fig. 4c, d). Cleaved caspase-3 at R6 h was significantly augmented by hepatic warm I/R in the NaHS(-) group, whereas it was significantly suppressed by NaHS treatment (Fig. 4e).

Oxidative stress

We calculated the sum of the values of malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE), the end products of lipid peroxidation, at R6 h. This value was augmented by hepatic warm I/R in the NaHS(-) group, whereas it was significantly reduced by NaHS treatment (Fig. 5a).

Anti-oxidative responses were evaluated by the translocation of Nrf2 and expression of the downstream enzymes, HO-1 and TRX-1. Immunohistochemistry of Nrf2 (Green) revealed homogeneous staining in the cytosol, but not in the nucleus, in the sham-operated group. In the NaHS (-) group, there was only faint staining of Nrf2 in the nucleus, whereas in the NaHS (+) group, almost all cells showed Nrf2-positive nucleus (pale blue; arrow), indicating that NaHS treatment augmented the nuclear translocation of Nrf2 at R6 h (Fig. 5b).

The western blot of nuclear proteins revealed that the sham group had the lowest value. The value in the NaHS(-) group at R6 h was increased significantly, and it was further increased significantly in the NaHS(+) group (Fig. 5c). The expression of HO-1 in the cytosol was increased by hepatic warm I/R in the NaHS(-) group, and it was further augmented significantly by NaHS treatment (Fig. 5d). The expression of TRX-1 in the cytosol showed

a tendency to decrease with hepatic warm I/R in the NaHS(-) group, whereas it was significantly augmented by NaHS treatment (Fig. 5e).

Proliferation

Hepatic proliferation was evaluated by PCNA staining at R24 h (Fig. 6a-d). In the sham group, the percentage of PCNA-positive hepatocytes was 47 ± 14 %. The positive rate was reduced significantly to 13 ± 15 % in the NaHS(-) group, whereas it was augmented significantly to 63 ± 14 % by NaHS treatment.

Discussion

We confirmed the beneficial effects of NaHS against warm I/R of the mouse liver, by demonstrating a reduction in tissue injury, apoptosis, oxidative damage, and inflammatory reactions, with stimulation of liver regeneration. These beneficial effects were at least in part due to the augmented nuclear translocation of Nrf2 and downstream activation of anti-inflammatory and anti-oxidant pathways. Activation of the pro-survival signals was demonstrated by the augmented phosphorylation of PDK-1, Akt, mTOR, and p70s6k in response to NaHS treatment. Simultaneous activation of anti-apoptotic, anti-inflammatory, anti-oxidative, pro-survival, and pro-proliferation cascades appeared to allow recovery of the liver subjected to warm I/R.

Hydrogen sulfide (H_2S) is produced endogenously from cysteine in the liver, kidney, vessels, brain, and nerves, and its exertion at low concentrations is biologically important [23]. Furthermore, H_2S has been reported to reduce IRI of the liver [13–16] and other organs [9–12]. Consistent with these reports, the present study showed a reduction in net injury by ALT leakage and histopathology.

Acute inflammation in hepatic IRI is initiated mainly in Kupffer cells and hepatocytes during warm ischemia [2, 3, 24]. These cells release TNF- α , IL-6, and IL-1 β , and stimulate inflammation, which in turn activates the cell death pathway, and ROS and protease release from neutrophils [3, 4]. Schlegel et al. [25] reported that serum TNF- α , IL-17, and the ratio of CD154-positive T cells were increased in a DCD liver graft after transplantation. IL-23 and Th17 cells, including NK, NKT, and $\gamma\delta$ T cells, play major roles in both acquired and innate immunity in organ transplantation [26]. In hepatic IRI, IL-23 stimulates Kupffer cells and CD4 +/Th17 cells. IL-6 released from Kupffer cells promotes further activation of Th17 cells to release IL-17, and stimulates neutrophil accumulation [27]. A recent report revealed that activation of CD40-CD40L (CD154) promoted oxidative stress-induced apoptosis in hepatocytes [28].

