# Superoxide dismutase gene transfer reduces portal pressure in $CCl_4$ cirrhotic rats with portal hypertension

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## ABSTRACT

**Background:** Increased intrahepatic vascular tone in cirrhosis has been attributed to a decrease of hepatic nitric oxide (NO) secondary to disturbances in the post-translational regulation of the enzyme eNOS. NO scavenging by superoxide ($O_2^-$) further contributes to a reduction of NO bioavailability in cirrhotic livers.

**Aim:** To investigate whether removing increased $O_2^-$ levels could be a new therapeutic strategy to increase intrahepatic NO, improve endothelial dysfunction and reduce portal pressure in cirrhotic rats with portal hypertension.

**Methods:** Adenoviral vectors expressing extracellular superoxide dismutase (SOD) (AdECSOD) or β-galactosidase (Adβgal) were injected intravenously in control and $CCl_4$-induced cirrhotic rats. After 3 days, liver $O_2^-$ levels were determined by dihydroethidium staining, NO bioavailability by hepatic cGMP levels, nitrotyrosinated proteins by immunohistochemistry and western blot, and endothelial function by responses to acetylcholine in perfused rat livers. Mean arterial pressure (MAP) and portal pressure were evaluated in vivo.

**Results:** Transfection of cirrhotic livers with AdECSOD produced a significant reduction in $O_2^-$ levels, a significant increase in hepatic cGMP, and a decrease in liver nitrotyrosinated proteins which were associated with a significant improvement in the endothelium-dependent vasodilatation to acetylcholine. In addition, in cirrhotic livers AdECSOD transfection produced a significant reduction in portal pressure (17.3 (SD 2) mm Hg vs 15 (SD 1.6) mm Hg; $p<0.05$) without significant changes in MAP. In control rats, AdECSOD transfection prevents the increase in portal perfusion pressure promoted by an ROS-generating system.

**Conclusions:** In cirrhotic rats, reduction of $O_2^-$ by AdECSOD increases NO bioavailability, improves intrahepatic endothelial function and reduces portal pressure. These findings suggest that scavenging of $O_2^-$ might be a new therapeutic strategy in the management of portal hypertension.

Increased intrahepatic vascular resistance to portal blood flow is the main cause of portal hypertension in cirrhosis and is the result of both structural changes and an increase in the hepatic vascular tone within the cirrhotic liver.[1] Sinusoidal endothelial cell dysfunction, characterised by impaired endothelium-dependent vasodilation, appears to be an important mechanism of the increased vascular tone of cirrhotic livers and has been attributed to increased release of cyclooxygenase-1-derived vasoconstrictive prostanoids[2] and to reduced nitric oxide (NO) bioavailability.[3–5]

Reduced NO bioavailability within cirrhotic livers is the result of a decrease in endothelial NO synthase (eNOS) activity due to alterations on its post-translational regulation.[6 7] However, recent data from our group have demonstrated that scavenging of NO by the increased levels of superoxide ($O_2^-$) found in cirrhotic livers contributes further to a reduction of NO bioavailability. This study suggests that removing increased $O_2^-$ levels in cirrhotic livers could be a new therapeutic strategy for improving intrahepatic NO bioavailability, reducing intrahepatic resistance and improving portal hypertension.[8]

Increased production of reactive oxidative species (ROS) in cirrhotic livers was shown to be the consequence of an increased production by several enzymatic systems (such as cyclooxygenase and xanthine oxidase)[8 9] but, in addition, to a reduced expression and activity of superoxide dismutase (SOD), a critical enzyme that metabolises $O_2^-$.[8 10]

Gene transfer of SOD has been shown to protect against oxidative stress and to improve endothelium-dependent relaxation in several situations, including myocardial infarction,[11] liver transplantation,[12] hypertension,[13 14] diabetes[15] and ageing.[16]

Therefore, the present study was aimed at investigating whether adenovirus-mediated gene transfer of SOD is able to decrease $O_2^-$ levels, increase NO bioavailability, and, consequently, improve hepatic endothelial dysfunction and reduce portal pressure in rats with cirrhosis and portal hypertension.

