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The effect of a hydrogen sulfide releasing molecule (Na_2S) on the cold storage of livers from cardiac dead donor rats. A study in an *ex vivo* model

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ABSTRACT

Liver transplantation is currently the preferred treatment option for end-stage liver disease. Donation after cardiac death was a common practice in the early years of organ donation before brain death criteria were established. Those organs were subjected to variable periods of warm ischemia that might intensify cold ischemia/reperfusion injuries. In the present, shortage of brain dead donors has led to the reassessment of organ donation after cardiac death.

Since many cytoprotective roles have been describe for H_2S during ischemia/reperfusion on a variety of tissues, we hypothesized that graft exposure to this bioactive gas might improve preservation of non-heart beating donated organs.

Therefore, to establish a rat model of donation post-cardiac arrest and using this approach to judge H_2S delivery effects on graft hypothermic preservation, were the main objectives of this investigation.

Cardiopulmonary arrest was induced in sedated rats by overload of potassium (K^+). Livers were surgically removed and subsequently stored in HTK Solution (Histidine–tryptophan–ketoglutarate) at 0–4 °C. After 24 h of hypothermic preservation, livers were rewarmed in an *ex vivo* model. Three experimental groups were established as follows: I – Livers procured before cardiac death and cold stored 24 h in HTK (BCD); II – Livers procured after cardiac death (45 min) and cold stored 24 h in HTK (ACD); III – Livers procured after cardiac death (45 min) and cold stored 24 h in HTK + 10 μM Sodium Sulfide (Na_2S) (ACD-SS). Data suggest that after 45 min of warm ischemia, viability parameters assessed during reperfusion in the *ex vivo* model were significantly impaired. Real time PCR revealed that after *ex vivo* reperfusion there is an increased expression of HO-1 and TNF-α and a modest drop in Bcl-2 mRNA, which could be interpreted as the cellular response to the hypoxic insult sustained during warm ischemia.

On the other hand, warm ischemic livers exposed to H_2S during cold storage, improved microcirculation, morphology and viability parameters during *ex vivo* reperfusion and showed significant modulation of HO-1 mRNA expression.

In conclusion, HTK supplementation with Na_2S arose as a potential treatment to recover non-heart beating harvested organs. Furthermore, an appropriate model of cardiac dead liver donors was successfully developed.

1. Introduction

Over the last five decades, liver transplantation has become a well-recognized treatment option for people with organ failure leading to a rapid increase in patient waiting lists. During liver transplantation procedures, interruption of the blood flow (ischemia) and subsequent reperfusion provokes the release of pro-inflammatory cytokines and the generation of reactive oxygen species that eventually cause cell damage and organ dysfunction [23]. Despite improvements in surgical techniques and perioperative management, liver failure remains one of the major drawbacks in the transplantation field [22].

A renewed interest in donation after cardiac death started in the nineties as a potential answer to the organ shortage problem. However, those grafts are frequently associated with a higher risk

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of early graft dysfunction and lower graft survival, due to the inevitable warm ischemia occurring between the declaration of death and organ retrieval process [9,24]. Warm ischemic injury induced prior to procurement is markedly exacerbated by cold ischemia when applying current conventional static storage solutions [22]. Thus, agents such as H_2S gaseous transmitter exhibiting vasoactivity [12,38], anti-inflammatory [11,33], anti-oxidant [6,16] and anti-apoptotic effects [32], arose as interesting approaches to improve preservation of marginal livers. Moreover, modulation of signaling cascades at low temperature and demonstration of the improvement in graft function has been previously documented [8]. Hydrogen sulfide donors (Na_2S, NaHS) have been extensively used in the study of cytoprotection mediated by H_2S but, until now, those assays relied almost entirely on models of ischemia–reperfusion *in vivo*, based on the occlusion of hepatic blood vessels [16,17].

In the present work, livers from cardiac dead donor rats were exposed to Na_2S during cold ischemia in a commercial preservation solution. These livers were then oxygenated and re-warmed in an *ex vivo* system to judge H_2S therapeutic potential.

2. Experimental procedures

2.1. Buffers and solutions

Histidine–tryptophan–ketoglutarate (HTK) solution (CUSTOPLEX®, Biogam, Argentina); Krebs Henseleit Buffer (KHB), composition: NaCl 118 mM, KCl 4.8 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.5 mM, heparin 2 IU/mL, glucose 5 mM; final pH of KHB solution after equilibration with carbogen (O_2:CO_2, 95:5%) was 7.40; perfusate solution was KHB + 2% Dextran-70. All reagents were of analytical grade.

2.2. Animals

Adult male Wistar rats (9- to 12-week-old, weighing 300–320 g) used in these studies were obtained from the Central Animal Building at Biochemistry and Pharmaceutical Sciences Faculty of National University of Rosario. Rats were maintained on standard food pellets and water *ad libitum*. All protocols were performed according to the guidelines of the National Council of Research in Argentina and a Local Ethics Committee from the Biochemistry and Pharmaceutical Sciences Faculty and they are in concordance with International regulations (EU Directive 2010/63/EU). All efforts were made to minimize the number of animals and their suffering.

2.3. Liver procurement

2.3.1. Procurement before cardiac death

Male Wistar rats were anesthetized with chloral hydrate (Parafarm, Argentina) (500 mg/kg, *i.p.*). The abdomen was opened by midline incision and the liver was freed from all ligamentous attachments. In brief, 0.1 mL of saline containing 1000 UI of Heparin (Sobrius, Argentina) was injected into the femoral vein, the bile duct was cannulated with a PE-50 catheter (Intramedic, USA) and a 14 G Abbocath catheter was introduced and fixed in the portal vein. Perfusion with KHB was started immediately at 15 mL/min, the suprahepatic inferior vena cava was cannulated with steel tubing (internal diameter 3 mm) and, after blood wash out, the liver was carefully removed [2].

