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Original Paper

A novel form of the human manganese superoxide dismutase protects rat and human livers undergoing ischaemia and reperfusion injury

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Abstract

Hepatic microcirculatory dysfunction due to cold storage and warm reperfusion (CS + WR) injury during liver transplantation is partly mediated by oxidative stress and may lead to graft dysfunction. This is especially relevant when steatotic donors are considered. Using primary cultured liver sinusoidal endothelial cells (LSECs), liver grafts from healthy and steatotic rats, and human liver samples, we aimed to characterize the effects of a new recombinant form of human manganese superoxide dismutase (rMnSOD) on hepatic CS + WR injury. After CS + WR, the liver endothelium exhibited accumulation of superoxide anion (O_2^-) and diminished levels of nitric oxide (NO); these detrimental effects were prevented by rMnSOD. CS + WR control and steatotic rat livers exhibited markedly deteriorated microcirculation and acute endothelial dysfunction, together with liver damage, inflammation, oxidative stress, and low NO. rMnSOD markedly blunted oxidative stress, which was associated with a global improvement in liver damage and microcirculatory derangements. The addition of rMnSOD to CS solution maintained its antioxidant capability, protecting rat and human liver tissues. In conclusion, rMnSOD represents a new and highly effective therapy to significantly upgrade liver procurement for transplantation.

Key words: cold storage, endothelium, liver sinusoidal endothelial cell (LSEC), oxidative stress, transplantation

INTRODUCTION

Liver transplantation is the only curative treatment for end-stage liver diseases. Although remarkable improvement in graft survival has been achieved during previous years, early organ damage continues to be an important problem and remains a major focus of therapeutic attention [1]. In addition, liver transplantation rates are limited by the shortage of adequate organs for clinical use, which have led to the use of steatotic liver grafts. Unfortunately, steatotic livers are more susceptible to ischaemia/reperfusion (I/R) injury, exhibit poorer outcome, and are associated with increased risk of primary graft dysfunction [2,3].

I/R injury is the phenomenon of deprivation and afterwards restoration of oxygen and blood-derived shear stress stimulation during the transplantation setting. In most liver transplant procedures, I/R injury derives from hypothermic preservation followed by warm reperfusion periods [cold storage and warm reperfusion (CS + WR)]. CS + WR is poorly tolerated by liver grafts, and liver sinusoidal endothelial cells (LSECs) represent one of the most affected cell types, which rapidly develop severe alterations including cell activation and apoptosis [4,5]. These

Abbreviations: Ach, acetylcholine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CS + WR, cold storage and warm reperfusion; DAF, 4-amino-5-methylamino-2′,7′-difluorofluorescein; DHE, dihydroethidium; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, haematoxylin and eosin; HFD, high-fat diet; ICAM-1, intracellular adhesion molecule-1; I/R, ischaemia/reperfusion; KLF2, Kruppel-like factor 2; LDH, lactate dehydrogenase; LSEC, liver sinusoidal endothelial cell; NOx, nitrites/nitrates; O_2^-, superoxide anion; rMnSOD, recombinant form of human manganese superoxide dismutase; ROS, reactive oxygen species; SOD, superoxide dismutase; vWF, von Willebrand factor.

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de-regulations cause acute endothelial dysfunction development, which correlates with poorer liver transplantation outcome [6,7].

Different mechanisms for endothelial damage during CS + WR have been described and include inflammation, vasoconstriction cascades and oxidative stress [5,8–11]. Indeed, during CS, lack of oxygen causes accumulation of respiratory chain intermediates which, during WR, are rapidly converted into reactive oxygen species (ROS) [12]. These ROS can cause important damages in DNA and protein structure and function. Furthermore, an excess of ROS acts as a scavenger of nitric oxide (NO), forming peroxynitrite [13] and further negatively affecting cell viability. Previous studies have investigated the role of reducing oxidative stress in liver grafts preserved for transplantation, showing partially positive results [14,15]; however, none of them has specifically evaluated the role of CS + WR-derived oxidative stress on the hepatic microcirculation.

Recently, a novel recombinant form of human manganese superoxide dismutase (rMnSOD) has been developed [16]. This new formulation of a key superoxide anoin (O_2^-)-degrading protein remains stable in solution, has a good biodistribution in all organs, effectively scavenges intra- and extra-cellular O_2^-, freely enters the cells and is constitutively active in the cytoplasm, nucleus and mitochondria [17,18]. The aim of the present study was to investigate whether rMnSOD could be a new therapeutic strategy to reduce hepatic and microcirculatory status of liver grafts preserved for transplantation.

MATERIALS AND METHODS

Animals and treatment

Male Wistar and Sprague–Dawley rats from the Charles River Laboratories weighing 300–325 g were used. Liver steatosis was induced by feeding animals with a safflower oil-based high-fat diet (HFD; 28% carbohydrates, 58% fat, 14% protein; #5ALX, TestDiet) for 7 days, as previously described by our group [11,19]. Livers from HFD-fed animals exhibited over 75% of hepatocytes with macro-vesicular fat, an 11.2-fold increase in lipid content determined by Oil Red staining, and significantly elevated levels of non-esterfied ('free') fatty acids (4.3 ± 0.5 compared with 6.0 ± 0.3 μmol/g) and triacylglycerol (1.0 ± 0.1 compared with 4.0 ± 0.4 mg/g). Animals were kept in environmentally controlled animal facilities at the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS). All experiments were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with the European Community guidelines for the protection of animals used for experimental and other scientific purposes (EEC Directive 86/609).

LSEC isolation and cold preservation

LSECs from control rats were isolated as described previously [20]. Highly pure and viable cells were used. After 1 h of isolation, LSECs were washed with PBS and incubated for 16 h at 37 °C (no CS group) or at 4 °C in Celsior solution (Genzyme) supplemented with rMnSOD (0.15 μM) or its vehicle (PBS). After this period, cells were incubated for 1 h at 37 °C in culture medium to partially mimic the WR period, and *in situ* determination of O_2^- and NO intracellular levels was performed as described in the following sections.

Liver vascular studies

Hepatic I/R injury was induced using the *ex vivo* model of liver CS + WR as previously described [5,21,22]. Although this experimental approach does not allow polymorphonuclear neutrophil infiltration, it indeed reproduces hepato-endothelial cell injury, inflammation, and microcirculatory dysfunction observed in transplantation models.