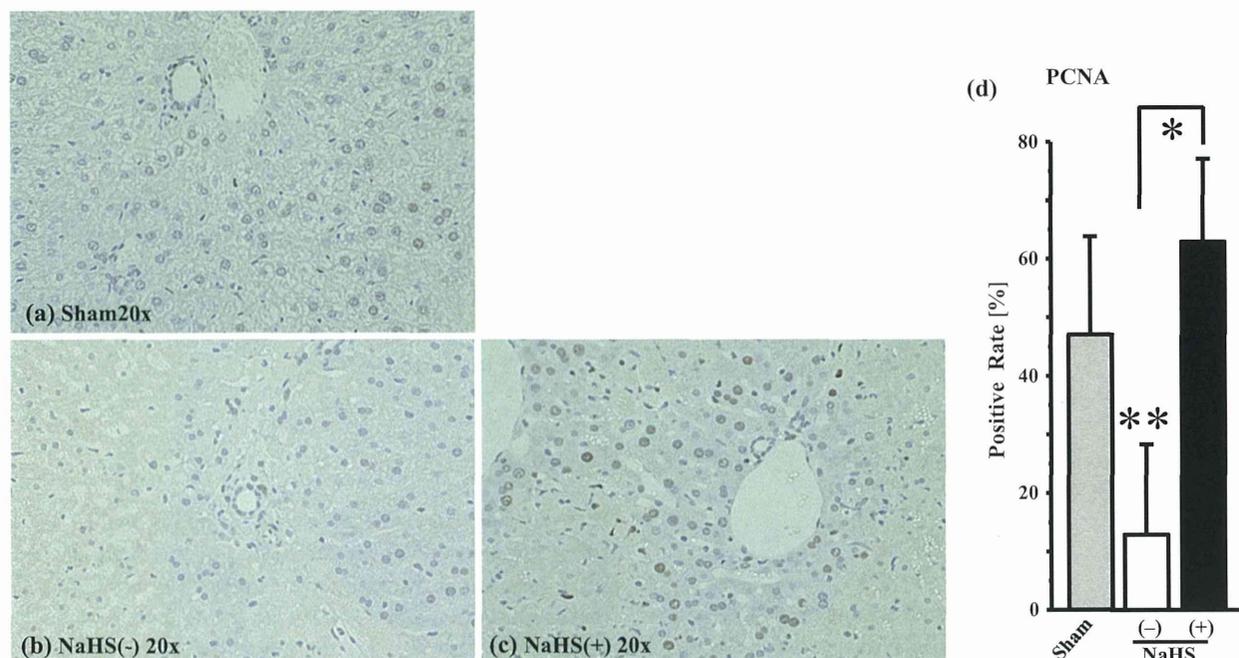


Fig. 6 Sodium hydrogen sulfide (NaHS) stimulates liver regeneration. Mice were subjected to partial warm ischemia for 75 min and subsequent reperfusion (I/R) for 24 h. Liver sections were stained by the anti-proliferating cell nuclear antigen (PCNA) antibody and hematoxylin as nuclear counterstaining. Representative photographs (20× magnification) are shown. In the sham group (a), the positive

rate was 47 %, but it was significantly decreased in the NaHS(-) group (b), and it was significantly augmented in the NaHS(+) group (c). The rate of PCNA-positive hepatocytes is shown in (d). Values are expressed as the mean ± SD. * $P < 0.05$, NaHS (-) vs. NaHS (+). ** $P < 0.05$ vs. Sham