2.3.2. Procurement after cardiac death

To obtain livers from non-heart beating rats we modified a protocol from García Valdecasas et al. [13], where they used an intravenous injection of concentrated KCl solution to induce cardiac arrest in pigs. Animals were prepared for surgery as explained previously. Catheters were fixed in the portal vein and the bile duct prior to femoral injection of Heparin 1000 UI (0.15 mL) and potassium chloride solution 2 M (0.15 mL). Heart beat cessation was detected with a stethoscope, usually within 25–30 s. Warm ischemia time extended up to 45 min while livers remained *in situ*. Following the warm ischemia period, perfusion with KHB was started and the surgery proceeded as mentioned above.

2.4. Cold storage protocol

Excised livers were flushed out with 20 mL of HTK solution and submerged in a reservoir (100 mL Boeco bottles) containing 80 mL of hypothermic preservation solution. Static cold storage extended up to 24 h at 0–4 °C.

2.5. H_2S delivery during cold storage

Sodium Sulfide (Na_2S) is a widely known source of H_2S, this salt dissociates spontaneously in aqueous solution releasing H_2S in the process [15]. HTK solution (100 mL) was supplemented with Na_2S at a final concentration of 10 μM at the beginning of cold storage. H_2S release from Na_2S in HTK solution was assessed using 5,5′-di thiobis-(2-nitrobenzoic acid) (DTNB) according to Nashef et al. [25]. Briefly, DTNB 10 mM (20 μL) and sample (2 mL) were incubated at RT for 2 min and absorbance was read at $\lambda = 412$ nm, $\varepsilon = 28.3\ mM^{-1}\ cm^{-1}$. In this method, 1 mol of H_2S reacts with one mole of DTNB and produces two moles of 5-thio-2-nitrobenzoate (NBT) and one mole of free sulfur.

2.6. Experimental design

Rat livers were divided into 3 experimental groups according to the ablation procedure and conditions of cold storage as depicted in Table 1. Four animals were included in each group.

2.7. Ex vivo reperfusion

This in vitro model (Fig. 1) provided the opportunity to assess cellular injury and liver function in an isolated setting [3,28]. After hypothermic storage, livers were fixed in a glass support and perfusion flow was increased gradually over a 10 min period to allow stabilization of the system. Livers were then perfused by means of a peristaltic pump (Masterflex®, USA) through the portal vein at a constant pressure of 100 mm H_2O (7.36 mmHg) in a recirculating perfusion system regulated at 37 °C, perfusion media consisted of a KHB (see Buffers and Solutions) (150 mL, pH 7.40) containing Dextran-70 (2%), and constantly oxygenated through a thin wall silicone tubing curled in a carbogen (5% CO_2/95% O_2) ventilated chamber, yielding a perfusate with a $pO_2 > 450$ mmHg. Intrahepatic resistance at a constant perfusion pressure was calculated through portal flow rate recordings every 15 min. A sodium taurocholate solution (300 μM) was infused into the perfusion medium at 0.2 mL/min throughout the experiment to sustain bile production. Bile was collected in pre-weighted tubes every 15 min and the bile flow was estimated gravimetrically assuming bile density to be equal to that of water. Perfusate samples were

Table 1
Experimental groups.

Cold storage/procurement	Before cardiac death	After cardiac death
Standard HTK	BCD	ACD
HTK + 10 μM Na_2S		ACD-SS

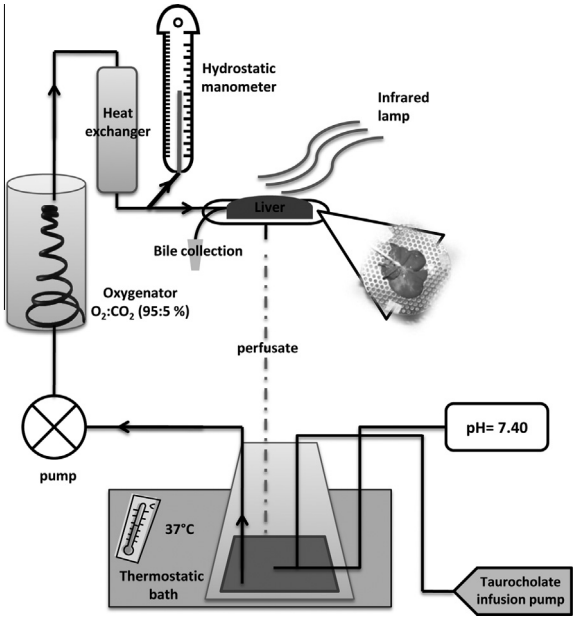

Fig. 1. Isolated rat liver perfusion system. This scheme shows the main components of the system operated to evaluate the livers response to different treatments. Oxygenated perfusate is pumped at constant pressure throughout the circuit, enters the liver via the portal vein catheter and exits through the suprahepatic vein catheter. Meanwhile, bile secretion is collected by means of a catheter fixed in the bile duct.

taken from the reservoir every 30 min for lactate dehydrogenase (LDH) determination. Oxygen consumption, calculated as in Pizarro et al. [28], was determined at 30 min intervals from samples of liver inflow and outflow perfusates using a YSI model 5300 Biological Oxygen Monitor (Yellow Springs, OH, USA), equipped with a Clark-type sensor (YSI 5331 oxygen probe). A single dose of bromosulfophthalein (BSP) (5 mg) was delivered into the reservoir at the beginning of reperfusion to evaluate the livers capacity to eliminate this dye from perfusate through bile excretion. BSP was determined in bile samples throughout 90 min of reperfusion by absorbance readings at $\lambda = 580$ nm in NaOH 0.1 M to obtain the percentage of excretion. After 90 min, perfusion was stopped and livers were weighed and cut into small blocks (4 mm thickness) that were fixed in 10% formaldehyde for subsequent histological studies. Also, tissue samples from different sections of each liver were taken and stored in LN_2 for further analysis.