Under anaesthesia with intraperitoneal ketamine (100 mg/kg, Merial Laboratories) and midazolam (5 mg/kg, Baxter), rats were treated via the femoral vein with rMnSOD (50 μg/kg for controls and 150 μg/kg for steatotic rats), or its vehicle, 30 min before liver isolation (doses based on preliminary results in the present study, results not shown).

Liver vascular responses were assessed in the isolated, *in situ* liver perfusion system, as described previously [5,11,23]. Baseline pressures at a constant portal flow of 30 ml/min were recorded after 20 min of stabilization; afterwards, livers were flushed with cold Celsior solution and then cold-stored for 16 h in Celsior solution.

After CS, livers were exposed at room temperature (22 °C) for 20 min to mimic the normothermic ischaemia period and reperfused through the portal vein with Krebs buffer (37 °C). The perfused livers were continuously monitored for 1 h. Afterwards, liver endothelial function was evaluated analysing endothelium-dependent vasorelaxation in response to incremental doses of acetylcholine (Ach: 10^{-7} to 10^{-5} M) after pre-constriction with methoxamine (10^{-4} M) [24].

Control livers (no CS) were perfused, harvested, and immediately reperfused *ex vivo*. Aliquots of the perfusate were sampled for the measurement of transaminases and lactate dehydrogenase (LDH) using standard methods at the Hospital Clinic of Barcelona's CORE laboratory.

Histological analysis

Liver samples were fixed in 10% formalin, embedded in paraffin, and sectioned, and slides were stained with haematoxylin and eosin (H&E) to analyse the hepatic parenchyma [25]. Hepatic histology was analysed and scored by a third researcher under blinded conditions. To detect neutral lipids, snap-frozen livers were fixed in a freezing medium (Jung, Leica Microsystems) and stained with Oil Red for 2 h at room temperature [19].

The samples were photographed and analysed using a microscope equipped with a digital camera and the assistance of AxioVision software (Zeiss). Five fields of each sample were randomly selected, photographed at a magnification of 40× with an inverted optical microscope equipped with a digital camera (Zeiss Axiovert) and then quantified using AxioVision software. The red-stained area per total area was determined using a morphometric method. The results were expressed as a steatosis ratio (%), calculated as the ratio of the Oil Red-positive area to the total area.

O_2^- and NO detection

In situ O_2^- and NO levels in LSECs and hepatic tissue were assessed with the oxidative fluorescent dye dihydroethidium (DHE; 10 μM; Molecular Probes) or with 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA; 10 μM; Molecular Probes) as described previously [13,26,27]. Specificity of the assays was ensured using superoxide dismutase (SOD; 200 units/ml) or N^G-nitro-L-arginine methyl ester (L-NAME) (1.5 mM) as negative controls [23,28]. Fluorescence images were obtained with a fluorescence microscope (Olympus BX51), and quantitative analysis of at least 20 images per condition was performed with ImageJ 1.44 m software (National Institutes of Health).

In addition, levels of cGMP, a marker of NO bioavailability, were analysed in liver homogenates using an enzyme immunoassay (Cayman Chemical) as previously described [5].

Hepatic nitrites/nitrates (NOx) production was assessed in aliquots of perfusate using specific microelectrodes (Lazar Laboratories).

SOD activity

Total SOD activity was determined using a commercially available kit (Superoxide activity assay kit, Cayman Chemical). Briefly, livers were homogenized in buffer containing 20 mM Hepes, 1 mM EDTA, 210 mM mannitol and 70 mM sucrose. After centrifugation at 1500 ***g*** for 5 min at 4 °C, the supernatant was collected and the protein concentration was quantified. SOD activity assay was performed according to the manufacturer's instructions.

Nitrotyrosine and von Willebrand factor immunohistochemistry

After antigen-retrieval procedure and endogenous peroxidase activity inhibition, sections were incubated with anti-nitrotyrosine (1:200 dilution; Millipore) or anti-von Willebrand factor (vWF; 1:400 dilution; Dako) for 1 h at room temperature. Horseradish peroxidase (HRP)-conjugated rabbit/mouse (Dako) secondary antibody was added. Colour development was induced by incubation with a diaminobenzidine (DAB) kit (Dako), and the sections were counterstained with haematoxylin. Sections were dehydrated and mounted. The specific staining was visualized, and images were acquired using a microscope equipped with a digital camera and the assistance of AxioVision software. vWF and nitrotyrosine relative volume was determined by point-counting morphometry on immunoperoxidase-stained sections, using a point grid to obtain the number of intercepts over vWF/nitrotyrosine-positive cells over the tissue. Six fields were counted in each liver. All measurements were performed by two independent blinded observers. The relative volume was calculated by dividing the number of points over that particular cell type by the total number of points over liver tissue.

Nitrotyrosine fluorohistochemistry

Quantitative tyrosine nitration detection was performed as previously described [29]. Briefly, slides were deparaffinized, hydrated, incubated with aqueous sodium dithionite solution (10 mM) for 10 min, washed with distilled water and then incubated overnight at 4 °C with an equimolar solution of aluminium chloride and salicylaldehyde (200 μM). Afterwards, the aqueous solution was removed, and sections were mounted in Fluoromount G medium (Southern Biotech). Negative and positive internal controls were included. Fluorescence images were obtained with a fluorescence microscope, and quantitative analysis of at least six images per sample was performed with ImageJ 1.44 m software.

Western blot analysis

Liver samples were processed and Western blot analysis was performed as described previously [23]. Primary antibodies against endothelial nitric oxide synthase (eNOS) (BD Transduction Laboratories) and intracellular adhesion molecule 1 (ICAM-1) (R&D Systems), both at 1:1000 dilution, were used. Blots were revealed by chemiluminescence, and protein expression was determined by densitometric analysis using the Science Lab 2001, Image Gauge (Fuji Photo Film). Blots were also assayed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology) content as standardization of sample loading.

Glutathione levels and catalase activity

Total hepatic glutathione was determined as previously described [30]. Briefly, in the presence of glutathione reductase (50 units/ml), total GSH reacts with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB; Sigma) to generate 2-nitro-5-thiobenzoic acid, a yellow compound absorbing at 412 nm.

