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ORIGINAL ARTICLE

# Beneficial effects of gaseous hydrogen sulfide in hepatic ischemia/reperfusion injury

Eelke M. Bos,[1,2] Pauline M. Snijder,[1,2] Henrike Jekel,[1] Michel Weij,[2] Jaklien C. Leemans,[3] Marcory C. F. van Dijk,[1] Jan-Luuk Hillebrands,[1] Ton Lisman,[2] Harry van Goor[1] and Henri G. D. Leuvenink[2]

1 Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
2 Department of Surgery, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
3 Department of Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

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apoptosis, heme oxygenase 1, hypometabolism, inflammation, necrosis.

**Correspondence**
Eelke M. Bos, Department of Pathology and Medical Biology, University Medical Center Groningen, PO Box 30.001, Hanzeplein 1, 9700RB Groningen, The Netherlands.
Tel.: +31 50 3614179;
fax: +31 50 3632796;
e-mail: e.m.bos@path.umcg.nl

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The authors report no conflict of interest regarding this paper.

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## Summary

Hydrogen sulfide ($H_2S$) can induce a reversible hypometabolic state, which could protect against hypoxia. In this study we investigated whether $H_2S$ could protect livers from ischemia/reperfusion injury (IRI). Male C57BL/6 mice were subjected to partial hepatic IRI for 60 min. Animals received 0 (IRI) or 100 ppm $H_2S$ (IRI + $H_2S$) from 30 min prior to ischemia until 5 min before reperfusion. Core body temperature was maintained at 37 °C. Animals were sacrificed after 1, 6 or 24 h. Hepatic ischemia caused extensive hepatic necrosis in the IRI animals which coincided with an increase in ALT and AST serum levels. Animals treated with $H_2S$ showed attenuated serum ALT and AST levels and reduced necrotic lesions after 24 h. IRI animals had increased Bcl-2 mRNA expression and increased active Caspase 3 protein, which were both significantly lower in $H_2S$ treated animals. Increased TNFα and IL-6 mRNA in the IRI livers was significantly attenuated by $H_2S$ treatment, as was hepatic influx of Ly-6G positive granulocytes. Hepatic superoxide production after ischemia was attenuated by $H_2S$ treatment. In hepatic ischemia/reperfusion injury, gaseous $H_2S$ treatment is highly protective, substantially reducing necrosis, apoptosis and inflammation. Gaseous $H_2S$ is therefore a very promising treatment for reducing IRI during hepatic transplantation.

## Introduction

Hepatic ischemia/reperfusion injury (IRI) is the result of transient blood flow deprivation, often seen during surgical intervention and liver transplantation. The detrimental effects of IRI have clinical consequences in the transplant setting, attributing to organ failure and rejection [1–3]. From animal experiments it has become clear that the hepatic response to IRI has different facets. In the early phase there is substantial production of reactive oxygen- and nitrogen species (ROS and RNS), causing necrosis and apoptosis [4,5]. In addition, the response to injury will result in upregulation of adhesion molecules, influx of leukocytes and further activation of Kupffer cells, which induces increased production of ROS and of pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) [5].

Hydrogen sulfide ($H_2S$), mostly known for its toxic properties [6], is a widely studied molecule. In recent years, however, $H_2S$ has matured from a dangerous and malodorous gas to a physiologically important signaling molecule [7]. The functions of $H_2S$ range from vasodilatation [8–11], inhibiting platelet aggregation [12], regulating bile excretion [13], stimulating angiogenesis [14] and neuromodulation [14–16] to scavenging of free radicals [17–19]. It is now, in addition to nitric oxide (NO) and carbon monoxide (CO), acknowledged as the third gasotransmitter, sharing many functions with these gases [20].

In addition to these physiological properties, one of the most captivating discoveries concerning $H_2S$ in recent

years is its ability to induce a reversible, hibernation-like state in naturally non-hibernating mammals [21]. Subtoxic concentrations of gaseous $H_2S$ rapidly reduce metabolic parameters in mice, slowing down $O_2$ consumption and $CO_2$ production by ~60% within 5 min, and more than 90% after 6 h of treatment. Core body temperature, heart rate and breathing frequency all decline to a great extent during treatment. When exposure to $H_2S$ is stopped, animals recover rapidly without any apparent toxic effects. Although the mechanism behind $H_2S$-induced hypometabolism is unknown as of yet, one of the involved proteins likely to be involved is cytochrome *c* oxidase, the terminal enzyme in the mitochondrial electron transport chain. $H_2S$ can reversibly inhibit mitochondrial oxygen consumption and ATP production by binding to cytochrome *c* oxidase [22,23]. In addition to protective effects of $H_2S$ mediated ROS scavenging, this process lowers the demand for $O_2$ and prevents mitochondrial ROS production.

To pharmacologically reduce the demand for $O_2$ is an attractive strategy to attenuate the effects of IRI, such as during the unavoidable periods of hypoxia during liver transplantation. The liver is very susceptible to IRI, and can only be preserved outside of the body without major influence on long term outcome for up to 16 h by using hypothermia and preservation solutions [24]. If metabolism in the liver can be reversibly lowered, the time the liver can be preserved might be increased, hepatic function after transplantation could be improved and acute and chronic rejection could be reduced. In this study, we therefore investigate whether hydrogen sulfide protects the liver from IRI in an experimental mouse model.

## Materials and methods

### Animals

Male C57BL/6 mice (6–8 week old, Harlan, Zeist, the Netherlands, $n = 5$ per group) were housed under standard conditions with a 12 h light:dark cycle at our animal research facility with *ad libitum* access to water and murine chow. Experimental procedures were in agreement with institutional and legislator regulations and approved by the local committee for animal experiments.

### Respirometry

Measurement of animal $CO_2$-production was performed using a modular respirometry system (TR-3 system, Sable Systems, Las Vegas, NV, USA). Air or $H_2S$/Air mixture was pushed through a mass flow controller (Model 840, Sierra Instruments, Egmond, The Netherlands) set to 200 ml/min. Animals were placed in a downstream, airtight respirometry chamber with heat pads. The excurrent gas was lead through a CA-10a dual wavelength infrared sensor $CO_2$-analyzer (Sable Systems). Data acquisition was performed using a UI-2 interface and ExpeData v1.0.24 software (Sable Systems). Compressed air and 500 ppm $H_2S/N_2$ (Air Products, Amsterdam, The Netherlands) were mixed in a 4:1 ratio, producing a 100 ppm $H_2S$/17% $O_2$ mixture. Control animals received room air. $CO_2$-production was corrected for body weight and normalized to mean control values.