In this study, serum TNF- α , IL-6, IL-1 β , IFN- γ , IL-17, IL-23, and soluble CD40L (CD154) increased significantly within 3 h of reperfusion, but these changes were inhibited by NaHS. Hydrogen sulfide reduced hepatic IRI with less production of TNF α and IL-6 [16]. Suppression of the IL23–IL17 axis reduced hepatic IRI [29]. Altogether, these facts suggest that NaHS reduced hepatic IRI by inhibiting the IL23–IL17 axis, thereby inhibiting the activation of Kupffer cells, neutrophils, and lymphocytes (CD4 +/Th17 cells) in the early phase of reperfusion. Although there are various sources of CD40L, the main source is the activated platelets [30], and CD40L was found to worsen hepatic IRI [28]. In this study, there was a massive release of cytokines and chemokines at R3 h. Multiple pro-inflammatory mediators from various cell types, platelet-endothelial adhesion, and platelet-leukocyte aggregation would stimulate the further activation of platelets [2], leading to the sustained high level of soluble CD40L.

Oxidative stress is another important factor in hepatic IRI [2–4]. Oxidative damage in the sinusoidal endothelial cells stimulates endothelin-1 (ET-1) production, leading to microcirculatory disturbance [31, 32]. Several distinct mechanisms of the anti-oxidant property of H₂S have been reported, namely, direct scavenging of ROS [33], reversible

inhibition of mitochondrial respiration [34], augmentation of glutathione (GSH) production through enhanced cystine/cysteine transport [35], and Nrf2-dependent expression of anti-oxidant and anti-inflammatory proteins [23]. Consistent with these reports, NaHS reduced lipid peroxidation and inflammation in this study.

Nrf2 exists in the cytosol with Keap-1 in the resting state, but it dissociates from Keap-1 on exposure to stimuli, such as oxidative stress and pro-survival signals, leading to up-regulation of HO-1 [36] and TRX-1 [37]. HO-1 reduces oxidative stress through the conversion of heme into biliverdin and carbon monoxide (CO) [38]. Furthermore, CO exerts anti-inflammatory and vasodilatory effects [39] and TRX-1 reduces protein thiol and/or hydrogen peroxide with the support of GSH [40]. In line with these reports, our study showed, for what we believe to be the first time, that NaHS augmented HO-1 and TRX-1 levels through augmented nuclear translocation of Nrf2, leading to reductions in oxidative stress and inflammation.

The anti-apoptotic and pro-survival effects of H₂S against mitochondria would result in an inhibition of intrinsic apoptosis [36]. H₂S activates this pathway in the I/R of hippocampal neurons [7, 41] and myocardium [5]. Insulin supplementation has been shown to stimulate

myocardial surviving expression via activation of PI3K-Akt-mTOR-p70s6k, resulting in anti-apoptotic effects [42]. In this study, pro-survival signals mediated by the PDK-1-Akt-mTOR-p70s6k axis were maintained by the higher phosphorylation in the NaHS-treated liver. Phosphorylation of p70s6k confers protection against IRI in the heart and small intestines through anti-apoptotic and anti-inflammatory effects [17, 18]. The enhanced PDK1-Akt signal reduced IRI through the augmented phosphorylation of PDK1 [43] and Akt [14], but not through de novo gene expression of these proteins, at least within 6 h of reperfusion. In line with these reports, this study is the first to show that NaHS supplementation ameliorated hepatic warm IRI by maintaining the phosphorylation of p70s6k and upstream kinases, including mTOR, Akt, and PDK-1. Since we did not assess any gene expression, further study is required to clarify the precise mechanism of NaHS-mediated protection during the early phase of reperfusion.

Another important anti-apoptotic signal in hepatic IRI is the signal transducer and activator of transcription 3 (STAT3) [9]. Ke et al. [29] recently reported that HO-1 ameliorated hepatic inflammation, apoptosis, and net injury after warm I/R by inhibiting NF-kappaB signals in the nucleus. They showed that STAT3 was indispensable for the HO-1-mediated down-regulation of TLR-4 and PTEN, and the augmentation of phospho-Akt. In the present study we observed a transient rise of IL-6 at 3 h, only slight increases of IL-6 and TNF- α at 6 h, and a significantly higher PCNA-positivity rate 24 h after reperfusion in the NaHS-treated group. Debonera et al. [44] reported that IL-6-mediated activation of STAT3 did not trigger liver regeneration in severely injured liver grafts. Although we did not evaluate STAT3 here, these observations suggest that the STAT3-mediated machinery of liver regeneration [45] would have functioned well in the NaHS-treated liver.