To measure catalase activity, liver homogenates containing same amount of protein were mixed with 30 mM hydrogen peroxide (H_2O_2) (Panreac) and 50 mM of phosphate buffer, and the absorbance was measured for 60 s. The enzymatic activity was calculated using the H_2O_2 molar absorbance coefficient ($\varepsilon = 0.0436\ mM^{-1} \cdot cm^{-1}$) [31].

rMnSOD as a CS solution supplement for rat and human livers

Rats were anaesthetized, the abdomen was opened and liver was washed with saline solution. Liver biopsies were taken and preserved 16 h at 4 °C in Celsior solution supplemented with rMnSOD (0.15 μM), or its vehicle. Afterwards, an *in vitro* WR period was mimicked incubating liver biopsies in complete culture medium for 1 h at 37 °C. At the end of the study, tissue was snap-frozen for O_2^- detection using DHE staining.

Furthermore, O_2^- levels were evaluated in human liver samples obtained from healthy donors accepted for liver transplantation. A biopsy from each donor was divided into the following two parts: (i) cold-stored for 16 h in Celsior solution and (ii) cold-stored for 16 h in Celsior solution with 0.15 μM rMnSOD. After this time, liver tissues were incubated for 1 h at 37 °C in culture medium, and O_2^- levels were determined. The present study was approved by the Ethical Committee of the Hospital Clinic de Barcelona.

Analysis of hepatic triacylglycerol and non-esterified fatty acids

Frozen livers samples were homogenized (1:3, w/v) in buffer composed of 50 mM Tris, 150 mM NaCl, and 5 mM EDTA, and

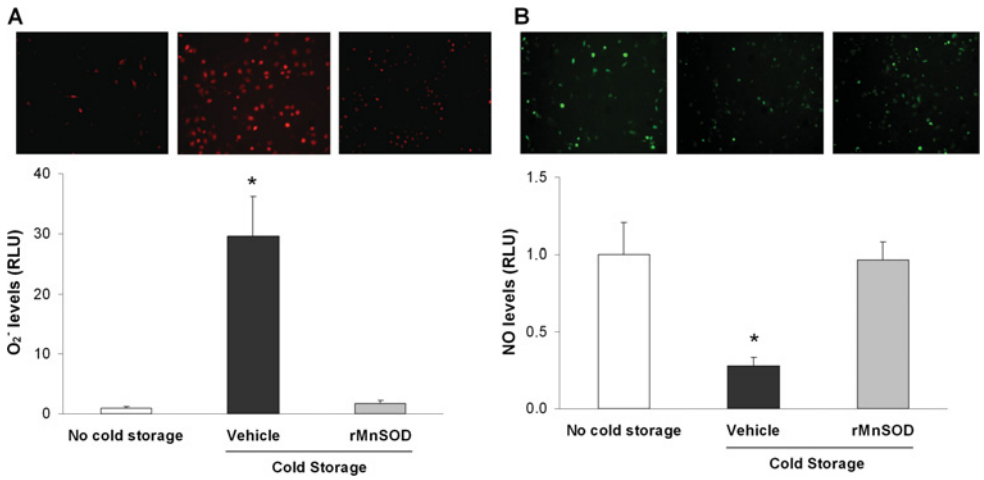

Figure 1 **rMnSOD prevents O_2^- accumulation and maintains NO levels in LSECs**
Freshly isolated LSECs were incubated for 16 h at 37 °C (control group) or at 4 °C in Celsior solution supplemented with rMnSOD, or its vehicle. (**A**) Endothelial oxidative stress was assessed as O_2^- levels. (**B**) LSEC NO levels were determined by DAF staining. Fluorescent intensity was divided by the total number of cultured cells. (Images ×20; $n = 5$ per group; $^*P < 0.01$ compared with no CS and rMnSOD).

triacylglycerols and non-esterified fatty acids were analysed with standard methods at the Hospital Clinic de Barcelona CORE lab.

Statistical analysis

Statistical analyses were performed with the IBM SPSS Statistics 19 for Windows statistical package. All results are expressed as means ± S.E.M. Comparisons between groups were performed with ANOVA followed by least-squares difference (LSD) test, or with Student's t test or Mann–Whitney U test when adequate. Differences were considered significant at $P < 0.05$.

RESULTS

rMnSOD prevents O_2^- accumulation and maintains NO levels in LSECs

Cold-stored and warm-reperfused LSECs exhibited significantly higher levels of O_2^- (Figure 1A) and reduced NO (Figure 1B) compared with no cold-stored cells. These detrimental effects of CS + WR were prevented in LSECs preserved with rMnSOD.

rMnSOD pre-treatment prevents O_2^- accumulation and improves viability of cold-stored and warm-reperfused control livers

As shown in Figure 2(A), CS + WR markedly increased O_2^- levels in hepatic tissue without significant changes in SOD activity (Figure 2B). Rats pre-treated with a single dose of rMnSOD exhibited significantly increased hepatic SOD activity, which led to reduced levels of O_2^-, demonstrating that intravenously administered rMnSOD reaches the liver where it is functionally active.

Furthermore, cold-stored and warm-reperfused livers exhibited hepatocellular lesions, mainly in centrilobular areas, defined by loss of cohesion of cell plates, hepatocyte necrosis, the presence of Councilman bodies and anoxia-derived small fat vacuoles (Figure 2A). Hepatocellular damage was accompanied by increased levels of ICAM-1 and a significantly greater release of transaminases and LDH in comparison with no cold-stored grafts (Figures 2C and 2D). Pre-treatment with rMnSOD significantly reduced, or even prevented, these parameters of liver injury (Figures 2A, 2C and 2D).

rMnSOD improves microcirculation and endothelial function in cold-stored and warm-reperfused control livers

Livers cold-stored for 16 h exhibited significantly deteriorated microcirculation upon reperfusion, as demonstrated by the markedly increased portal perfusion pressure compared with no cold-stored livers. Hepatic microcirculation de-regulation was prevented in liver grafts from rats pre-treated with rMnSOD (Figure 3A).

In addition, cold-stored and warm-reperfused livers exhibited endothelial dysfunction defined as a significant reduction in the endothelium-derived vasodilatation in response to Ach in comparison with no cold-stored livers. Liver vasorelaxation was significantly improved in rats pre-treated with rMnSOD (Figure 3A).