2.8. LDH release into perfusate

The enzyme LDH serves as a general tissue-damage marker [3]. Lactate dehydrogenase (LDH) activity in perfusate samples were determined by kinetic spectrophotometry (BIOCHROM LIBRA S22, UK). LDH activity was determined by measuring NADH oxidation at $\lambda = 340$ nm in a reaction mixture containing 0.6 mM pyruvate and 0.2 mM NADH in 50 mM phosphate buffer pH 7.50.

2.9. Liver glycogen content

Liver glycogen was determined in biopsies taken after normothermic reperfusion in order to obtain information about differential energy substrate utilization between groups, reduced tolerance of livers from non-heart beating donors might be explained by scarce glycogen stores [34]. Liver tissue was disrupted by an Ultraturrax® homogenizer (IKA® Works, USA) in 0.1 M Citrate Buffer, pH 5.00. Glycogen content was calculated from the amount of glucose released by treatment of homogenized tissue with α-amyloglucosidase [7]. Homogenates were denatured at 100 °C and subsequently treated with α-amyloglucosidase resulting in the complete hydrolysis of glycogen to glucose. The free glucose content of the homogenate supernatant is then determined with the aid of a Glycemia assay kit (Wiener Lab, Argentina).

2.10. Urea synthesis

Urea synthesis is the main elimination pathway of Nitrogen from the organism, enzymes of Urea cycle begin to lose their activity when the liver is challenged by some stress or pathology [5]. Urea concentration in perfusate after 90 min of *ex vivo* reperfusion was determined by a colorimetric assay with diacetyl monoxime according to Rahmatullah et al. [29] and Butler et al. [4]. Briefly, perfusate samples were deproteinized by perchloric acid (5%) treatment and incubated 10 min at 100 °C with diacetyl monoxime (0.5%)/thiosemicarbazide (0.01%) reactive. Finally, absorbance was read at $\lambda = 525$ nm.

2.11. Glutathione levels

Glutathione has been shown to be of critical importance in the protection against oxidative injury following ischemia reperfusion [35]. Liver tissue samples were collected at the end of *ex vivo* reperfusion and stored in LN_2 until processing. Liver tissue was disrupted by an Ultraturrax® homogenizer in 0.1 M phosphate buffer (1:15), pH 7.50, 0.1% Triton X-100. Deproteinization of liver homogenates was performed with 2% Perchloric acid followed by supernatant neutralization with potassium carbonate. Total and oxidized glutathione (GSH) concentrations were determined through enzymatic recycling method [14]. The assay is based on the reaction of GSH with DTNB that produces TNB and oxidized glutathione–TNB adducts (GS–TNB). The rate of formation of TNB, measured at 412 nm, is proportional to the concentration of total GSH in the sample. The disulfide product (GS–TNB) is then reduced by GR in the presence of NADPH, recycling GSH back into the reaction.

2.12. Catalase activity

Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen, supporting endogenous defense against oxidative stress [27]. Liver tissue sample was collected at the end of *ex vivo* reperfusion and store in LN_2 until processing. For assaying the catalase activity, frozen tissues were homogenized by an Ultraturrax® homogenizer in 0.1 M phosphate buffer (1:15), pH 7.50, 0.1% Triton X-100. Samples were then centrifuge 20 min (4 °C, 20,000*g*) to obtain clear supernatants. Ten microliters of sample were assayed by following the decrease in absorbance of H_2O_2 (10 mM) at $\lambda = 240$ nm. The decomposition of H_2O_2 initially follows that of a first-order reaction with a rate constant (k) where, $k = s^{-1}$. $\ln(A_0/A_t)$. Catalase activity in liver tissues was expressed as k g^{-1} liver [1].

2.13. Malondialdehyde content

Malondialdehyde (MDA) is an end-product of the oxidative decomposition of polyunsaturated fatty acids; therefore, it is frequently used as a biomarker of oxidative stress [27]. Liver tissue sample was collected at the end of *ex vivo* reperfusion and store in LN_2 until processing. The concentration of MDA was determined by an HPLC technique [18]. Frozen tissue was homogenized with Ultraturrax® homogenizer in 0.1 M phosphate buffer (1:15), pH 7.50, 0.1% Triton X-100 and deproteinized with 2% Perchloric acid. Sample homogenates were centrifuged 15 min (4 °C, 20,000*g*), diluted 3:10 in distilled H_2O and filtered through a 0.2 mm PES

filter (Nalge, cat. 0001801320). Briefly, a 50 μL aliquot of the filtered supernatant was injected into an HPLC system (loop 20 μL), consisting of a Gilson 307 pump, a guard column (Phenomenex – AJO 4287), a Luna 5 μ C18(2) column (250 mm × 4.6 mm) (Phenomenex – 00G-4252-E0) and a Gilson 151 UV–VIS detector set at $\lambda = 254$ nm. The samples were eluted with a mobile phase of 65% KH_2PO_4: 35% Metanol, pH 4.00 at a flow rate of 0.8 mL/min, resulting in a typical retention time of 10.60 min for MDA. The peaks were identified and quantified using 1,1,3,3 tetraethoxypropane based MDA standards as in Karatas et al. work [18]. The data were analyzed and processed using the 712 HPLC Control Software package from Gilson (Villiers-Le-Bel, France).

2.14. Histology

Hematoxilin and Eosin (H/E) staining followed by light microscopy was performed to judge the morphological integrity of the parenchyma. We analyzed 30 microscopic images (400× magnification) of different biopsies from each one of the experimental groups. Fields were chosen randomly within the liver parenchyma and the images were captured by a Cannon Power Shot A 620 digital camera. Histopathological observations were semiquantified by a scoring method consisting on 80 point grids superposed over the images (software ImageJ 1.44p, National Institutes of Health, USA). Points that were in contact with injured morphology were computed and divided by total number of points on the grid to obtain a histological score. Every point was analyzed for the occurrence of sinusoidal dilatation, vacuolated cytoplasm and sinusoidal endothelial cell injury [28].