### $H_2S$ treatment

Animals were treated with 100 ppm $H_2S$ or air for 25 min in our respirometry system (as described above) to induce a hypometabolic state. They were then transferred to the operating table, where they received 100 ppm $H_2S$ or air through a standard anesthesia cap during the procedure until 5 min before reperfusion. After that, all animals received only ambient air.

### Hepatic ischemia/reperfusion

The left hepatic artery and portal vein were clamped for 60 min using non-traumatic vascular clamps through a midline abdominal incision under general anesthesia (75 mg/kg ketamine, 1 mg/kg domitor), causing ischemia in the median and left lateral hepatic lobes. After removing the clamps, the liver was inspected for restoration of blood flow and the muscle and skin layers were sutured with 5–0 stitches. Body temperature was monitored with a rectal probe and maintained at ~37 °C using a heating pad and lamps. Sham-operated animals were subjected to the same procedure, excluding the placement of the clamps. Subsequent to closure of the abdomen, all mice received a subcutaneous injection of 50 μg/kg buprenorphin (Schering-Plough, Kenilworth, NJ, USA) for analgesic purposes and were allowed to recover from surgery at 35 °C in a ventilated incubator. Mice were sacrificed 1, 6 or 24 h after reperfusion. At the time of sacrifice mice were anesthetized using 2.5% isoflurane in $O_2$, blood was collected in EDTA containing tubes, centrifuged for 10 min at 1000 rcf and serum was collected and stored at −80 °C. Livers were perfused with 0.9% NaCl solution and ischemic and non-ischemic lobes were collected. Half of each lobe was fixed in 4% paraformaldehyde, processed for paraffin embedding and used for immunohistochemical analysis. The other half was snap frozen in liquid nitrogen and stored at −80 °C.

### Dihydroethidine (superoxide) staining

Frozen liver sections (4 μm) were dried for 20 min under a room temperature blower and subsequently incubated

for 30 min at 37 °C with 12,5 μM dihydroethidine (Invitrogen, Carlsbad, CA, USA) dissolved in PBS. Images were acquired at 20× magnification and analyzed using NCBI ImageJ.

### Plasma biochemical analysis

Hepatic damage was assessed by measuring aspartate transaminase (AST), alanine transaminase (ALT) and lactate dehydrogenase (LDH) in plasma samples using standard methods by our hospital research services.

### Histopathological scoring

The extent of hepatocellular damage and necrosis was determined in haematoxylin-eosin (HE) stained sections. Whole slides were scanned using an Aperio ScanScope GS (Aperio Technologies, Vista, CA, USA). Total hepatic area and necrotic hepatic area were determined using the Aperio Imagescope software, and the ratio of necrotic hepatic surface area to total hepatic surface area was determined. The necrotic area calculated in this manner correlated linearly with serum AST (24 h) with an $r^2$ of 0.9252 and with serum ALT (24 h) with an $r^2$ of 0.8792.

### Immunohistochemistry for active Caspase 3 and Ly-6G

Immunohistochemical staining for active Caspase 3 and granulocytes was performed as described before [25]. In short, for active Caspase 3, paraffin embedded sections were stained using rabbit anti-human active Caspase 3 polyclonal antibody (Cell Signaling, Beverly, MA, USA), followed by HRP-conjugated goat-anti-rabbit IgG (Immunovision Technologies, Hillsborough, CA, USA). For granulocytes, paraffin embedded sections were stained for Ly-6G using rat-anti-mouse Ly6G/C-FITC IgG2b antibody (AbCam, Cambridge, MA, USA), followed by rabbit-anti-FITC and HRP-conjugated goat-anti-rabbit antibodies. Full slides were scanned using an Aperio ScanScope GL (Aperio Technologies, Vista, CA, USA) and analyzed for positive pixel area (Ly-6G) or positive pixel intensity (Caspase 3) using the Aperio Positive Pixel Analysis v9.1 algorithm.

### Qualitative real-time polymerase chain reaction

RNA was extracted from frozen livers using the TRIZOL method (Invitrogen, Carlsbad, CA, USA). DNAse treatment was performed using Turbo DNAse-free (Ambion, Austin, TX, USA). cDNA was synthesized using Superscript II RT and random hexamer primers (Invitrogen). A relative quantification PCR was performed to determine gene expression (Applied Biosystems, Foster City, CA, USA). β-actin was used as housekeeping gene. The primers used were: Heme Oxygenase-1 (HO-1) – Forward: CGAGGGAAACCCCAGATCA, Reverse: TTGCCAACAG GAAGCTGAGA; B-Cell Lymphoma-2 (Bcl-2) – Forward: CTGGGATGCCTTTGTGGAA, Reverse: TCAGAGAC AGCCAGGAGAAATCA; Bcl-2 Associated X protein (BAX) – Forward: CAAGAAGCTGAGCGAGTGTCTC, Reverse: AATCATCCTCTGCAGCTCCATATT; Tumor Necrosis Factor alpha (TNFα) – Forward: ACAAGGC TGCCCCGACTAC, Reverse: TGACTTTCTCCTGGTATG AGATAGCA; Interleukin-6 (IL-6) – Forward: CGCTATG AAGTTCCTCTCTGCAA, Reverse: GTAGGGAAGGCCGT GGTTGT; alpha Smooth Muscle Actin (αSMA) – Forward: GAGAAAATGACCCAGATTATGTTTGA, Reverse: GGACAGCACAGCCTGAATAGC; Collagen 1a – Forward: GGAGAGTACTGGATCGACCCTAAC, Reverse: CTGACC TGTCTCCATGTTGCA; Hypoxia Inducible Factor-1 alpha (HIF-1α) – Forward: CTCAGAGGAAGCGAAA ATGGA, Reverse: CAGTCACCTGGTTGCTGCAATA. PCR was performed in a total volume of 20 μl containing 10 ng cDNA template and 10 μl PCR-mastermix (Eurogentec, Seraing, Liège, Belgium). The Thermal Profile was 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The average Ct-values for the target genes were subtracted from the average β-actin Ct-values to yield the delta Ct. Results were expressed as $2^{-\Delta\Delta Ct}$.