## MATERIAL AND METHODS

### Induction of cirrhosis

Cirrhosis was induced in male Wistar rats (175–200 g) by inhalation of carbon tetrachloride ($CCl_4$) three times a week. Phenobarbital (0.3 g/l) was added to the drinking water as previously described.[17] When cirrhotic rats had developed ascites, 12–15 weeks after inhalation of $CCl_4$, administration of $CCl_4$ and phenobarbital was stopped and experiments were performed 1 week later. Control animals received only phenobarbital. The animals were kept in environmentally controlled animal facilities at the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS).

### Adenoviral vectors and gene transfer in vivo

Replication-deficient adenoviral constructs, under the control of the human cytomegalovirus (CMV)

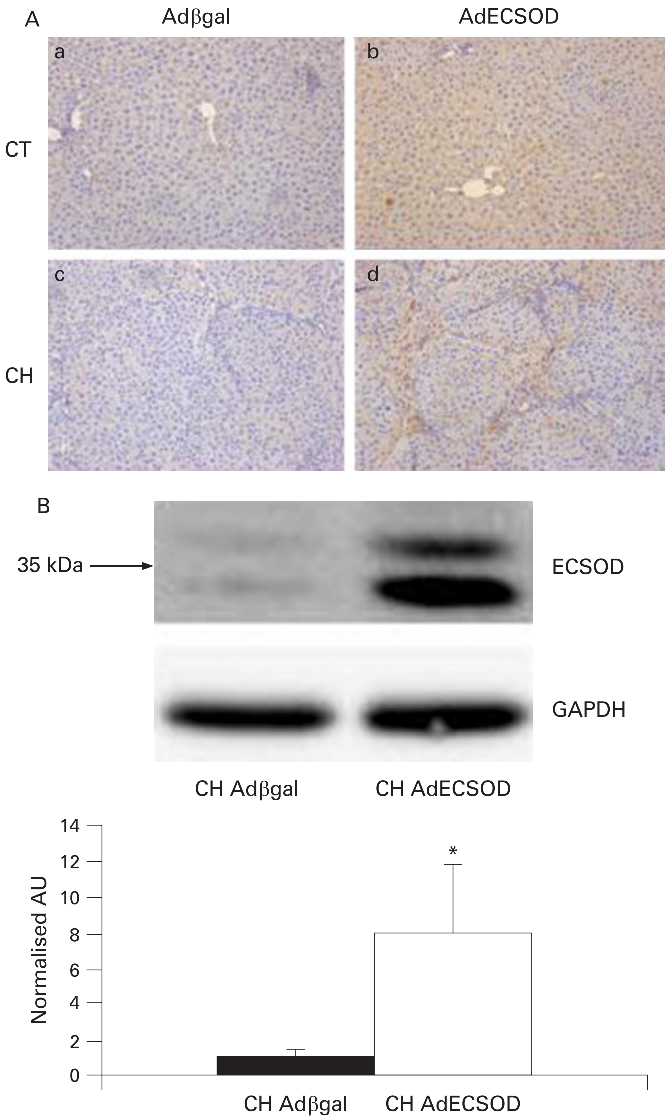


**Figure 1** (A) Representative histological images of liver tissues immunostained for human extracellular superoxide dismutase (ECSOD) from control (CT) and cirrhotic (CH) rats, 3 days after intravenous injection of adenoviral vectors expressing either β-galactosidease (Adβgal) (a and c) or ECSOD (AdECSOD) (b and d) ($5\times10^{11}$ viral particles per rat) (n = 4 animals per group). (B) *Top*. Representative western blot of ECSOD in livers from cirrhotic (CH) rats transfected with Adβgal or AdECSOD. *Bottom*. Densitometry analysis of ECSOD expression in cirrhotic rat livers transfected with Adβgal (n = 4) or AdECSOD (n = 6) (normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); normalised arbitrary units (AU) (SEM). *$p<0.05$ vs CH Adβgal).