2.15. RNA isolation and real-time PCR analysis

Total RNA was isolated from liver tissues using TriPure reagent (Roche, Germany) following manufacturer's instructions. Tissue extraction was performed with a tissue-grinding tube and pestle, using 1 mL of TriPure reagent per 10 mg of liver tissue. RNA concentration and purity were determined by absorbance readings at 260 and 280 nm, whereas RNA integrity was assessed by the 18S and 28S band intensity ratio after 1.5% agarose gel electrophoresis visualized by ethidium bromide staining.

Retro-transcription of total RNA (1 μg) was performed with High Capacity cDNA Reverse Transcription kit with random hexamer primers and MultiScribe™ reverse transcriptase (Applied Biosystems, USA) following manufacturer's instructions. Retro-transcription reaction was performed in a thermal cycler at 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min. cDNA was stored at −20 °C.

Quantitative analysis of gene expression was performed by real-time PCR on 30 ng of cDNA. The gene-specific sense and anti-sense primers (Table 2) (usually 250 nM, 150 nM for *18S* primers) were added with iQ SYBER Green Supermix in an i-Cycler iQ thermocycler (Bio-Rad Laboratories, Hercules, CA, USA), the thermal cycler conditions consisted of 3 min at 95 °C and 40 cycles at 95 °C for 20 s, 60 °C for 20 s and 72 °C for 30 s. In order to verify the specificity of the amplification, a melt-curve analysis was performed, immediately after amplification protocol, under the following conditions: 1 min denaturation at 95 °C, 1 min annealing at 55 °C, and 80 cycles of 0.5 °C increments (10 s each) beginning at 55 °C. The relative quantification was made using the iCycler iQ software, version 3.1 (Bio-Rad Laboratories, Hercules, CA, USA) by the ΔΔCt method [37], normalizing the results to two housekeeping genes (β-actin and 18S). The levels of mRNA were expressed relative to samples in BCD group.

2.16. Statistical analysis

Mean ± Standard Deviation (SD) are presented unless otherwise stated. Statistical significance of the differences between groups was assessed by Analysis of variance (ANOVA) combined with Tukey HSD multiple range tests. Kruskal–Wallis test followed by Dunn's post hoc analysis was applied when normality criteria were not met. A p value of less than 0.05 was considered statistically significant (Statgraphics Plus 5.0, Statistical Graphics System, US).

3. Results

3.1. Hydrogen sulfide (H_2S) release by Sodium Sulfide (Na_2S) in HTK solution

A few minutes after the administration, H_2S concentration in HTK solution was 7.05 ± 0.15 μM ($n = 3$); and 24 h later, toward the end of hypothermic preservation period, H_2S concentration in HTK solution was 4.58 ± 0.26 μM ($n = 3$).

3.2. Liver viability parameters during ex vivo reperfusion

The *Intrahepatic resistance (IR)* progression of 4 livers in each group, was followed during *ex vivo* reperfusion and is shown in Fig. 2(A). This parameter reflects microcirculatory alterations as a consequence of tissue damage and vasoconstriction. Livers excised after cardiac death (ACD group) showed a significant increase in IR values when compared to livers procured before cardiac death (BCD group) ($p < 0.05$). On the other hand, when such livers were cold stored in HTK with a source of H_2S (ACD-SS group), IR levels diminished appreciably in the first hour of reperfusion ($p < 0.05$ vs. ACD group) with a behavior comparable to that of livers harvested without a warm ischemic period.

The *Oxygen consumption* of 4 livers in each group, was followed throughout *ex vivo* reperfusion as a viability indicator, and displayed in Fig. 2(B). O_2 uptake from perfusate in ACD group was considerable inferior when compared to BCD group ($p < 0.05$), revealing the consequences of warm ischemia at the time of procurement. However, when warm ischemic livers were exposed to H_2S during cold storage (ACD-SS group), O_2 uptake during reperfusion was appreciably recovered ($p < 0.05$ vs. ACD group).

The *Bile production* of 4 livers in each group was evaluated in the isolated system and illustrated in Fig. 2(C). This important marker of liver function was initially impaired in livers procured after

Table 2
List of primers used in quantitative polymerase chain reaction.

Target gene	Accession number	Amplicon length (bp)	Forward primer sequence (5′ → 3′)	Reverse primer sequence (5′ → 3′)
18S	X_01117	150	TAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
β-actin	NM_031144	79	CAACCTTCTTGCAGCTCCTC	GACGAGCGCAGCGATATC
HO-1	NM_012580	76	GGTGATGGCCTCCTTGTA	ATAGACTGGGTTCTGCTTGT
TNF-α	NM_012675	139	TGTCTACTGAACTTCGGGGTGATC	CGTGGGCTACGGGCTTGTC
Bcl-2	L_14680	123	TCTGTGGATGACTGAGTA	AGCCAGGAGAAATCAAAC
GSR	NM_053906	125	CGTGGATTACGGCTTTCA	CGATGTGGGACTTGGTTA

Abbreviations: 18S, 18S ribosomal RNA; HO-1, heme oxygenase-1; TNF-α, tumor necrosis factor alpha; Bcl-2, B-cell lymphoma 2; GSR, glutathione-disulfide reductase.