### Statistical analysis

Data were analyzed using GraphPad PRISM 5.0 (GraphPad, San Diego, CA, USA) or SPSS 14.0 (SPSS Inc., Chicago, IL, USA) using, the Mann–Whitney *U* test or the Kruskall–Wallis test where appropriate. Dunns postcorrection was applied where multiple comparisons were made. Normality was tested using the Kolmogorov–Smirno test. $P < 0.05$ was considered statistically significant. All data are expressed as the mean ± SEM (Standard Error of the Mean) unless otherwise indicated.

## Results

### Respirometry

Measurement of $CO_2$ production using the respirometry system indicated that mice enter a hypometabolic state within 5–10 min after the start of exposure to 100 ppm $H_2S$ (Fig. 1a). Our experiments indicate that $CO_2$ production is stable over longer periods of time when core body temperature is maintained. On the basis of these results, we chose a pretreatment period of 30 min for our ischemia experiments, to ensure that the mice would enter a stable state of hypometabolism (Fig. 1b). $CO_2$ production of the animals just prior to the start of the ischemia

procedure was measured in control and $H_2S$ treated animals, verifying that mice had entered a hypometabolic state in our IRI experiments. Relative $CO_2$ production was reduced by 38% ($P < 0.001$) in $H_2S$ treated animals (Fig. 1c).

### Necrosis

Necrotic hepatic surface area in the ischemic lobes was significantly increased after 24 h of reperfusion in the IRI group ($P < 0.05$), while $H_2S$ treatment attenuated the onset of necrosis (Fig. 2a). On average, after 6 h of reperfusion, IRI animals had 7.4% necrotic surface area, while $H_2S$-treated animals had 13.2% ($P$ = ns). After 24 h, the IRI group had 46.2% necrotic area, while treatment with $H_2S$ had only 7.5% necrotic area ($P < 0.01$). Examples of the relative necrotic areas are shown in Fig. 2b, in which necrosis was artificially colored red.

### Serum AST, ALT and LDH

Serum levels of AST and ALT were both significantly higher in the IRI group at 1, 6 and 24 h after reperfusion compared to sham-operated animals (Fig. 3a and b). This increase was significantly lower in the IRI + $H_2S$ group at 1 and 24 h, but not at 6 h after reperfusion. The reduction in AST was 68.2% at 1 h ($P < 0.05$) and 75.4% after 24 h ($P < 0.05$). The reduction in ALT was 50.9% after 1 h ($P < 0.05$) and 87.5% after 24 h ($P < 0.05$). LDH levels in the serum of IRI mice was significantly increased at 1 and 6 h after reperfusion, which was significantly attenuated by $H_2S$ treatment at 1 h (25.1% lower, $P < 0.05$), but not at 6 or 24 h of reperfusion (Fig. 3c).

### Apoptosis

Immunohistochemistry for active Caspase 3 showed a substantial increase in apoptotic cells after 24 h of reperfusion, especially in the peri-necrotic areas. This increase was fully prevented by $H_2S$ treatment ($P < 0.05$, Fig. 4a). Livers from $H_2S$-treated animals had active Caspase 3 staining intensity comparable to sham-operated animals. Representative examples of active Caspase 3 staining are shown in Fig. 4b–e. mRNA expression for the anti-apoptotic gene Bcl-2 were also significantly increased in the ischemic lobes of IRI animals after 24 h (Fig. 4f), while this increase was not seen in IRI + $H_2S$ treated animals ($P < 0.05$), indicating a sustained mitochondrial integrity. BAX gene expression was also significantly lower in $H_2S$ treated animals (Fig. 4g).

### Inflammation

TNFα mRNA expression was significantly increased in the ischemic hepatic lobes of IRI animals 24 h after reperfusion ($P < 0.05$, Fig. 5a). No significant increase of TNFα mRNA was seen in IRI + $H_2S$ treated animals ($P < 0.05$). Hepatic IL-6 mRNA levels were massively increased in IRI animals, while no increase was detected in IRI + $H_2S$ treated animals ($P < 0.05$, Fig. 5b). Similarly, IL-1β mRNA levels were induced 24 h after ischemia, and this increase was completely prevented by $H_2S$ treatment (Supplementary Table S1). IL-18 levels were not differentially expressed in any group (Supplementary Table S1). Massive influx of Ly-6G positive granulocytes as assessed by immunohistochemistry was observed at 24 h after reperfusion in IRI animals, while IRI + $H_2S$ treated animals

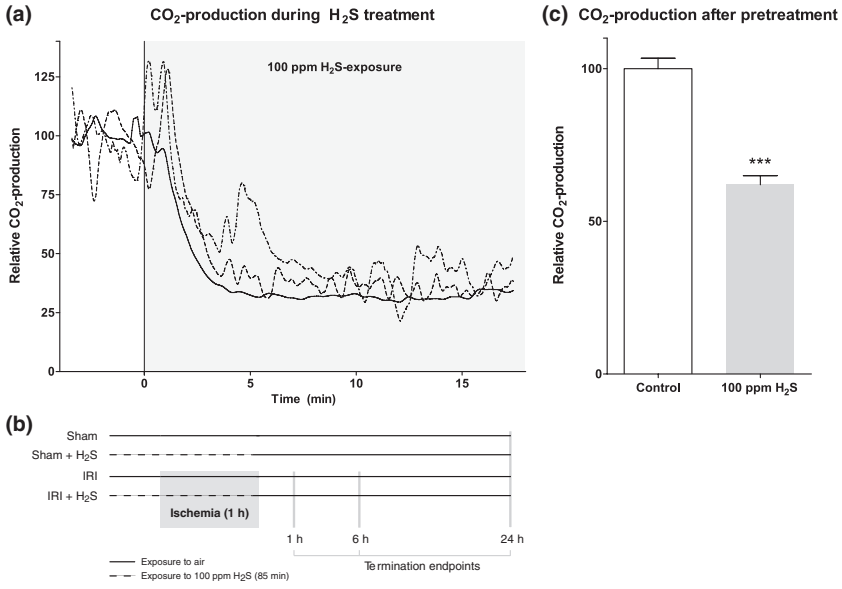


**Figure 1** $H_2S$ induced hypometabolism and experimental design. (a) $CO_2$-production of three separate, awake mice before and during treatment with 100 ppm $H_2S$. Exposure to $H_2S$ rapidly reduces $CO_2$ production in these animals, indicating the induction of a hypometabolic state. (b) Experimental design, showing the $H_2S$ treatment regimens used (dashed line indicates treatment with 100 ppm $H_2S$). (c) Average $CO_2$ production of anesthetized control and $H_2S$-treated mice at the end of the pretreatment period, just before ischemia. $H_2S$ treated animals had significantly lower $CO_2$-production, indicating a successful induction of hypometabolism (***$P < 0.001$).