promoter/enhancer were used. One, expressing human extracellular SOD (AdECSOD) constructed by Dr Chu, was provided by the Vector Core at the University of Iowa, and the other, expressing β-galactosidase (Adβgal), was provided by Dr CB Newgard (Duke University, Durham, North Carolina, USA). The viruses were propagated in the human embryonic kidney (HEK) 293 cell line. Purification and titres were determined using established protocols.[18] These ECSOD adenoviruses are capable of infecting and expressing enzymatically active SOD in endothelial cells.[13]

Gene transfer in vivo was performed in rats anaesthetised with isoforane (Abbott, Madrid, Spain) (2–5%). The AdECSOD or the control virus, Adβgal ($5\times10^{11}$ particles in 3% sucrose in phosphate-buffered saline) were injected into the penile vein of control and cirrhotic animals and all studies were performed 3 days later. Three days after intravenous injection of AdECSOD ($5\times10^{11}$ particles), plasma alanine aminotransferase was not modified and no gross changes were revealed in the histological examination of the liver.[14]

## ECSOD protein expression

To confirm the expression of human ECSOD in transfected livers, immunohistochemistry and western blot for ECSOD were performed.

Immunohistochemistry was performed in paraffin-embedded liver sections (8 μm thick) from control and cirrhotic rats, after transfection with AdECSOD or Adβgal, as previously described (n = 4 animals per group).[14] Immunohistochemistry was performed using a polyclonal rabbit anti-human ECSOD antibody that specifically recognises human ECSOD (1:500, 4°C, overnight), which was a generous gift from Professor JD Crapo (National Jewish Medical Research Center, Denver, Colorado, USA), followed by horseradish peroxidase (HRP)-conjugated goat–anti-rabbit immunoglobulin G (IgG) antibody (1:200). Binding was visualised using 3′,3′-diaminobenzadine (DAB) (Dako, Carpinteria, California, USA) and 0.01% $H_2O_2$ as the chromogen. For the negative control, phosphate-buffered saline was used instead of the primary antibody. All sections were air dried, counter-stained with haematoxylin and examined by light microscopy using a ×10 objective (Axiovert 135; Carl Zeiss, Göttingen, Germany).

Immunoreactivity for human ECSOD was quantified with an image-analysis system (AxioVision Release 4.6.3; Carl Zeiss) that allows a semiquantitative grading from 0 to 3 (0, minimal staining; 1, weak staining; 2, moderate staining; and 3, strong staining). Preparations were blindly assessed by the same investigator (BL).[19]

Western blot was performed in frozen liver samples from cirrhotic rats transfected with AdECSOD (n = 6) or Adβgal (n = 4). Samples were crushed to a powder and subsequently homogenised in Triton–lysis buffer as previously described.[20]

Aliquots from each sample containing equal amounts of protein (20 μg) were run on a 12% sodium dodecyl sulfate–polyacrylamide gel, and transferred to a nitrocellulose membrane. After transfer, the blots were subsequently blocked for 1 h with Tris-buffered saline containing 0.05% (vol/vol) Tween 20 and 5% (wt/vol) nonfat dry milk and probed with a rabbit anti-extracellular SOD (1 μg/ml) antibody (ECSOD; Stressgen, Victoria, British Columbia, Canada) overnight at 4°C followed by incubation with anti-rabbit HRP-conjugated secondary antibody (1:10 000, 1 h, room temperature; Stressgen).

Protein expression was determined by densitometric analysis using the Science Lab Image Gauge (Fuji Photo Film, Düsseldorf,

**Table 1** Semiquantitative data of extracellular superoxide dismutase immunohistochemistry studies

| | Score | | | | |
|---|---|---|---|---|---|
| **Group** | **0 (%)** | **1 (%)** | **2 (%)** | **3 (%)** | **Staging score** |
| CT Adβgal | 100 | 0 | 0 | 0 | 0 |
| CT AdECSOD | 0 | 0 | 30 | 70 | 2.7 (0.15)* |
| CH Adβgal | 0 | 0 | 0 | 0 | 0 |
| CH AdECSOD | 0 | 0 | 70 | 30 | 2.3 (0.15)† |

Data are mean (SEM). n = 4 animals per group.
*$p<0.05$ vs CTAdβgal; †$p<0.05$ vs CHAdβgal.
AdECSOD, adenoviral vectors expressing extracellular superoxide dismutase; Adβgal, adenoviral vectors expressing β-galactosidase; CH, cirrhotic rats; CT, control rats.