Recently, Zhang et al. [46] reported that NaHS administration before ischemia inhibited mitochondrial permeability transition pore opening and activated Akt-GSK3 β . Exogenously administered hydrogen sulfide disappeared rapidly from the blood and tissues through oxidation and thiol-binding [47], with half-lives of 2.0 and 5.4 min in the aerobic and anaerobic liver, respectively [48]. Therefore, we administered NaHS before reperfusion to maintain enough concentration at reperfusion. In contrast to the previous report [46], we failed to show the efficacy by administration before ischemia in our preliminary study. In relation to dosage, Kang et al. [15] reported the effective dose to be 0.78 mg/kg. Since 0.5 mg/kg was less effective than 1 mg/kg, and 3 mg/kg resulted in animal death by respiratory dysfunction in our preliminary study, we adopted 1 mg/kg as the optimal dose (data not shown). The controversy might be due to the differences in ischemia time, species, and strain. Although further investigation is necessary

to establish the optimal mode of administration for liver graft protection, it is encouraging that hydrogen sulfide proved effective when administered before ischemia [46], before reperfusion, and during cold preservation [49].

In conclusion, NaHS treatment against hepatic warm I/R resulted in high phosphorylation levels of PDK1, Akt, mTOR, and p70S6k, and nuclear translocation of Nrf2, leading to anti-oxidant, anti-inflammatory, anti-apoptotic, pro-survival, and pro-proliferative effects, and eventually reduced net IRI with rapid liver regeneration.

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Conflict of interest Shingo Shimada and his co-authors have no conflicts of interest.

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Postoperative Assessment of Hepatic Asialoglycoprotein Receptor Function with Tc-99m GSA: The Safety Margin of Resection Size in Living Donor Liver Transplantation

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- Background:** Living liver donation is associated with size-dependent complications. The resectable size and its safety margin should be defined for the safety of donors. The purpose of the present study was to determine if the current partial hepatectomies are done under the safety margin of the resectable size, by measuring asialoglycoprotein receptor (ASGPR) function of donor's remnant liver.
- Material/Methods:** Seventy-four living donors (age 35±11 years) underwent Technetium-99m-diethylenetriaminepentaacetic acid-galactosyl-human serum albumin (Tc-99m GSA) scintigraphy at postoperative week 1. We evaluated the scintigraphic results using established parameters of GSA uptake (LHL15) and its clearance from the blood pool (HH15). Based on the literature, we consider HH15 <0.55 to indicate normal ASGPR function, and 0.55≤ HH15 <0.65 to indicate mild impairment. In terms of the hepatic uptake, we consider LHL15>0.93 to indicate normal ASGPR function, and 0.87< LHL15 ≤0.93 to indicate mild impairment.
- Results:** The average resected size was 337±170 mL, corresponding to 28±12% of the original donor's whole liver volume. No donors showed 0.65≤ HH15 or LHL15 <0.87, suggesting moderate or severely impaired ASGPR function. However, larger resection size (35–53%) was positively associated with higher HH15 values (R=0.53, p<0.001). In the range of HH15 (0.35–0.64) among present donors, higher HH15 values did not affect the regeneration volume (R=0.03, p=NS).
- Conclusions:** Larger partial resection (≥35% of the original liver volume) may impair postsurgical ASGPR function, but smaller resection (<35%) was considered to be under the safety margin of the hepatectomy. Although mildly impaired postsurgical ASGPR function did not indicate poor prognosis, careful attention may be required for donors undergoing larger (≥35%) partial resection.
- MeSH Keywords:** **Asialoglycoprotein Receptor • Hepatectomy • Liver Function Tests • Liver Regeneration • Liver Transplantation • Living Donors**
- Full-text PDF:** <http://www.annalsoftransplantation.com/abstract/index/idArt/892490>