 

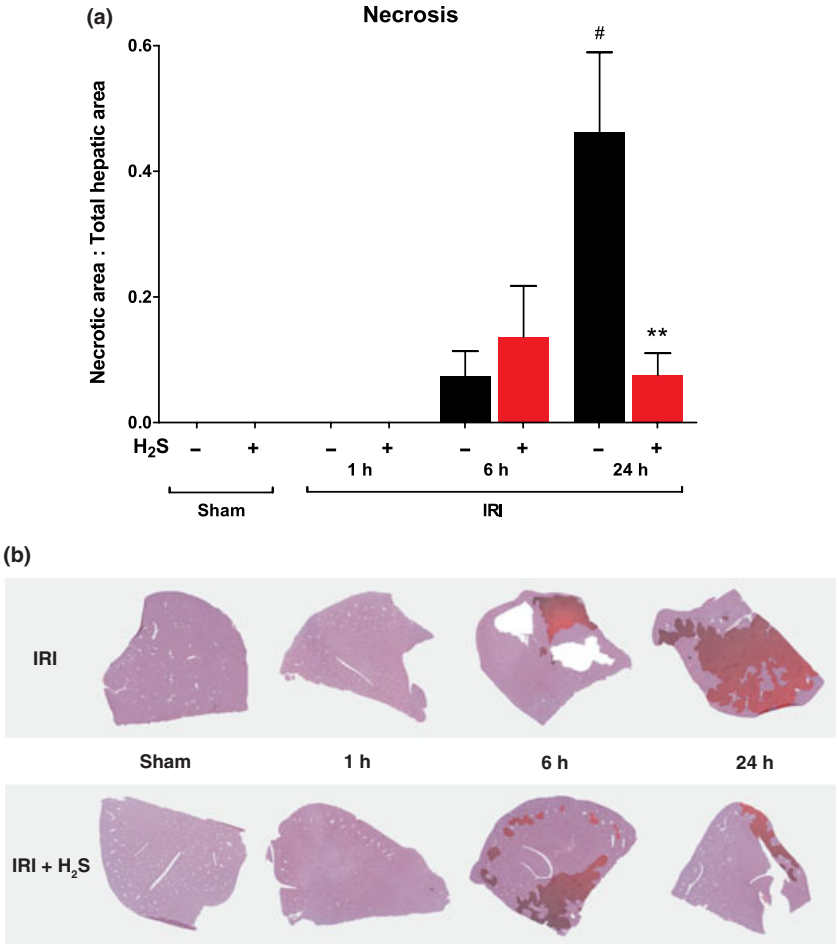


**Figure 2** $H_2S$ reduces hepatic necrosis induced by ischemia. (a) Relative necrotic area in haematoxylin-eosin (HE) stained hepatic sections, showing massive necrosis in the IRI group after 24 h of reperfusion, which was significantly lower in the livers of $H_2S$ treated animals. (b) Representative images of HE stained hepatic sections from all different treatment groups. Necrotic area was artificially colored red (*$P < 0.01$ vs. IRI, #$P < 0.05$ vs. Sham).

had greatly reduced increase in granulocytes in the ischemic hepatic lobes ($P < 0.01$, Fig. 5c). Figure 5d–g show representative sections of Ly-6G stained hepatic sections, demonstrating the extent of granulocyte influx into the necrotic areas in IRI animals.

### Superoxide production

Superoxide levels in the hepatic sections were significantly increased in IRI animals at 6 and 24 h after reperfusion (Fig. 6, $P < 0.01$). $H_2S$ treatment significantly attenuated the increase in superoxide at 6 and 24 h ($P < 0.01$).

### HO-1 gene expression

The expression of heme oxygenase-1 (HO-1) mRNA was increased after 6 h of reperfusion, in ischemic lobes as well as in non-ischemic lobes. $H_2S$ did not significantly modulate this increase at the 6 h time point. At 24 h, mRNA expression was significantly increased in ischemic lobes of IRI animals ($P < 0.05$), but not in non-ischemic lobes (Fig. 7a and b). $H_2S$ treatment significantly reduced the expression of HO-1 at the 24 h time point in ischemic lobes, and relative to the non-ischemic lobe ($P < 0.05$, Fig. 7c).

### Gene expression of αSMA and Collagen-1a

Early expression of pro-fibrotic genes was assessed by investigating mRNA expression of αSMA and Collagen-1a (Supplementary Table S1). The expression of both genes in the ischemic lobes was reduced in IRI + $H_2S$ treated animals compared to IRI after 24 h of reperfusion ($P < 0.01$), although the changes in expression relative to the non-ischemic lobes were not significantly modulated.

### HIF-1α gene expression

No significant differences in HIF-1α gene expression were detected in ischemic or non-ischemic lobes at all time points (Supplementary Table S1).

## Discussion

Hepatic ischemia/reperfusion injury in mice can be prevented to a great extent by inducing a hypometabolic state with gaseous $H_2S$. In all of the dimensions of hypoxic injury that were investigated – including necrosis, apoptosis, inflammation and reactive oxygen species – $H_2S$ treatment reduced deleterious effects, in some parameters to such an extent that damage was comparable to sham levels.

Several studies have reported on the use of $H_2S$ in hypoxic injury, and the majority of studies show beneficial effects of $H_2S$ treatment in models of shock [26], cardiac arrest [27] and in cardiac- [22,28], intestinal- [29], pulmonary- [30] and renal ischemia [31,32]. Two studies have shown beneficial effects of $H_2S$ in hepatic IRI [33,34]. These studies use soluble salts as donors of $H_2S$ in solution ($Na_2S$ or NaHS), and show that $H_2S$ can reduce apoptosis and hepatic damage after IRI. They differ in the time of administration, however, one paper injecting before ischemia, and the other just before reperfusion. The unique perspective our study offers is the use of gaseous administration of $H_2S$ and the induction of a hypometabolic state, linking the protective properties of $H_2S$ to the reduction in the $O_2$-demand of the liver.