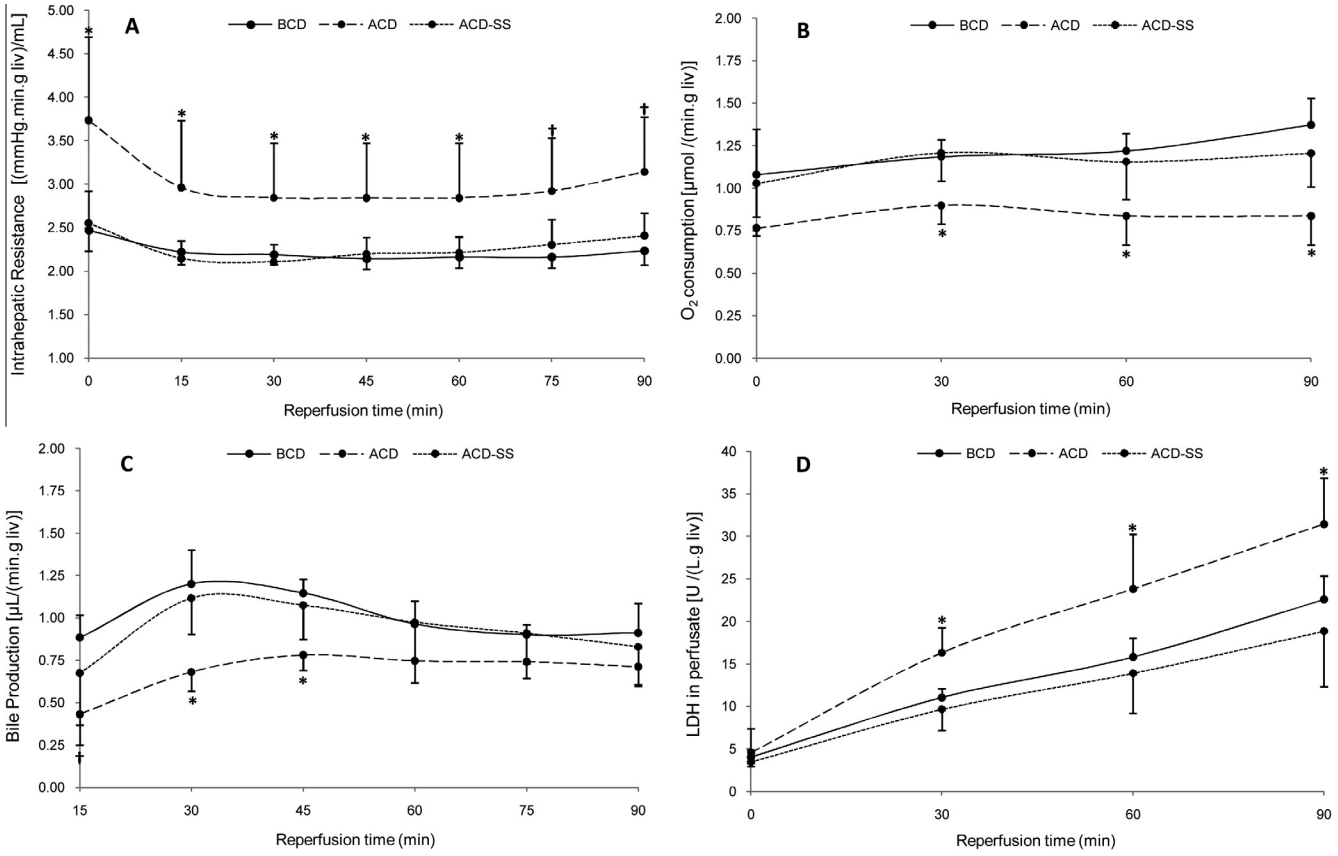

Fig. 2. Liver viability parameters during 90 min of *ex vivo* reperfusion. (A) *Intrahepatic resistance* (mmHg min g liv mL^{-1}) was calculated every 15 min as a function of perfusion flow at a constant perfusion pressure of 7.36 mmHg. (B) *Oxygen consumption* (µmol min^{-1} g liv^{-1}) was determined at 30 min intervals from samples of liver inflow and outflow perfusate. (C) *Bile production* (µL min^{-1} g liv^{-1}) was estimated gravimetrically by successive collection of bile in pre-weighed tubes during 15 min. (D) *Lactate dehydrogenase (LDH) in perfusate* (U L^{-1} g liv^{-1}) was assayed in samples taken at 30 min intervals. Results are expressed as the mean ± SD ($n = 4$). A significant difference from BCD and ACD-SS groups was denoted by an asterisk ($^{*}p < 0.05$), and a significant difference from BCD group only was denoted by a dagger ($^{\dagger}p < 0.05$). Statistical analysis was performed by ANOVA, followed by Tukey's HSD post hoc analysis. *Abbreviations:* BCD, before cardiac death; ACD, after cardiac death; ACD-SS, after cardiac death – Sodium Sulfide administration.

cardiac death (ACD group) ($p < 0.05$ vs. BCD group). However, when Na_2S was added to the preservation media (ACD-SS), bile secretion significantly increased in comparison to non-treated warm ischemic livers ($p < 0.05$ vs. ACD).

The values of *LDH in perfusate* of 4 livers in each group was periodically assayed during isolated perfusion and illustrated in Fig. 2(D). Livers excised after cardiac death (ACD group) showed substantial cell damage during the reperfusion stage when compared with livers excised before cardiac death that did not experience a warm ischemic period (BCD group) ($p < 0.05$). Again, livers from the ACD-SS group were better preserved during cold storage, as indicated by the significant decrease in LDH release into perfusate ($p < 0.05$ vs. ACD).