Germany). After stripping, blots were assayed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Santa Cruz, California, USA) expression as standardisation of sample loading. Quantitative densitometric values of all proteins were normalised to GAPDH.

### Measurement of $O_2^-$ content in liver tissue

The generation of $O_2^-$ in situ was evaluated in fresh liver cryosections taken from nine cirrhotic livers (three transfected with AdECSOD, three with Adβgal or three treated with vehicle (3% sucrose in phosphate-buffered saline) and three control livers with the oxidative fluorescent dye dihydroethidium (DHE) (Molecular Probes, Eugene, Oregon, USA). Ten fields for each animal, using a ×40 objective, were chosen at random and quantified as previously described.[8] DHE specifically reacts with intracellular and extracellular $O_2^-$[21] and is converted to the red fluorescent compound ethidium bromide (EtBr), which then binds irreversibly to double-stranded DNA and appears as punctate nuclear staining.

### Nitric oxide bioavailability

Measurements of cGMP, a marker of NO bioavailability, were performed in control (n = 5 per group) and cirrhotic (n = 7 per group) rat liver homogenates transfected with AdECSOD or Adβgal using an enzyme immunoassay (Cayman Chemical, Ann Arbor, Michigan, USA), as previously described.[20]

### Nitrotyrosine protein detection

Immunohistochemistry for nitrotyrosine (NT) was performed in paraffin-embedded liver sections (8 μm thick) from cirrhotic rats, transfected with AdECSOD (n = 3) or Adβgal (n = 3), using a polyclonal rabbit anti-nitrotyrosine antibody (Upstate, Lake Placid, New York, USA) (1:100 dilution), as described above. A semiquantitative analysis was performed as previously described.

In addition, in cirrhotic rat livers transfected with AdECSOD (n = 4) or Adβgal (n = 4) protein nitrotyrosination was determined by western blot using a mouse anti-nitrotyrosine (1 μg/ml) antibody (Cayman Chemical) as previously described.[8]

After stripping, blots were assayed for GAPDH (Santa Cruz Biotechnology) expression as standardisation of sample loading. Quantitative densitometric values of all proteins were normalised to GAPDH.

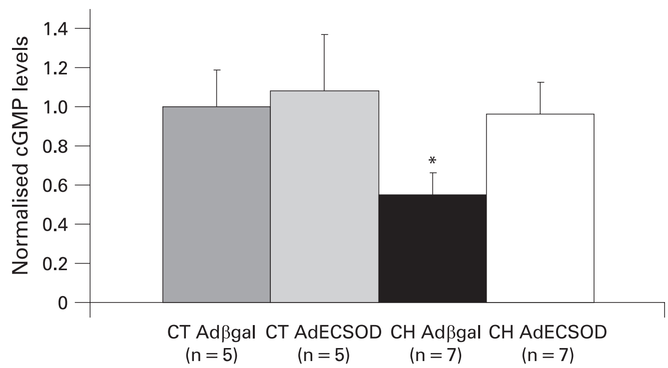


**Figure 3** Intrahepatic cGMP levels in control (CT) and cirrhotic (CH) rats transfected with adenoviral vectors expressing β-galactosidase (Adβgal) or adenoviral vectors expressing extracellular superoxide dismutase (AdECSOD). Data are expressed as normalised mean (SEM). *p<0.05 vs CT Adβgal.

### Effects on portal perfusion pressure and superoxide levels promoted by the superoxide generating system NADPH/NADPH oxidase in control rat livers

Control livers transfected with Adβgal (n = 5) or AdECSOD (n = 7) were isolated and perfused by a flow-controlled perfusion system as previously described.[17] Briefly, livers were perfused with Krebs buffer in a recirculation fashion with a total volume of 100 ml at a constant flow rate of 35 ml/min with an ultrasonic flow probe (T201; Transonic System, Ithaca, New York, USA). A pressure transducer was placed immediately