The increase in serum ALT, AST and LDH levels at 6 h of reperfusion was equal in IRI and IRI + $H_2S$ treated animals. This finding may well be related to the hepatic necrosis that was found at 6 h of reperfusion, and suggests that $H_2S$ cannot prevent all damage caused by hepatic IRI. Alternately, this could be a toxic effect of $H_2S$. Unfortunately, we did not include a group of sham-operated animals terminated 6 h after the procedure, which would likely have shed more light on this observation. However, we believe it is unlikely that $H_2S$ would induce such a massive increase in ALT, AST and LDH without showing any histological signs of necrosis, apoptosis or inflammation in sham operated animals.

The reduction in active Caspase 3 staining seen in $H_2S$ treated animals indicated that $H_2S$ can prevent the activation of apoptosis pathways. Since $H_2S$ has been shown to protect mitochondrial integrity during hypoxia [22,31], we investigated if the mitochondrial apoptosis pathway would be activated in IRI animals. For this purpose, we

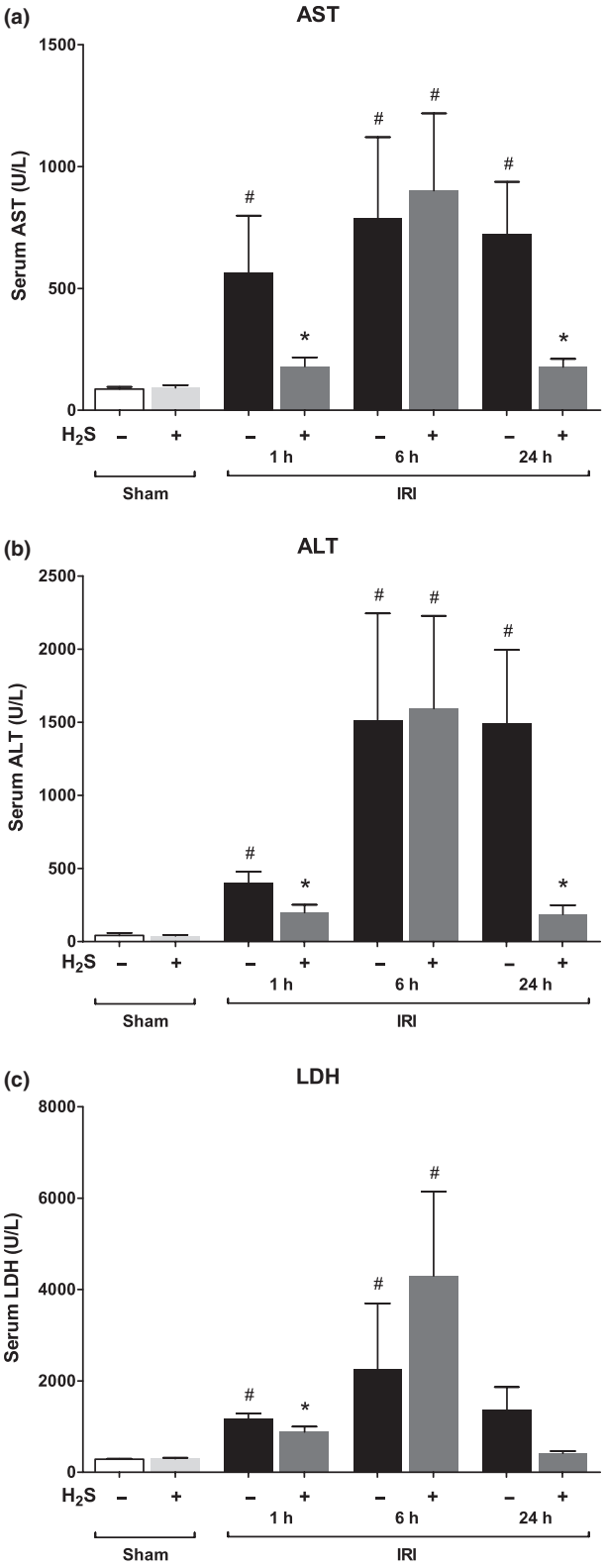


**Figure 3** Serum AST, ALT and LDH levels are attenuated by $H_2S$ treatment. (a and b) Serum AST and ALT levels show an increase at 1, 6 and 24 h after ischemia, which was significantly reduced by pretreatment with $H_2S$ at 1 and 24 h of reperfusion. At 6 h there was no significant difference between IRI + $H_2S$-treated animals and the IRI group. (c) Serum LDH levels were increased in IRI animals at 1 and 6 h after reperfusion. $H_2S$ significantly attenuated the increase at 1 h of reperfusion, but not at 6 h (*$P < 0.05$ vs. IRI, #$P < 0.05$ vs. Sham).