Interestingly, development of acute endothelial dysfunction caused by CS + WR was accompanied by a decrease in eNOS protein expression (Supplementary Figure S1 at http://www.clinsci.org/cs/127/cs1270527add.htm) and diminished NO production and bioavailability, measured by the release of NOx and cGMP respectively (Figure 3B), together with increased intrahepatic accumulation of nitrotyrosinated proteins (Figure 3C and Supplementary Figure S1). rMnSOD pre-treatment was effective in improving NO bioavailability, most probably through a reduction in its scavenging as demonstrated by diminished levels of nitrotyrosinated proteins (Figure 3C and Supplementary Figure S1).

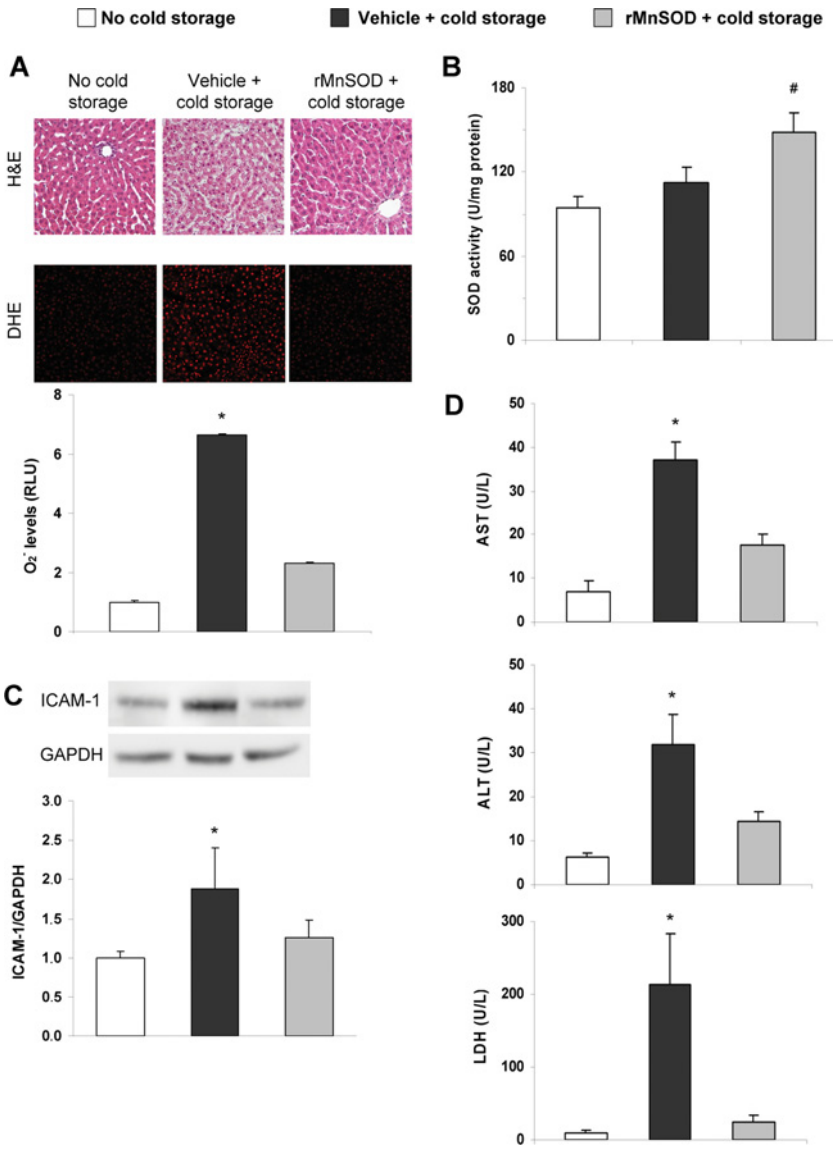

Figure 2 **rMnSOD improves hepatic I/R injury in control grafts**

Hepatic damage was evaluated at the end of WR in grafts not cold-stored (control group) and in livers from rats receiving rMnSOD, or its vehicle, after 16 h of CS. (**A**) Upper: hepatic morphological changes were assessed by H&E staining; lower: representative images of oxidative stress detection using DHE staining and quantitative analysis (all images ×20). (**B**) SOD activity determined in liver tissue. (**C**) Representative hepatic ICAM-1 immunoblot and densitometric analysis normalized to GAPDH. (**D**) Hepatic injury measured as release of transaminases (AST and ALT) and LDH. ($n = 8$ per group; $^{*}P < 0.05$ compared with no CS and rMnSOD; $^{\#}P < 0.05$ compared with no CS and vehicle). RLU, relative light units.

CS + WR induced a significant increase in the hepatic expression of the LSEC capillarization marker vWF, which was prevented by administering rMnSOD (Figure 3C).

rMnSOD prevents hepatic O_2^- accumulation and improves liver microcirculation and endothelial function in rats with steatosis

As shown in Figure 4(A), analysis of hepatic histology showed that HFD-fed rat livers exhibited massive micro- and macrovesicular fat deposition in all cases, characterized by the presence of multiple small vacuoles surrounding the nuclei of hepatocytes and large fat vacuoles distorting the nuclei respectively (Figure 4A).

Although rMnSOD was effective in maintaining SOD activity during CS + WR and, therefore, preventing hepatic O_2^- accumulation when administered to dietary-induced steatotic rats (Figures 4B and 2C), it was not associated with a reduction in hepatocellular damage biochemical markers (AST: 122.8 ± 28.2 units/l in vehicle compared with 110.1 ± 15.5 units/l in rMnSOD; ALT: 65.3 ± 18.2 units/l compared with 42.6 ± 8.5 units/l; LDH: 2688 ± 662 units/l compared with 2678 ± 614 units/l]. The lack of a reduction in liver injury after rMnSOD treatment may be explained by intrahepatic accumulation