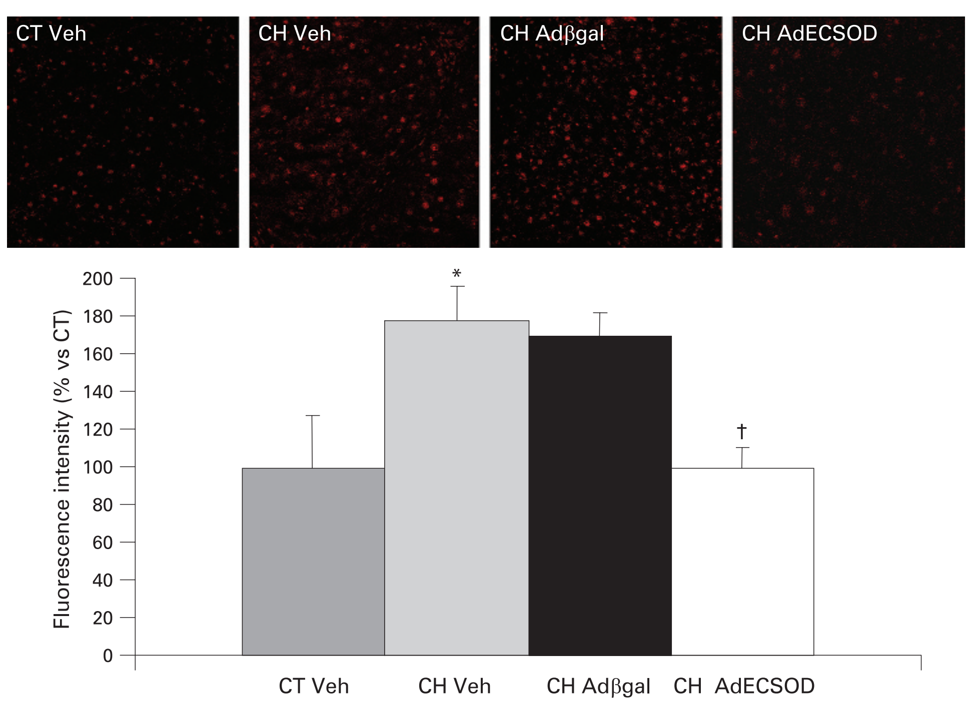


**Figure 2** (A) Representative confocal fluorescence microscopy images of in situ detection of superoxide with the oxidative dye dihydroethidium (DHE) in fresh liver sections from control (CT) and cirrhotic (CH) rats 3 days after adenoviral vectors expressing extracellular superoxide dismutase (AdECSOD), adenoviral vectors expressing β-galactosidase (Adβgal) transfection or vehicle (Veh). (B) DHE fluorescence intensity (% vs CT Veh) of three independent experiments. *p<0.05 vs CT Veh; †p<0.05 vs CH Adβgal.