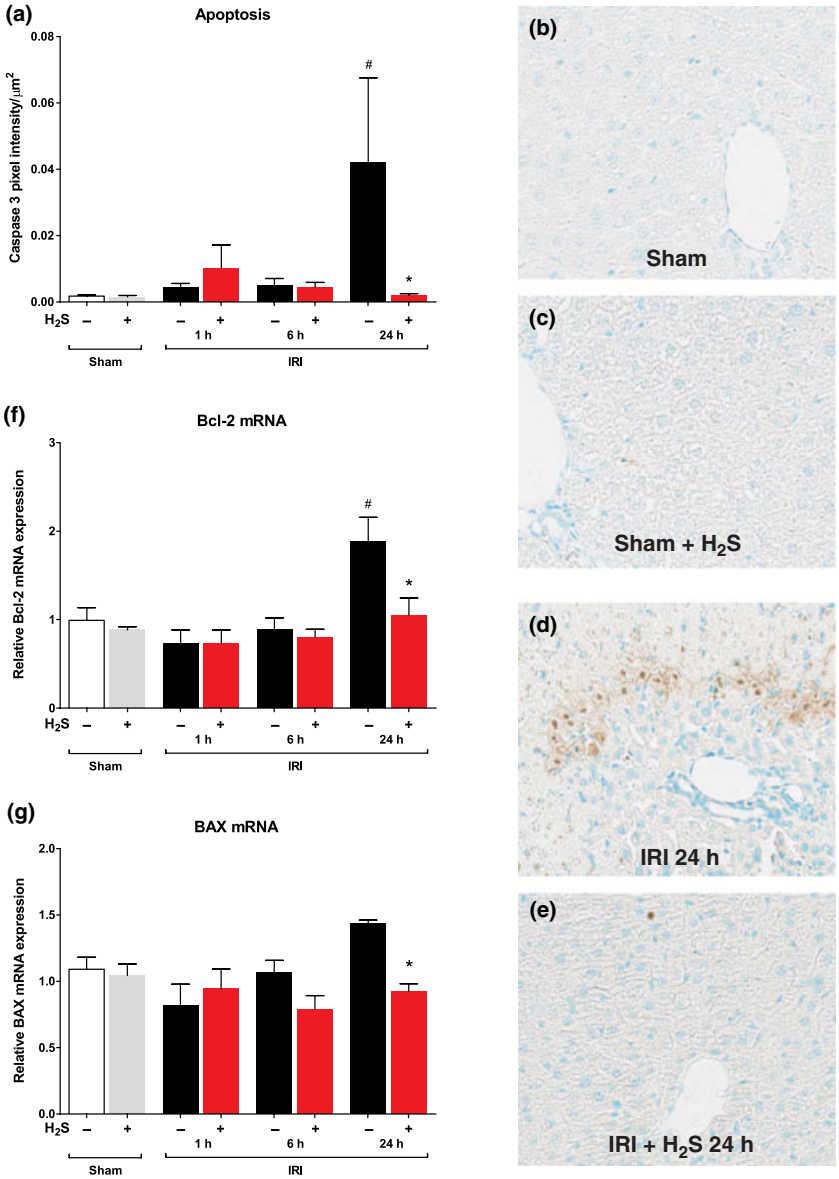


**Figure 4** $H_2S$ treatment reduces apoptosis of hepatocytes. (a) IRI induced massive apoptosis of hepatocytes, as measured by active Caspase 3 immunohistochemistry. $H_2S$ treatment prevented the onset of apoptosis in the ischemic lobes. (b–e) Representative images of hepatic sections stained for active Caspase 3 using immunohistochemistry. mRNA levels of the anti-apoptotic gene Bcl-2 (f) and pro-apoptotic BAX (g) indicate the activation of the mitochondrial apoptosis pathway in livers exposed to IRI. mRNA levels were significantly lower in $H_2S$ treated animals (*$P < 0.01$ vs. IRI, #$P < 0.05$ vs. Sham).

measured the expression of Bcl-2 and found a significant increase in Bcl-2 mRNA after IRI. This increase was not found in livers from the IRI + $H_2S$ group, indicating that the onset of mitochondrial degradation by IRI was prevented by $H_2S$ treatment.

Treatment with $H_2S$ prevented the production of TNFα and IL-6 mRNA after ischemia. This reduction in the production of cytokines may have contributed to the massively reduced influx of granulocytes to the necrotic areas in the liver. Treatment with $H_2S$ lowered the influx of granulocytes after renal ischemia/reperfusion injury [31]. $H_2S$ has also been implicated before in modulating leukocyte adhesion [35] through a mechanism involving the activation of $K_{ATP}$ channels, although we did not test the role of $K_{ATP}$ channels in this study. Nevertheless, there is no increase in granulocyte influx in $H_2S$ treated animals after 24 h of reperfusion, while there is an increase in necrotic area, indicating that although there is necrotic damage to these livers, there is reduced activation of signals that cause the influx of leukocytes.

The reduction in DHE fluorescence after IRI in $H_2S$ treated animals indicates a reduced amount of ROS in the liver. $H_2S$ can have direct scavenging effects on ROS, but can also have indirect effects, through increasing the amount of reduced glutathione (GSH). Whether direct or indirect ROS-scavenging mechanisms are at play here was not investigated. Another mechanism that could be involved is that reduction in oxidative metabolism and mitochondrial activity by $H_2S$ inhibited the generation of ROS in the mitochondria.