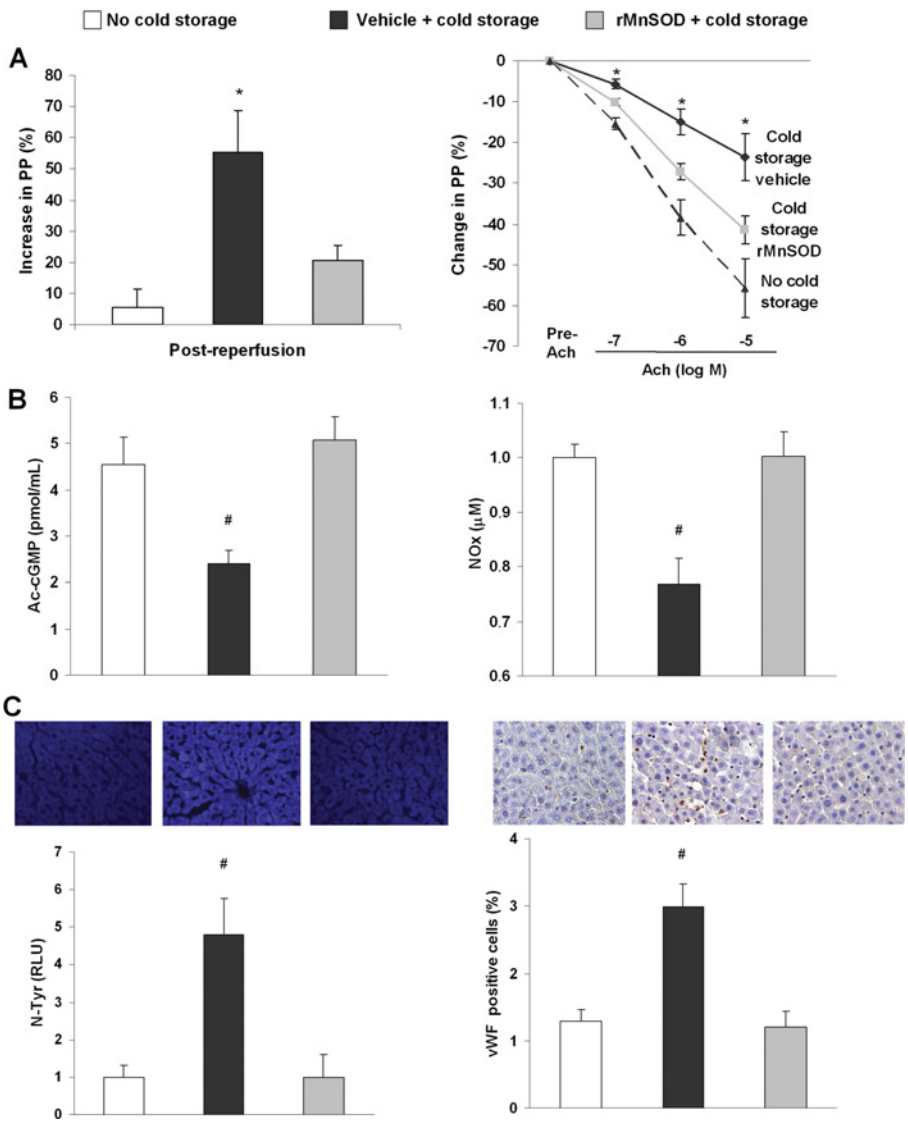

Figure 3 rMnSOD improves hepatic microcirculation and endothelial function in control livers

(**A**) Left: portal pressure increment observed during WR in livers not cold-stored or livers pre-treated with a single dose of rMnSOD, or its vehicle, and afterwards cold-stored for 16 h. Right: hepatic endothelial function evaluation by relaxation to incremental doses of Ach. (**B**) cGMP levels (left) and NOx release (right) in livers described in (**A**). (**C**) Representative images and quantitative analysis of nitrotyrosinated proteins fluorohistochemistry (left) and vWF immunohistochemistry (right) determined in livers described in (**A**) (×40 magnification). ($n = 8$ per group; $^{*}P < 0.05$ compared with no CS and rMnSOD; $^{\#}P < 0.01$ compared with no CS and rMnSOD).

of the SOD final product H_2O_2. Indeed, H_2O_2-scavenger systems glutathione and catalase were significantly diminished in steatotic livers undergoing CS + WR (Supplementary Figure S2 at http://www.clinsci.org/cs/127/cs1270527add.htm).

Cold-stored steatotic livers exhibited increased portal pressure upon reperfusion, together with acute endothelial dysfunction development compared with no cold-stored grafts (Figure 5A). These negative microcirculatory effects of CS + WR were significantly improved when steatotic rats were pre-treated with a single dose of rMnSOD.

In addition, an increase in hepatic nitrotyrosinated proteins and vWF-positive cells due to CS + WR was prevented in rMnSOD pre-treated rats (Figure 5B).

rMnSOD addition to cold-storage solution prevents oxidative stress accumulation in rat and human livers

The potential beneficial effects of rMnSOD as a supplement of a commercially available CS solution were evaluated in liver tissue from rats and humans. Figures 6(A) and 6(B) show significantly lower levels of O_2^- in control and steatotic rat hepatic tissues

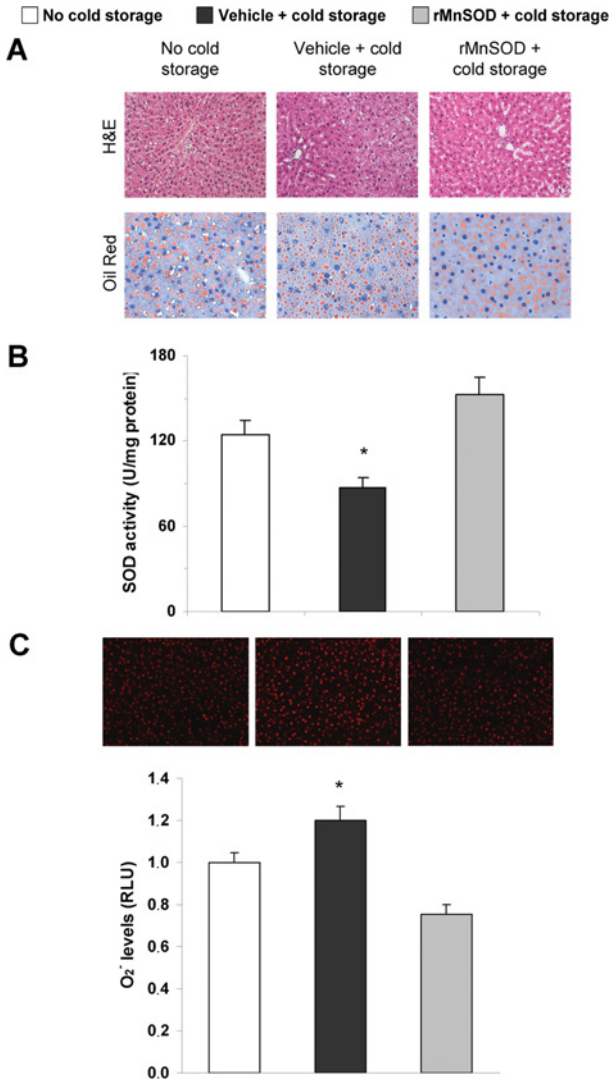

Figure 4 rMnSOD maintains SOD activity preventing O_2^- accumulation in severe steatotic liver grafts