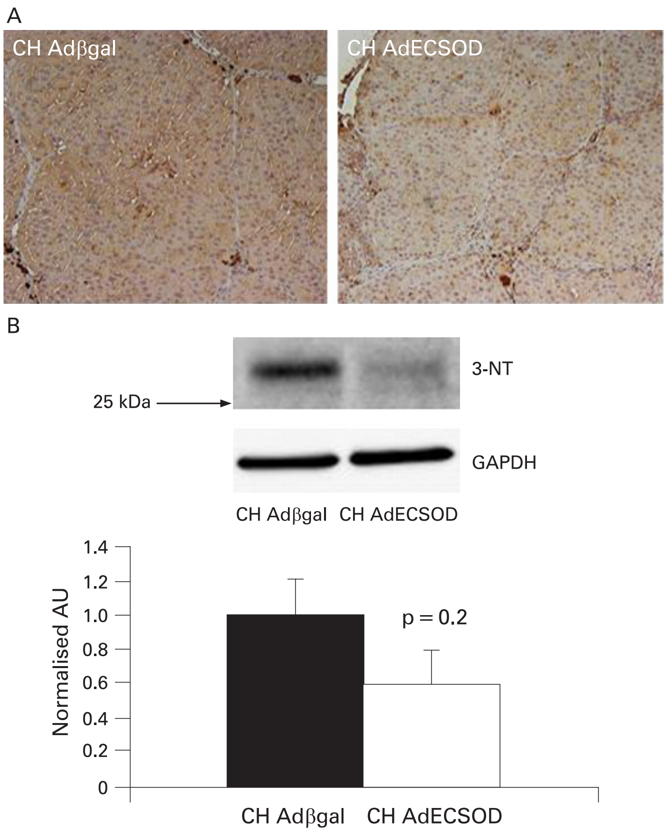


**Figure 4** (A) Representative histological images of liver tissues immunostained for nitrotyrosine from cirrhotic (CH) rats transfected with adenoviral vectors expressing β-galactosidase (Adβgal) or adenoviral vectors expressing extracellular superoxide dismutase (AdECSOD) (three different animals for each treatment). (B) *Top*. Representative western blot of nitrotyrosinated proteins (3-NT) of livers from cirrhotic (CH) rats transfected with Adβgal or AdECSOD. *Bottom*. Densitometry analysis of nitrotyrosinated proteins in cirrhotic rat livers transfected with Adβgal (n = 4) or AdECSOD (n = 4) (normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); normalised arbitrary units (AU) (SEM)).

before the portal inlet cannula to continuously monitor portal flow and portal perfusion pressure. The flow probe and the pressure transducers were connected to a Powerlab (4SP) linked to a computer using the Chart v5.0.1 for Windows software (ADInstruments, Mountain View, Louisiana, USA).

The perfused rat liver preparation was allowed to stabilise for 20 min before nicotinamide adenine dinucleotide phosphate (NADPH 100 mmol/l; Applichem, Darmstadt, Germany), the substrate of NADPH oxidase, or its vehicle (Krebs buffer) was added. The gross appearance of the liver, portal perfusion pressure, and buffer pH (7.4 (SD 0.1)) were observed during this period. Responses to NADPH were calculated as a per cent change in portal perfusion pressure.

Measurements of $O_2^-$ in liver tissue were evaluated as described above in two independent experiments.

### Splanchnic and systemic in vivo haemodynamics of control and cirrhotic rats

Control (n = 6) and cirrhotic (n = 10) rats were anaesthetised with ketamine hydrochloride (100 mg/kg intraperitoneally; Merial Laboratories, Barcelona, Spain) plus midazolam (5 mg/kg intramuscularly; Laboratorios Reig Jofré, Barcelona, Spain). The temperature was maintained at 37 (SD 0.5)°C. A tracheotomy was performed to maintain adequate ventilation.

**Table 2** Semiquantitative data of nitrotyrosine immunohistochemistry studies

| | Score | | | | |
|---|---|---|---|---|---|
| Group | 0 (%) | 1 (%) | 2 (%) | 3 (%) | Staging score |
| CH Adβgal | 0 | 25.00 | 33.33 | 41.67 | 2.17 (0.24) |
| CH AdECSOD | 0 | 66.67 | 33.33 | 0 | 1.33 (0.14)* |

Data are mean (SEM). n = 4 animals per group.
*$p<0.05$ vs CHAdβgal.
Adβgal, adenoviral vectors expressing β-galactosidase; AdECSOD, adenoviral vectors expressing extracellular superoxide dismutase; CH, cirrhotic rats.

The femoral artery and ileocolic vein were cannulated to continuously monitor mean arterial pressure (MAP; mm Hg) and portal pressure (mm Hg). Measurements were taken after 20 min of stabilisation.

### Evaluation of endothelial function in control and cirrhotic perfused rat livers

After haemodynamic measurements in vivo, livers were quickly isolated and perfused by a flow-controlled perfusion system as described above. The perfused rat liver preparation was allowed to stabilise for 20 min before vasoactive substances were added. The intrahepatic microcirculation was preconstricted by adding the $\alpha_1$-adrenergic agonist methoxamine (Sigma, St Louis, Missouri, USA) to the reservoir to achieve a final concentration of $10^{-4}$ mol/l. After 5 min, concentration–response curves to cumulative doses of acetylcholine, $10^{-7}$, $10^{-6}$ and $10^{-5}$ mol/l (Sigma), were evaluated. The concentration of acetylcholine was increased by one log unit every 1.5 min interval. Responses to acetylcholine were calculated as per cent change in portal perfusion pressure, as previously described.[4 22]

### Statistical analysis

Statistics were performed using the SPSS 14.0 for Windows statistical package. All results are expressed as mean (SD) unless otherwise specified in the figure legends. Comparisons between two groups were performed with the Student t test for unpaired data. The ANOVA test for repeated measurements was used when appropriate. Significance was established at the 0.05 level.