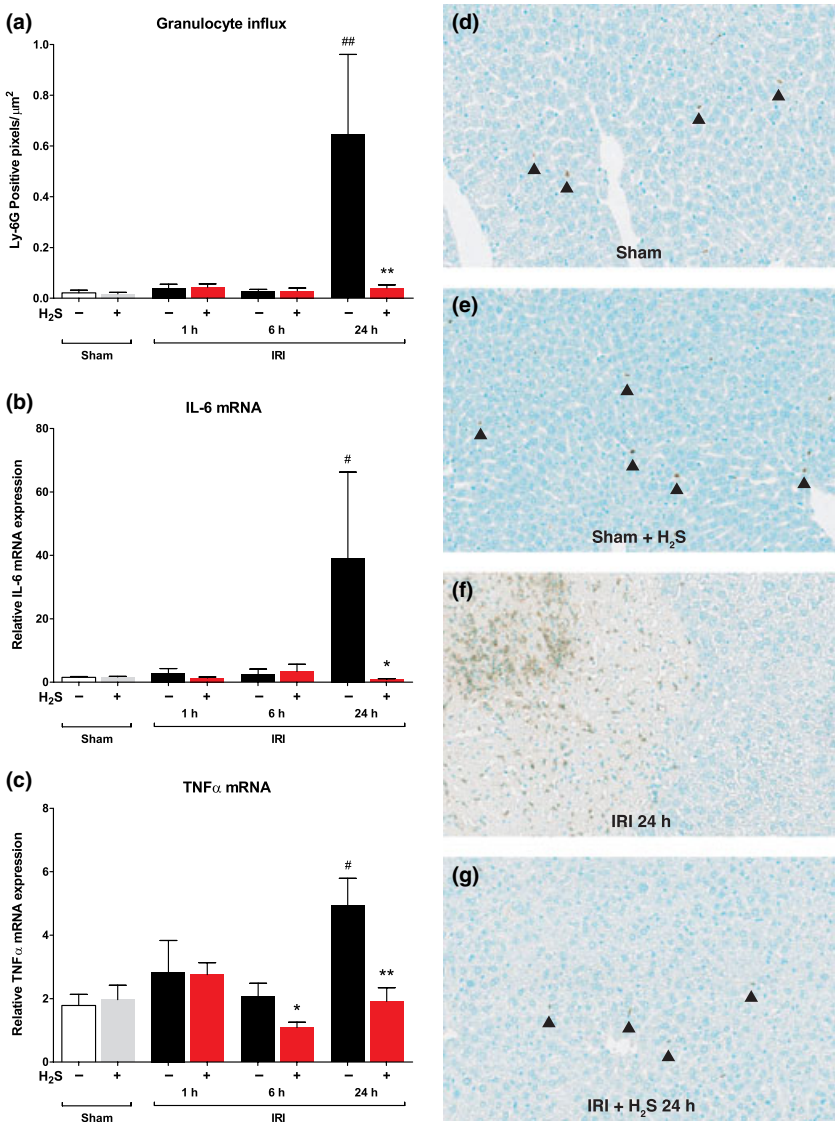


**Figure 5** $H_2S$ treatment reduces inflammation and influx of granulocytes. (a) Influx of Ly-6G positive granulocytes was assessed using immunohistochemistry. Influx of granulocytes was very high and was completely prevented by $H_2S$ pretreatment. (b) Hepatic expression of TNFα mRNA is increased 24 h after reperfusion and is significantly lower in $H_2S$ treated animals at 6 and 24 h of reperfusion. (c) IL-6 mRNA expression is massively higher after 24 h of reperfusion and is not induced in $H_2S$ treated animals. (d–g) Representative images of hepatic sections stained for granulocytes using Ly-6G antibody. Similar levels of granulocytes were seen in sham and $H_2S$ treated animals, while the IRI group had very high influx of granulocytes, especially in the necrotic areas, as can be clearly seen in 5f. Arrowheads depict granulocytes in 5d–e, g (*$P < 0.05$, **$P < 0.01$, both vs. IRI. #$P < 0.05$, ##$P < 0.01$, both vs. Sham).

Heme oxygenase-1 converts heme into biliverdin and CO, and is known as an enzyme that can be protective in models of oxidative stress [36]. It is known that gene expression of HO-1 is rapidly induced after hepatic ischemia/reperfusion injury [37]. Increased expression of HO-1 has been implicated as one of the mechanisms behind $H_2S$ mediated protection in models of ischemia [28,38,39]. $H_2S$ treatment did not induce the expression of HO-1 in sham-operated animals. We found a large increase in expression of HO-1 in ischemic as well as non-ischemic lobes 6 h after the ischemia/reperfusion procedure, which was not modulated by $H_2S$ treatment. This effect on HO-1 gene expression is most likely not caused by ischemia of the liver, but by the stress of the surgical procedure, anesthetics and/or analgesics, since expression in ischemic and non-ischemic lobes was similar. The significant increase in HO-1 expression in ischemic lobes after 24 h of reperfusion, however, was attenuated by $H_2S$ treatment, indicating that HO-1 expression is not implicated in the protective mechanism of $H_2S$ in this model, but can be regarded as a marker of cellular or oxidative stress.

To investigate the main components of ischemia/reperfusion injury – necrosis, apoptosis, inflammation and fibrosis – we also investigated pre-fibrotic processes by looking at αSMA and Collagen-1a mRNA expression. There was significant modulation of the expression of both genes by $H_2S$ in ischemic lobes, which indicated that treatment with $H_2S$ might be able to prevent the onset of fibrotic mechanisms. However, when we corrected for the expression in the non-ischemic lobe there were no significant differences in expression. Also, the expression

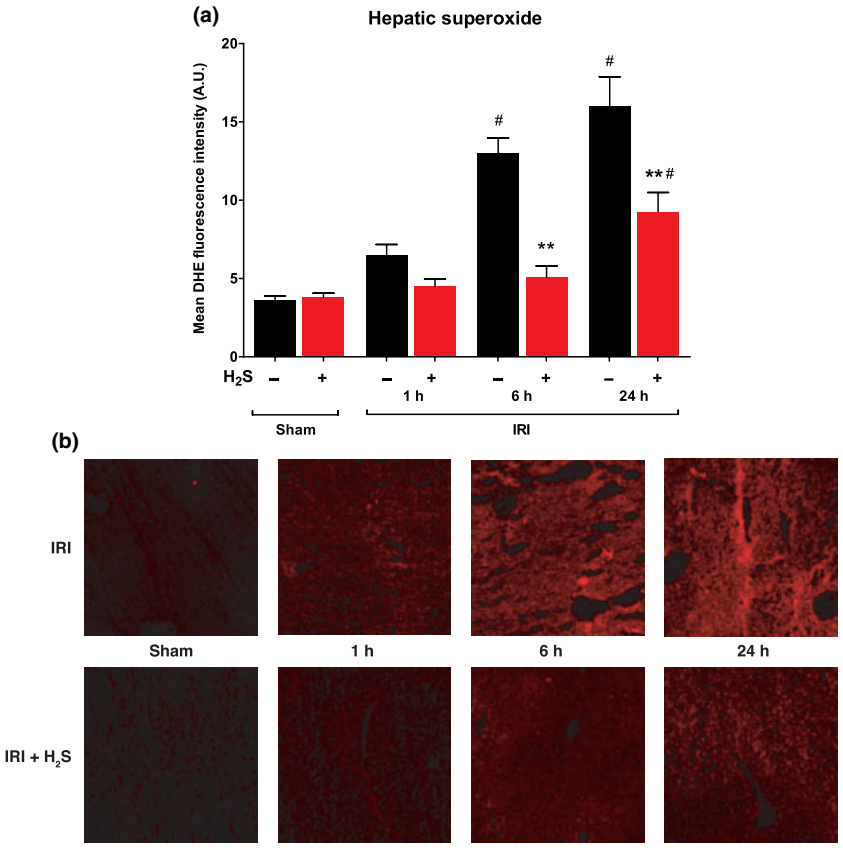


**Figure 6** Hepatic superoxide is reduced by $H_2S$ treatment. (a) Superoxide production in hepatic sections as assessed by DHE fluorescent imaging is significantly increased after IRI at 6 and 24 h. Treatment with $H_2S$ significantly reduced the extent of ROS at these timepoints. (b) Representative images of DHE fluorescence at all timepoints (**$P < 0.05$ vs. IRI, #$P < 0.05$ vs. Sham).

compared to sham-operated animals was not significantly changed, indicating that 24 h of reperfusion is probably too early to assess ischemia induced fibrotic mechanisms after ischemia.