Rats with severe steatosis due to 7-day HFD received a single intravenous dose of rMnSOD, or its vehicle, 30 min before graft explant, 16 h of CS and 1 h of WR. A control group of steatotic animals without the CS period was included. (**A**) Hepatic architecture status was assessed by H&E staining (upper panel; ×20) and advanced liver steatosis was confirmed by Oil Red staining (lower panel; ×40). (**B**) SOD activity evaluated in liver tissue. (**C**) Representative images of hepatic oxidative stress (O_2^-) detection using DHE (×20) and its corresponding quantification. ($n = 7$ per group; $^*P < 0.05$ compared with no CS and rMnSOD). RLU, relative light units.

cold-stored in Celsior solution supplemented with rMnSOD and afterwards warm reperfused, compared with livers cold stored in non-supplemented Celsior solution.

In addition, human liver biopsies preserved in rMnSOD-supplemented CS solution exhibited significantly lower intrahepatic oxidative stress levels than those preserved in standard Celsior solution, demonstrating the effectiveness of rMnSOD in human liver tissue (Figure 6C).

DISCUSSION

Endothelial phenotype de-regulation due to CS + WR injuries during transplantation is probably the first event in the development of graft dysfunction post-transplantation, which is followed by neutrophil recruitment and parenchymal damage [32,33]. Several features inherent to CS + WR negatively affect the endothelial phenotype. These include loss of haemodynamic stimulation and oxygen supply during cold ischaemia [8,34], as well as the production and accumulation of ROS upon reperfusion [35,36]. Previous studies from our group have focused on improving liver graft viability by maintenance of the Kruppel-like Factor 2 (KLF2) pathway [5,25]. Nevertheless, this novel therapeutic strategy had a limited effect in improving hepatic ROS accumulation. Previous studies aimed at decreasing oxidative stress in experimental models of liver ischaemia and reperfusion, including some using native SOD or derivatives, showed promising results; however, none of them reached clinical application probably because of poor stability of the antioxidant molecules and/or controversy in the use of adenoviral vectors in humans [15,37–40]. A new rMnSOD, which is constitutively active, has a good biodistribution, is stable in solution and freely enters cells, has been proposed as novel therapeutic agent for humans [17,18,41]. Therefore, the present study aimed at evaluating the effects of rMnSOD-lowering O_2^- accumulation as a new strategy to improve graft circulation, endothelial function and viability in experimental models of CS + WR.

We first characterized the impact of CS + WR, and the possible benefits of rMnSOD, on oxidative stress levels and NO bioavailability in primary cultured LSECs. In the present study, we demonstrate for the first time that CS + WR induces a marked increase in O_2^- levels in LSECs that is accompanied by a reduction in NO bioavailability. Importantly, rMnSOD blunts the O_2^- burst, which results in the maintenance of NO levels. These *in vitro* data suggest that rMnSOD may effectively improve hepatic vascular function and thereby viability of livers undergoing I/R injury.

This hypothesis was tested by administering a single dose of rMnSOD 30 min before graft procurement for transplantation in control and steatotic rats and determining the microcirculatory status and graft injury/viability after WR.

In control animals, and confirming previously reported findings [5,11,22], CS + WR induced liver damage, as shown by histological findings, increased aminotransferases and LDH release, which were accompanied by increased oxidative stress and inflammation, together with microcirculatory de-regulation and endothelium dysfunction manifested by loss of KLF2 and eNOS expression and abnormal endothelium-dependent vasorelaxation in response to incremental doses of Ach. Notably, our data show that rMnSOD pre-treatment almost entirely prevented endothelial dysfunction, microcirculatory damage and liver injury after CS + WR, probably due to the inhibition of ROS-mediated cell injury and ICAM-1 activation.

As stated above, endothelial protection during CS + WR is a key step in maintaining graft viability after transplantation; thus, we investigated the molecular mechanisms responsible for liver microcirculation protection by rMnSOD. The vasodilator

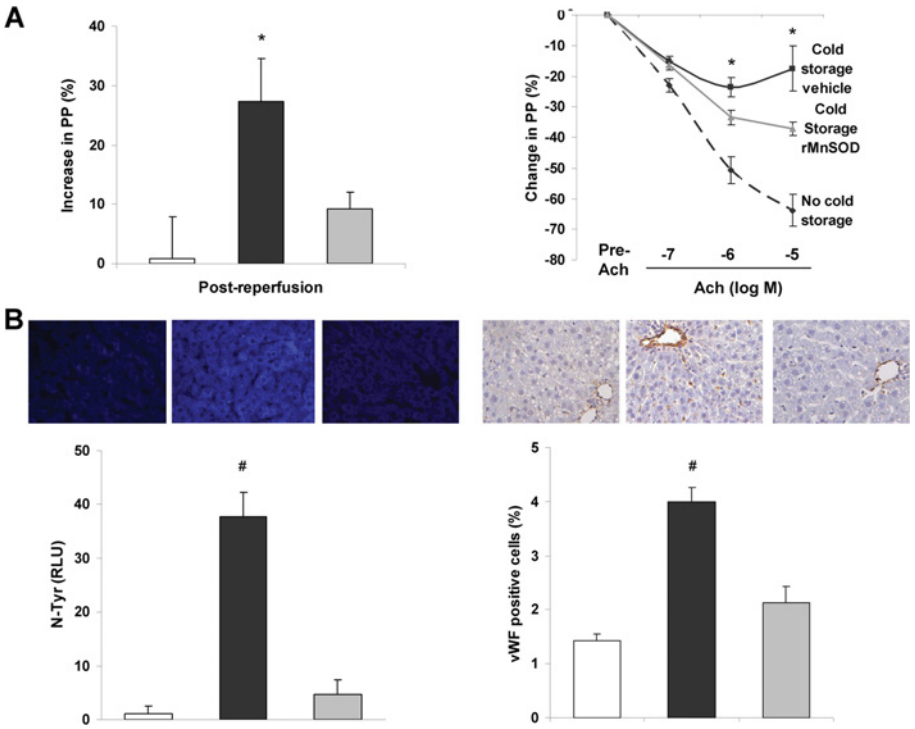

Figure 5 **rMnSOD ameliorates microcirculatory and endothelial dysfunction in severe steatotic liver grafts**
(**A**) Microcirculatory dysfunction was estimated as portal pressure increment during WR (left) and endothelial function as relaxation to incremental doses of Ach (right). (**B**) Detection of hepatic nitrotyrosinated proteins by fluorohistochemistry (left) and vWF expression by immunohistochemistry (right), with their corresponding quantifications ($n = 7$ per group; $^{*}P <$ 0.05 compared with no CS and rMnSOD; $^{\#}P <$ 0.01 compared with no CS and rMnSOD; images $\times 40$).