3.3. Parameters of liver physiology after ex vivo reperfusion

Livers were able to synthesize urea, eliminate BSP from perfusate, and maintain glycogen stores as shown in Table 3. There were no statistical differences in the urea synthesis ratio between different experimental groups ($p = 0.06$). Still, livers from cardiac dead donors (ACD group) presented the lowest rates of Urea synthesis revealing that the presence of a H_2S source in the storage media might help preserve liver metabolic function. This was also reflected on the BSP excretion feature, a pathway that requires various enzymatic steps and functional transporters; livers harvested after cardiac death and stored in standard HTK solution (ACD group) showed a noticeable impairment when compared to BCD and ACD-SS groups ($p < 0.05$). Finally, we observed that glycogen stores were severely diminished in livers that underwent a warm ischemic period as expected, moreover, H_2S treatment did not change this fact (groups ACD and ACD-SS) ($p < 0.01$ vs BCA group).

Table 3
Parameters of liver physiology after 90 min of *ex vivo* reperfusion: urea synthesis, percentage of BSP excreted in bile and glycogen content.

Parameter	Group BCD	Group ACD	Group ACD-SS
nmol Urea min^{-1} g liv^{-1}	216 ± 26	160 ± 37	207 ± 26
% of BSP in bile*	97.65 ± 1.57	85.80 ± 5.52†	95.50 ± 5.39
mg glycogen g liv^{-1}	5.00 ± 1.24‡	1.57 ± 0.45	2.16 ± 0.58

Note: Data are expressed as the mean ± SD, ($n = 4$).
Abbreviations: BSP, bromosulfophthalein; BCD, before cardiac death; ACD, after cardiac death; ACD-SS, after cardiac death – Sodium Sulfide administration.
* Initial dose of BSP was 5 mg (100%).
† Significantly different from groups BCD and ACD-SS: $p < 0.05$, ANOVA, followed by Tukey's HSD post hoc analysis.
‡ Significantly different from groups ACD and ACD-SS: $p < 0.01$, ANOVA, followed by Tukey's HSD post hoc analysis.

3.4. Parameters of oxidative stress after ex vivo reperfusion

Samples of 4 livers of each experimental group, obtained after *ex vivo* reperfusion, were tested for signs of oxidative stress such as glutathione depletion, malondialdehyde accumulation, and catalase activity (Table 4). None of these parameters revealed a

Table 4
Parameters of oxidative stress in liver biopsies after 90 min of *ex vivo* reperfusion: total glutathione, malondialdehyde content and catalase activity.

Parameter	Group BCD	Group ACD	Group ACD-SS
Total GSH [μmol g liv^{-1}]	4.87 ± 0.83	5.60 ± 0.70	4.52 ± 0.48
MDA content [nmol g liv^{-1}]	399.76 ± 62.87	426.16 ± 25.83	424.50 ± 87.05
Catalase activity (k) [s^{-1} g liv^{-1}]	17.89 ± 4.07	22.89 ± 4.40	20.96 ± 3.75

Note: Data are expressed as the mean ± SD, ($n = 4$). There were no statistical differences in any case, ANOVA.
Abbreviations: GSH, glutathione; MDA, malondialdehyde; BCD, before cardiac death; ACD, after cardiac death; ACD-SS, after cardiac death – Sodium Sulfide administration.

visible change in the redox balance between livers procured before (BCD group) or after cardiac death in presence or absence of Na_2S (ACD and ACD-SS groups).

3.5. Histopathological studies

Representative photomicrographs of Hematoxylin/Eosin stained liver sections are presented in Fig. 3. All experimental groups showed a rather conserved histology with organized hepatocyte plates, lack of necrotic/apoptotic areas and well-structured portal triads. At first glance, parenchyma of livers obtained after cardiac death (ACD group) presented a further deteriorated aspect when contrasted to BCD group, but only the endothelial cell damage score was significantly increased ($p < 0.05$). Nevertheless, livers from cardiac dead donors treated with Na_2S (ACD-SS group), revealed a significant decrease in sinusoid widening score when compared to non-treated grafts (ACD group) ($p < 0.01$).

3.6. Quantitative Real-Time PCR analysis

Samples from 4 livers in each group were taken and processed to obtain total RNA. Results obtained from mRNA expression analysis are shown in Fig. 4.

We found that HO-1 mRNA levels were highly increased after cold storage/reperfusion in livers from cardiac dead donors (ACD group) ($p < 0.01$ vs. BCD group). On the other hand, when warm ischemic grafts were stored in HTK with a source of H_2S (ACD-SS group) HO-1 mRNA expression was significantly reduced ($p < 0.01$ vs. ACD group), and even matched BCD group levels (Fig 4(A)).

TNF-α mRNA levels were 2 times higher after reperfusion in livers that experienced a warm ischemic period of 45 min before cold storage (groups ACD and ACD-SS) ($p < 0.05$ vs. BCD group); which means that H_2S exposure in this setting, did not promote any appreciable changes regarding anti-inflammatory signaling (Fig 4(B)).

Bcl-2 mRNA levels were diminished in post-reperfusion tissue samples of livers excised after cardiac death (ACD group) ($p < 0.05$ vs. BCD group). However, when these organs were cold stored in HTK with Na_2S (ACD-SS group), Bcl-2 mRNA expression did not differ from that of BCD group (Fig 4(C)).

GSR mRNA levels were not differentially expressed in any group (Fig 4(D)).