## RESULTS

### Effect of ECSOD gene transfer on $O_2^-$ levels and NO bioavailability in rat livers

Control and cirrhotic rat livers transfected with AdECSOD effectively expressed human ECSOD, as shown by semiquantitative analysis of ECSOD immunostainings (fig 1A and table 1) and western blot (fig 1B) in comparison to control and cirrhotic rat livers transfected with Adβgal.

Superoxide levels were significantly higher in cirrhotic rat livers than in control rat livers (fig 2). AdECSOD, but not Adβgal, transfection produced a marked and significant reduction of $O_2^-$ levels in cirrhotic rat livers to a point that was similar to those found in control livers (fig 2). cGMP levels, a surrogate marker of NO bioavailability, were significantly lower in cirrhotic than in control livers (fig 3). cGMP content did not change after AdECSOD transfection in the control livers but significantly increased in those cirrhotic rat livers transfected with AdECSOD. In addition, in cirrhotic rat livers AdECSOD transfection produced a reduction of nitrotyrosinated proteins, as shown by semiquantitative analysis of nitrotyrosine immunostaining (fig 4A and table 2) and western blot analysis (fig 4B).

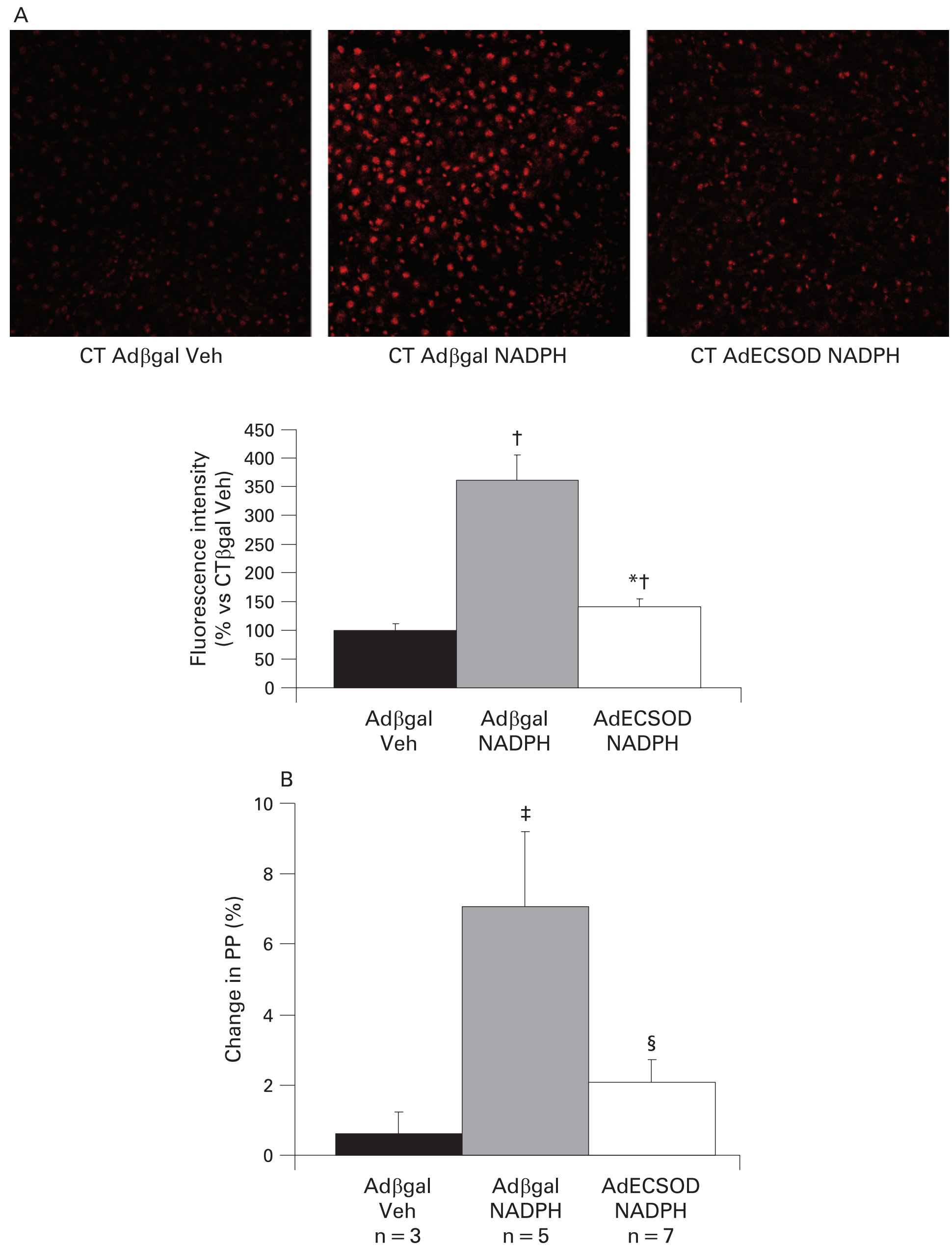


**Figure 5** (A) *Top*. Representative confocal fluorescent microscopy images of in situ detection of $O_2^-$ with dihydroethidium (DHE) in fresh liver sections from control (CT) rats transfected with adenoviral vectors expressing extracellular superoxide dismutase (AdECSOD) or adenoviral vectors expressing β-galactosidase (Adβgal) in response to nicotinamide adenine dinucleotide phosphate (NADPH) or its vehicle (Veh). *Bottom*. DHE fluorescence intensity (% vs CT Adβgal Veh). (†$p<0.05$ vs CT Adβgal Veh; *$p<0.05$ vs CT Adβgal NADPH). (B) Portal perfusion pressure change (PP) after NADPH or its vehicle, in control (CT) rats transfected with Adβgal (n = 5) or AdECSOD (n = 7). Results are expressed as mean (SEM). ‡$p<0.05$ vs CT Adβgal Veh; §$p<0.05$ vs CT Adβgal NADPH.