NO plays a critical role in modulating liver microcirculation, and its decreased availability is a marker of endothelial dysfunction [24,42]. It has been demonstrated that, when large amounts of O_2^- are found in the same environment as NO, this is rapidly scavenged to form peroxynitrite, which reduces NO bioavailability and increases vascular tone [13,43]. Therefore, we characterized the NO pathway in liver grafts included in the present study. These experiments showed that rMnSOD administration led to an increase in hepatic NO bioavailability as measured by two different final products, cGMP and NOx, without changes in eNOS expression, or in the expression of the vasoprotective transcription factor KLF2 that orchestrates eNOS expression (results not shown). In fact, the increase in NO was associated with a reduction in hepatic nitrotyrosinated proteins, a marker of peroxynitrite formation, thus confirming lower O_2^--mediated NO scavenging in livers from rMnSOD-treated rats. Together with low NO, elevated levels of the glycoprotein vWF have been associated with liver sinusoidal endothelial phenotype de-regulation [44,45]. Indeed, vWF is not expressed in healthy LSECs but, after acute or chronic injury, LSECs change their phenotypic pattern and express vWF. Thus, an increase in this protein is associated with endothelial dysfunction. Interestingly, in the present study, we demonstrate that vWF levels within the liver are rapidly increased after CS + WR, reinforcing the relatively novel concept of acute endothelial dysfunction development due to organ procurement for transplantation, and more importantly that rMnSOD pre-treatment prevents vWF up-regulation. Taken together, our findings suggest that rMnSOD efficiently maintains liver endothelial function after CS + WR, which ultimately will positively contribute to preserve global liver function and viability.

It is well known that liver steatosis is increasingly found in liver grafts donated for transplantation. Although moderate steatosis (30–60%) does not prevent transplantation, these grafts may exhibit worse post-operative viability and function [46]. Therefore, new therapeutic options to improve steatotic graft procurement are desirable, especially due to the rising trend in obesity affecting most developed countries. Accordingly, in the present study, we also investigated the possible beneficial effects of rMnSOD in the procurement of liver grafts with severe steatosis [11]. Our experiments confirm previous reports demonstrating that steatotic livers undergoing CS + WR exhibit enhanced parenchymal damage as shown by a more severe degree of histological changes, elevated transaminases and LDH release, and increased oxidative stress [47]. Furthermore, these steatotic grafts show impaired hepatic microcirculation after CS + WR [11], together with exacerbations in O_2^--mediated NO scavenging, and endothelial phenotype de-regulation. Pre-treatment with rMnSOD significantly reduced hepatic oxidative stress levels, which were associated with a reduction in peroxynitrites and vWF levels and improvement in hepatic microcirculation and endothelial function. Nevertheless, in spite of the beneficial effects of rMnSOD, no reduction in hepatic parenchymal damage was observed. These partially positive effects of rMnSOD

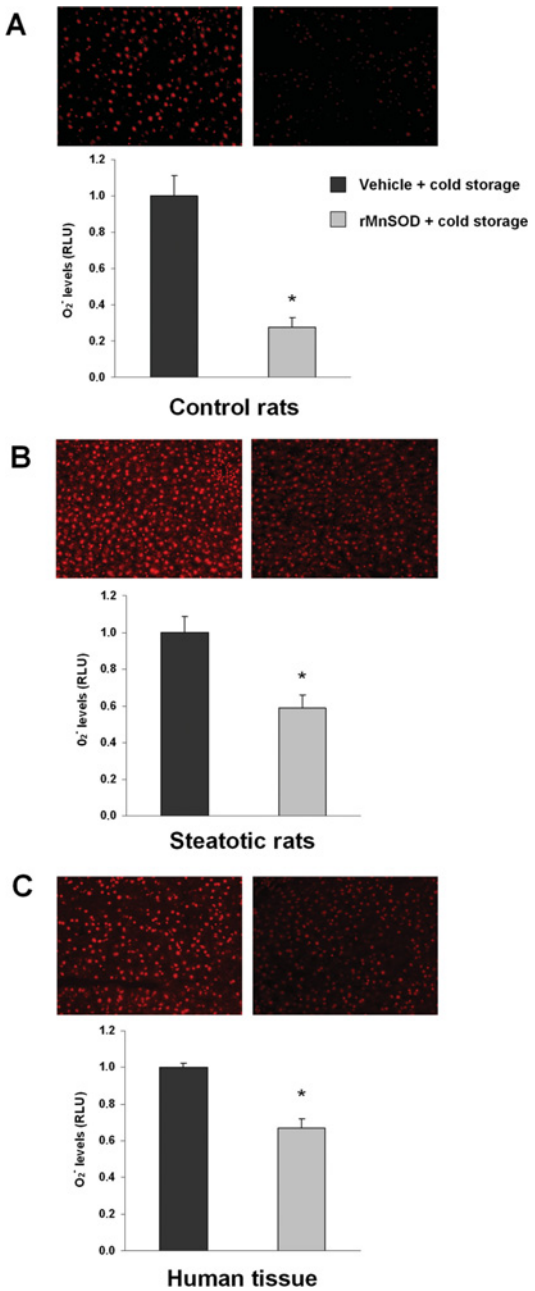

Figure 6 rMnSOD addition to cold storage solution prevents oxidative stress accumulation in rat and human livers

In situ hepatic O_2^- levels were determined in liver biopsies from control rats (**A**), advanced steatotic rats (**B**) and healthy humans (**C**) preserved for 16 h in Celsior solution supplemented with rMnSOD, or its vehicle, and afterwards warm-incubated for 1 h. (×20 magnification; $n = 5$ per condition; $^*P < 0.01$ compared with vehicle). RLU, relative light units.

in liver grafts with steatosis can be explained by an undesirable accumulation of H_2O_2 within the hepatocytes, partly due to insufficient activity/levels of catalase and glutathione, which may ultimately damage the parenchyma [48]. Future experiments evaluating the effects of combined antioxidant therapies are desirable.