4. Discussion

The present work focused on the potential cytoprotective role of H_2S during cold storage of marginal grafts. For that purpose, we modified a methodology from García Valdecasas et al. [13] to obtain rat livers after cardio-respiratory arrest, those grafts were stored in cold preservation solution (24 h) in the presence or absence of a H_2S

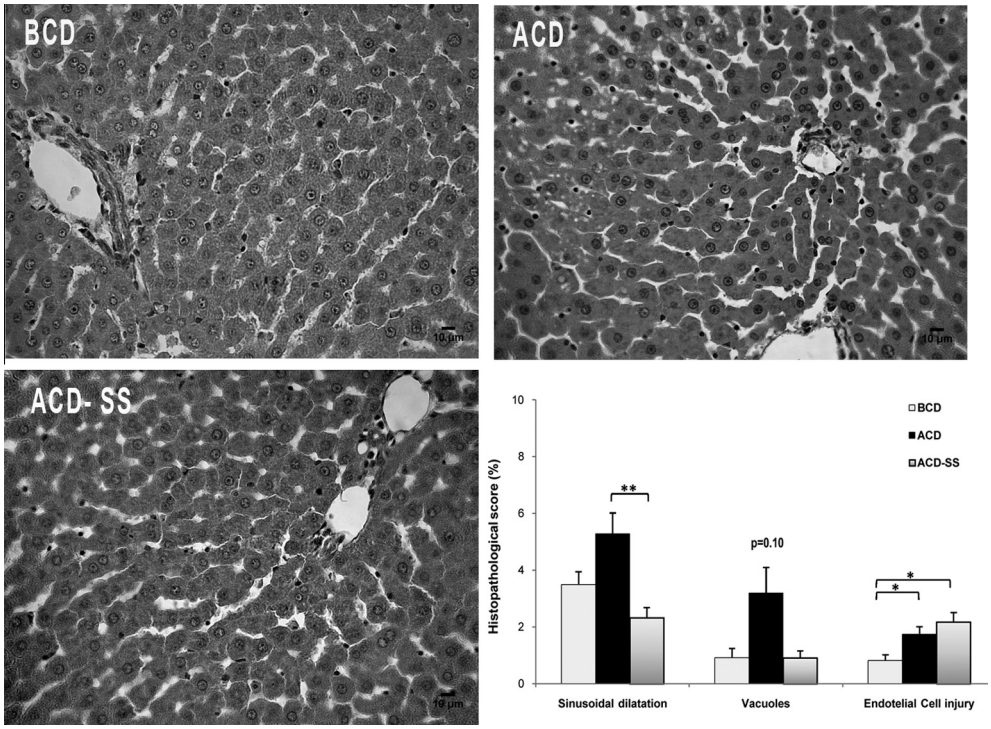

Fig. 3. Histopathological studies. Hematoxilin/eosin stained liver sections after 90 min of *ex vivo* reperfusion were analyzed by light microscopy. Representative photographs (400× magnification) from the 3 experimental groups are shown. Histopathological observations such as sinusoidal dilatation, vacuolated cytoplasm and endothelial cell rounding and retraction were semi-quantified by a scoring method. Results are expressed as the mean ± SEM ($n = 30$). A significant difference was denoted by one or two asterisk ($^{*}p < 0.05$ and $^{**}p < 0.01$). Statistical analysis was performed by Kruskal–Wallis test, followed by Dunn's post hoc analysis. *Abbreviations:* BCD, before cardiac death; ACD, after cardiac death; ACD-SS, after cardiac death – Sodium Sulfide administration.

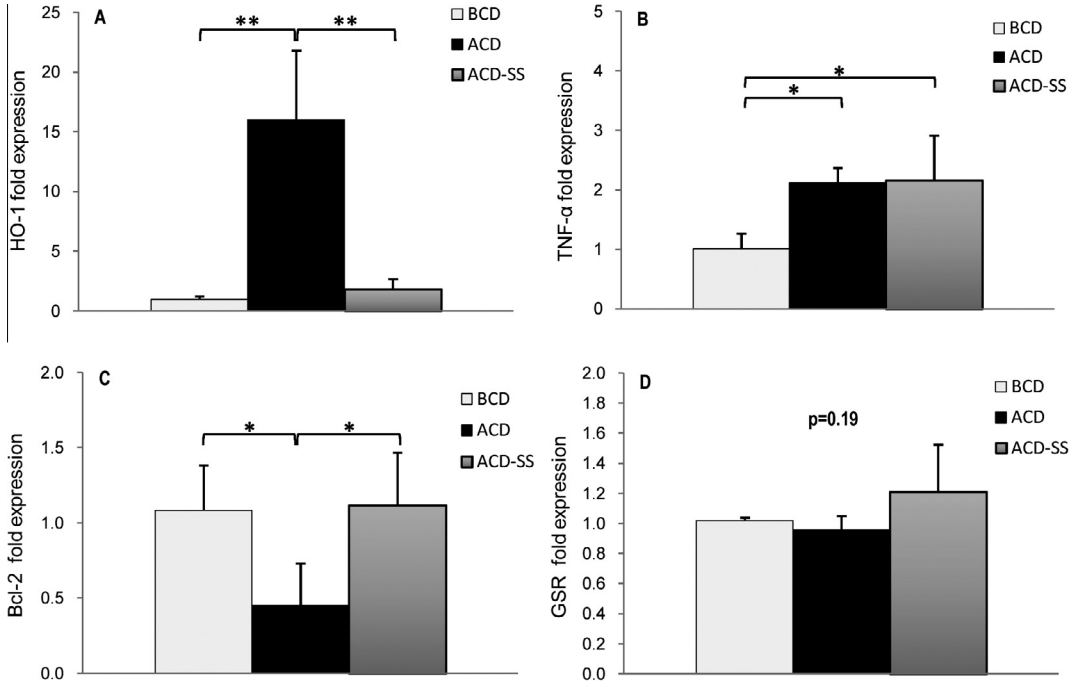

Fig. 4. Quantitative Real-Time PCR analysis of mRNA encoding (A) HO-1, (B) TNF-α, (C) Bcl-2 and (C) GSR proteins. Data were normalized to 2 housekeeping genes (β-actin and 18S) and mRNA expression levels were expressed relative to BCD group (arbitrarily set to 1). Results are expressed as the mean ± SD ($n = 4$). A significant difference was denoted by one or two asterisk ($^{*}p < 0.05$ and $^{**}p < 0.01$). Statistical analysis was performed by ANOVA, followed by Tukey's HSD post hoc analysis. *Abbreviations:* BCD, before cardiac death; ACD, after cardiac death; ACD-SS, after cardiac death – Sodium Sulfide administration; HO-1, Heme oxygenase-1; TNF-α, tumor necrosis factor alpha; Bcl-2, B-cell lymphoma 2; GSR, glutathione-disulfide reductase.

donor and subsequently reperfused in an isolated system to evaluate different parameters of hepatic patho-physiology. Furthermore, liver samples were collected at the end of reperfusion to assay oxidative stress markers and to study the level of expression of genes related to ischemic insult.