The mechanism behind the protective effects of hydrogen sulphide in hypoxic conditions might be related to its inhibitory effects on mitochondria. $H_2S$ treatment can protect mitochondria from degradation, and improve viability in models of hypoxia and ischemia [22,31]. This could well be related to the inhibition of cytochrome *c* oxidase, or the scavenging of mitochondrial ROS. Fascinatingly, the induction of a hypometabolic state using $H_2S$ can protect mice from an hypoxic atmosphere; where control mice died within 20 min after the onset of hypoxia, $H_2S$ treated mice could survive for up to 6 h [40]. This implies that the protection that we have seen during ischemia are possibly due to a reduced $O_2$ demand, thereby providing defense against hypoxia in the livers. In addition, it appears that $H_2S$ can be used as an electron donor in mammalian cells [41]. When $H_2S$ inhibits mitochondrial cytochrome *c* oxidase, cells might be able to use $H_2S$ as an energetic substrate for anaerobic metabolism, allowing a low level of energy production during hypoxia. In addition, $H_2S$-induced vasodilatation might have a role in the protective effects of $H_2S$. Nevertheless, from the study presented in this paper it is impossible to say which combination of the many known effects of $H_2S$ is responsible for the effects seen. We were unable to provide direct evidence towards the mechanisms that underlie $H_2S$ mediated protection, only associative data.

The clinical potential of $H_2S$ is diverse, and $H_2S$ donors or $H_2S$-releasing compounds are being tested in different clinical settings now, including myocardial infarction, cardiopulmonary bypass and inflammatory conditions such as arthritis and inflammatory bowel disease. Ischemic conditions could greatly benefit from $H_2S$ treatment. During surgical intervention $H_2S$ might be valuable to protect tissue from ischemia caused by temporary arterial clamping. However, some caution is required before these results can be translated to the human setting, since some of the effects of $H_2S$ on mice could not be reproduced in larger animals, such as sheep [42]. However, others were able to induce lower core body temperature, $O_2$-consumption and $CO_2$-production in pigs [43].

In our view, one of the first feasible clinical applications of gaseous $H_2S$ lies in the pretreatment of the brain dead (heart beating) organ donor. In addition to protecting the organ from ischemic damage, $H_2S$ might also

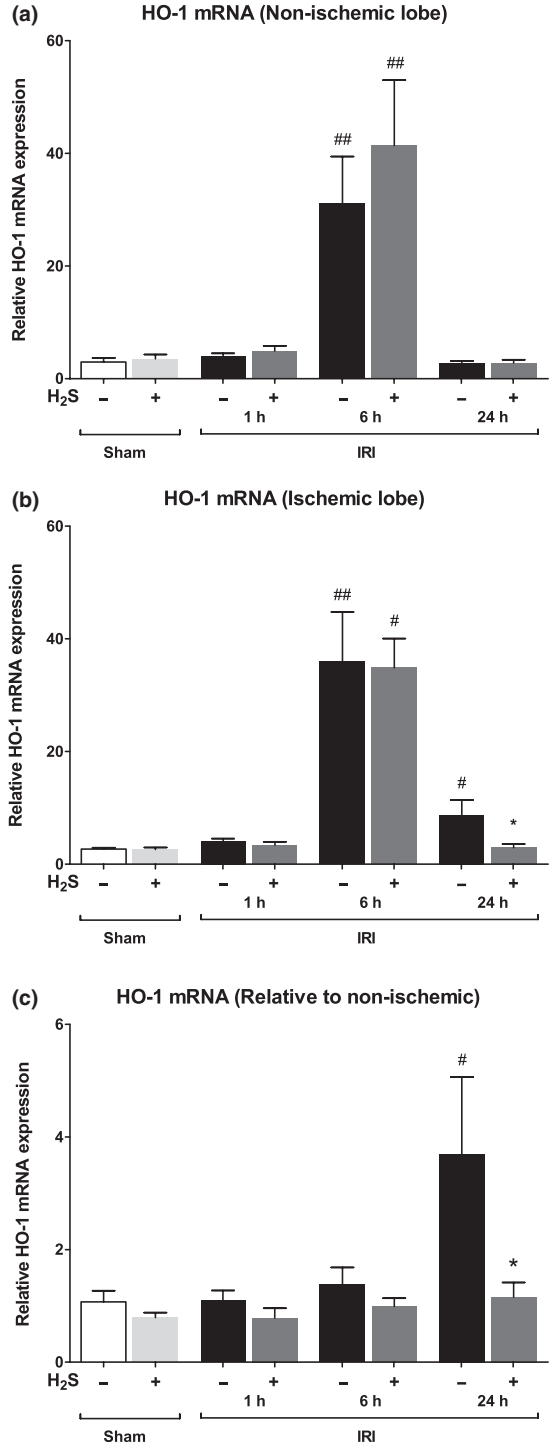


**Figure 7** HO-1 gene expression levels in ischemic and non-ischemic hepatic lobes. (a and b) HO-1 mRNA expression is induced in non-ischemic and in ischemic lobes at 6 h of reperfusion, indicating an effect not caused by ischemia. At 24 h, expression of HO-1 is significantly higher in IRI compared to the IRI + $H_2S$ group. (c) The ratio of ischemic to non-ischemic lobe expression of HO-1 indicates that induction of HO-1 is not involved in the protective mechanism of $H_2S$. (*$P < 0.05$ vs. IRI, #$P < 0.05$, ##$P < 0.01$, both vs. Sham).

modulate the deleterious effects brain death has on organs that are to be transplanted [44]. However, the effects of brain death on $H_2S$-induced protection against IRI will need to be investigated. The fact that $H_2S$ has protective effects in diverse organs makes $H_2S$ a promising candidate to treat multi-organ donors prior to procurement. $H_2S$ treatment will perhaps not only limit the extent of injury that occurs during the process of transplantation, but it might also increase the time livers can be preserved between explantation from the donor and implantation into the recipient. However, the potentially toxic effects of $H_2S$ should be carefully monitored when used in a clinical setting.

In all, these data indicate that pretreatment with gaseous $H_2S$ is a highly protective method to prevent ischemia/reperfusion injury of the liver, which makes it a promising candidate for use in a transplantation setting.

## Authorship

EB: designed and performed the research, collected and analyzed the data, and wrote the paper. PS: performed the research and collected data. HJ and MW: performed the research. JL: contributed important reagents. MD: contributed important reagents and collected data. J-LH: designed the research. TL, HG and HL: designed the research and wrote the paper.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Table S1**. mRNA expression of multiple genes in ischemic and non-ischemic lobes.

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