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Background

Living donor liver transplantation is an established surgical approach to treat patients with severe hepatic disorders, with patient survival rates that are equivalent to those obtained with deceased donor liver transplantation [1]. In geographic regions in which deceased donor rates are low, living donor liver transplantation reduces the wait-list death rate and improves survival from the time of listing compared to deceased donor liver transplantation [2]. Adult living liver donation is associated with significant donor complications. Despite technical advances and considerable experience with liver resection at specialized centers, partial hepatectomy is still burdened by relatively high rates of postoperative morbidity (4.09–47.7%) and mortality (0.24–9.7%) [3]. A more cautious approach should be taken regarding the use of this procedure, particularly in right-lobe living donor surgery [4].

The rates of hepatocellular dysfunction and serious complications significantly increase as the volume of the resected liver increases [5]. In practice, however, factors related to the recipient determine the size of the liver to be transplanted [6]. Therefore, the resectable size and its safety margin should be defined for the safety of donors undergoing partial hepatectomy. The volume of liver that can be resected safely is not well understood, but several studies have suggested that the parenchymal hepatic resection rate measured by computed tomography (CT) is a significant predictor of postoperative liver failure.

In general, resection resulting in a 25% volume of remnant liver or a volume of 250 mL/m² was considered to be the safe limit for living human transplantation surgery [7,8]. Since experimental resection in humans is not possible, postsurgical risk assessment is a practical alternative to investigate the safety margin of the resected size for living human transplantation surgery.

The purpose of the present study was to determine if the current partial hepatectomies are done under the safety margin of the resectable size. We measured the donors' hepatic asialoglycoprotein receptor (ASGPR) function immediately (within 7 postoperative days) after the transplantation surgery using the Technetium-99m-diethylenetriaminepentaacetic acid-galactosyl-human serum albumin (Tc-99m GSA) [9] and correlated the

function with resected size and regeneration volume of the remnant liver. To the best of our knowledge, this is the first imaging study to investigate postoperative ASGPR function immediately after partial hepatectomy in living human donors.

Material and Methods

Subjects and study design

The study population retrospectively included 84 living donors who underwent Tc-99m GSA scintigraphy after hepatectomy between March 2004 and December 2012. According to a standard institutional protocol, all of the donors underwent a postoperative evaluation using Tc-99m GSA scintigraphy and x-ray CT at 1 week after the hepatectomy. All donors who completed the required postoperative evaluation were included in the present study population, but the patients for whom an injection leakage of Tc-99m GSA (n=10) was suspected were excluded. The final study population included a total of 74 donors (47 men and 27 women), and mean age of 35.4±10.9 years, ranging from 18 to 62 yrs old (Table 1).

Tc-99m GSA scintigraphy was scheduled 1 week after hepatectomy to determine the residual liver's ASGPR function soon after the transplantation. We measured morphological liver volumes using contrast-enhanced x-ray computed tomography (CECT). The volume measurements were done before the operation, 1 week after the operation, and 1 year after the operation to evaluate the postoperative changes associated with partial hepatectomy. We analyzed the sizes of the resected livers in relation to ASGPR function and the regeneration volume of the donors' residual liver. The Institutional Review Board at Hokkaido University Hospital, Sapporo, Japan approved the analyses and the publication of the study results (#012-0032).

Operative procedures

Living donor liver transplantation was indicated for the treatment of cholestatic liver disease, chronic parenchymal liver disease (caused mainly by viral hepatitis), fulminant liver failure, metabolic liver disease, and hepatobiliary malignancy. Donors were selected according to the following standards: second-degree relation to or spouse of the recipient, 18 years old or

Table 1. Characteristics of the 74 living human liver transplantation donors.

	Left lobectomy	Lateral lobectomy	Right lobectomy
n	46	11	17
Age (yr old)	37.7±1.7	31.9±3.7	31.6±11.1
Gender (M: F)	34:12	1:10	12:5