H_2S was originally recognized for its toxicity as a consequence of its binding to cytochrome c oxidase [30]. The degree of mitochondrial impairment depends on the experimental approach, in intact cells mitochondrial respiration is decreased 50% when exposed to 30 μM NaHS (normal value: 4.36 ± 0.14 nmol $O_2/10^6$ cells/min) [20]. Nonetheless, H_2S physiological significance became clear when it was demonstrated that it is naturally produced in several organisms by constitutively expressed enzymes. We added Na_2S to HTK preservation solution (10 μM) at the time of graft procurement and determined that final concentrations of H_2S were around 7–5 μM from the beginning until the end of cold storage. This range of concentrations is close to the *in vivo* normal blood level of H_2S reported for Wistar rats (~10 μM) [21].

In our rat model of procurement post cardiac death, warm ischemia was initiated with heart beat cessation and continued for 45 min. This model allowed us to study the patho-physiological response of marginal livers treated with a H_2S donor salt (Na_2S) to confer extra protection during cold ischemia and normothermic reperfusion. Grafts from cardiac dead donors preserved in HTK without Na_2S, exhibited microcirculation disturbances along with poor bile and urea production, impaired BSP elimination and lower oxygen consumption. There were also signs of tissue congestion and cell injury according to histological analysis and LDH release levels. Glycogen stores were noticeably affected by previous warm ischemia, as anaerobic metabolism rapidly depletes energy substrates. On the other hand, when livers from non-heart beating donors were exposed to H_2S during cold storage, results showed improved microcirculation parameters, prevention in the sinusoid widening, which may be related to the effect of H_2S on intrahepatic resistance, and higher LDH retention. Previous studies reported that this gaseous transmitter exerted vasoactive properties through KATP-channel opening [26], vaso-relaxation decreases flow resistance inside liver sinusoids, which would diminish tissue congestion, alleviating damage and ensuring better oxygen and nutrient delivery to maintain functioning hepatic cells. H_2S has been recognized by its respiration regulating properties that preserves mitochondria in hypoxia/reoxygenation events [10]; this might explain how oxygen consumption rates seemed to be recovered in Na_2S treated grafts. In addition, these treated grafts displayed a well recuperated bile secretion function along with BSP elimination percentages that did not differ from heart-beating procurated livers.

Real time PCR studies revealed that after *ex vivo* reperfusion of cardiac dead donated livers there is an increased expression of HO-1 and TNF-α, and a modest drop in Bcl-2 mRNA, which could be interpreted as the cells response to the previous warm ischemia insult sustained by heart arrested donors. Particularly, HO-1 known by its cytoprotective role in oxidative stress [31], was strongly up-regulated (16-fold) with respect to control livers after reperfusion. As reported previously [36], while HO-1 is a critically important cytoprotective gene, the degree of up-regulation in response to an insult may serve as a marker of injury. Therefore, the decreased level of expression of HO-1 in warm ischemic livers exposed to H_2S during cold storage might support evidence of cytoprotective activity upon reperfusion. Apoptotic cell death during reperfusion has been implicated as an important contributor to reperfusion-induced injury. Therefore, targeting anti-apoptotic mechanisms may offer a potential strategy to attenuate reperfusion-induced cell death. Antiapoptotic signaling, represented by Bcl-2 mRNA, was rather superior in warm ischemic livers preserved in HTK + Na_2S when compared to those stored in standard HTK. Nevertheless, H_2S graft exposure was not able to limit pro-inflammatory cytokine (TNF-α) induction, involved in ischemia/reperfusion injuries in transplantation [19]. We would expect that TNF-α mRNA to decrease due to anti-inflammatory

properties attributed to H_2S, but in our experimental conditions there might be other factors affecting the inflammatory cascade. Glutathione reductase (GSR) catalyzes the reduction of glutathione disulfide to the sulfhydryl form glutathione, a critical molecule to ensure the reducing environment of the cell. In our experimental conditions GSR levels of expression did not change as a consequence of previous warm ischemia but neither did other biomarkers of oxidative stress such as lipid peroxidation derivatives (MDA), total GSH depletion or catalase activity.

It is possible that 45 min of previous warm ischemia are not enough to engage in a redox imbalance such that it could be measured by the selected parameters that were assayed in biopsies taken up to 90 min of *ex vivo* reperfusion. Furthermore, endogenous cytoprotective mechanisms triggered by hypoxia (e.g., HO-1 up-regulation) could be responsible, to some extent, for oxidative stress protection. Extended reperfusion times might be needed to reveal oxidative liver injuries in our model.

In conclusion, we have adapted a rat model of liver procuration post cardiac death. This experimental approach allowed us to study the potential benefits of H_2S treatment during routine static cold storage of organs. Na_2S reconditioning strategy improved liver microcirculation, morphology and function during *ex vivo* reperfusion. Moreover, expression of a stress related gene like HO-1 was modulated during cold ischemia/reperfusion of warm ischemic livers.

These promising findings are meant to be the groundwork for new alternatives in the organ preservation field, making use of therapeutic properties of gaseous transmitters molecules to expand the pool of transplantable organs. Further studies regarding delivery systems, dose–response, and experimental models are crucial in order to translate this concept into the clinical practice.

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Conflicts of interest

None.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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