Owing to the possible controversies caused by pharmacological pre-treatment of the donor, we evaluated the option to supplement a commercially available CS solution with rMnSOD to prevent ROS accumulation in liver tissues from healthy and steatotic rats and, importantly, also from healthy humans. In all cases, we found that addition of rMnSOD to the preservation solution prevents O_2^- accumulation derived from CS + WR. These results, comparable with those observed with the pre-treatment strategy, demonstrate the effectiveness of this new recombinant protein preventing oxidative stress accumulation in human liver, thus suggesting a global improvement in hepatic CS + WR injury.

Conclusions

The results of the present study demonstrate that donor pre-treatment with rMnSOD shortly before graft procurement protects the liver parenchyma in healthy rat donors and maintains a correct microcirculatory status in both control and steatotic rats. Although additional experimental transplantation studies, where polymorphonuclear neutrophils and other relevant inflammatory cells will contribute to CS + WR injury, are required, we propose rMnSOD as a new and highly effective supplement of the preservation solution for the procurement of liver grafts for transplantation.

CLINICAL PERSPECTIVES

- Liver transplantation is the unique solution for several end-stage liver diseases. Non-steatotic, but especially steatotic, grafts are highly susceptible to I/R injury, exhibiting poor viability and function upon transplantation. One of the main underlying mechanisms of I/R injury is oxidative stress accumulation.
- A single intravenous administration of rMnSOD to the donor shortly before organ procurement significantly improves graft function and prevents microcirculatory derangements, in both non-steatotic and steatotic rats. Addition of rMnSOD to a commercially available CS solution retains its antioxidant properties, inhibiting oxidative stress accumulation in rat and human livers procured for transplantation.
- Pre-treatment of healthy and extended-criteria organ donors with a single dose of rMnSOD could improve the clinical results of liver transplantation and, more importantly, it would increase organ donor pool for transplantation.

AUTHOR CONTRIBUTION

Diana Hide designed the research, conceived the study, performed the experiments, analysed the data and wrote the manuscript. Martí Ortega-Ribera and Anabel Fernández-Iglesias performed the experiments and analysed the data. Constantino Fondevila provided essential materials and critically revised the manuscript. Josepa Salvadó analysed the data and critically revised the manuscript. Lluís Arola and Juan García-Pagán critically revised the manuscript. Aldo Mancini provided essential reagents and critically revised the manuscript. Jaime Bosch conceived the study, critically revised the manuscript and obtained funding. Jordi Gracia-Sancho designed the research, conceived the study, wrote the manuscript, obtained funding and directed the study. All authors edited and reviewed the final manuscript.

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Original Paper

SUPPLEMENTARY ONLINE DATA

A novel form of the human manganese superoxide dismutase protects rat and human livers undergoing ischaemia and reperfusion injury

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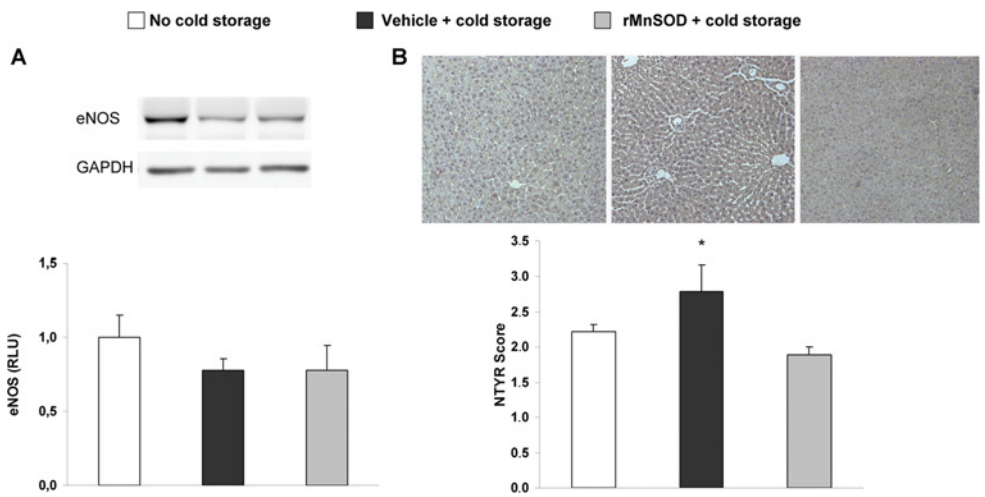

Figure S1 **Effects of CS + WR and rMnSOD on eNOS and nitrotyrosination in control rat livers**
(**A**) Representative images of hepatic eNOS immunoblot and densitometric analysis normalized to GAPDH. (**B**) Detection of hepatic nitrotyrosinated (NTYR) proteins by immunohistochemistry with the corresponding quantification (×20). ($n = 8$ per group; $^*P < 0.05$ compared with no CS and rMnSOD).

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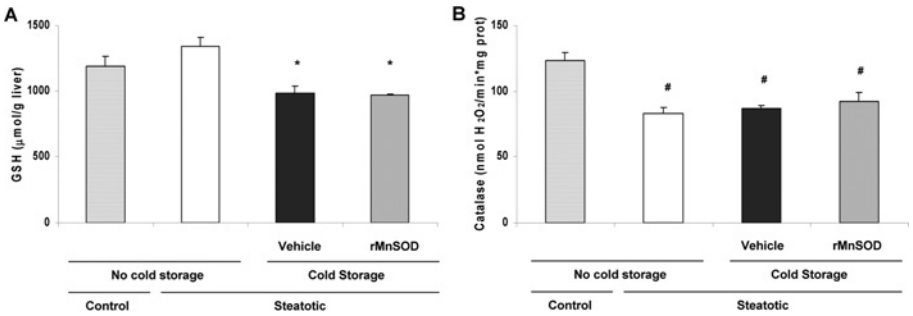

Figure S2 **Effects of CS + WR on H_2O_2-scavenger systems in steatotic rat livers**
Total glutathione (GSH) levels (**A**) and catalase activity (**B**) were evaluated in liver homogenates ($n = 7$ per group; $^{*}P < 0.05$ compared with no CS; $^{\#}P < 0.05$ compared with control).

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