### Effects on portal perfusion pressure and superoxide levels promoted by the superoxide generating system NADPH/NADPH oxidase in control rat livers transfected with AdECSOD

NADPH produced a significant increase in hepatic $O_2^-$ levels and in portal perfusion pressure (7 (SD 5)% vs 0.6 (SD 0.8)% increase, $p<0.05$) in control rats transfected with Adβgal in comparison to those treated with vehicle. AdECSOD transfection attenuated both the increase in hepatic $O_2^-$ levels and the elevation of portal perfusion pressure produced by NADPH (2 (SD 2)% increase in portal perfusion pressure, $p<0.05$ vs Adβgal+NADPH) (fig 5B).

### Effects of ECSOD gene transfer on portal pressure in control and cirrhotic rats

The cirrhotic rats transfected with Adβgal showed arterial hypotension and portal hypertension when compared with those control rats transfected with Adβgal.

MAP or portal pressure was not significantly different in control rats transfected with AdECSOD or Adβgal (fig 6A). However, in contrast, cirrhotic rats transfected with AdECSOD had significantly lower portal pressure than those transfected with Adβgal (15 (SD 1.6) mm Hg vs 17.3 (SD 2) mm Hg; $p<0.05$), without significant differences in MAP (97 (SD 17) mm Hg vs 102 (SD 15) mm Hg; not significant) (fig 6B).

### Effect of ECSOD gene transfer on endothelial function in rat livers

To further characterise the effects of AdECSOD transfection on the liver vasculature, control and cirrhotic rat livers transfected with AdECSOD or Adβgal were isolated and perfused. Baseline portal perfusion pressure was significantly greater in Adβgal cirrhotic rat livers than in Adβgal control rat livers (10.9 (SD 2.3) vs 4.8 (SD 1.1) mm Hg; $p<0.0001$). In addition, in accordance with results obtained from previous studies,[4 22]

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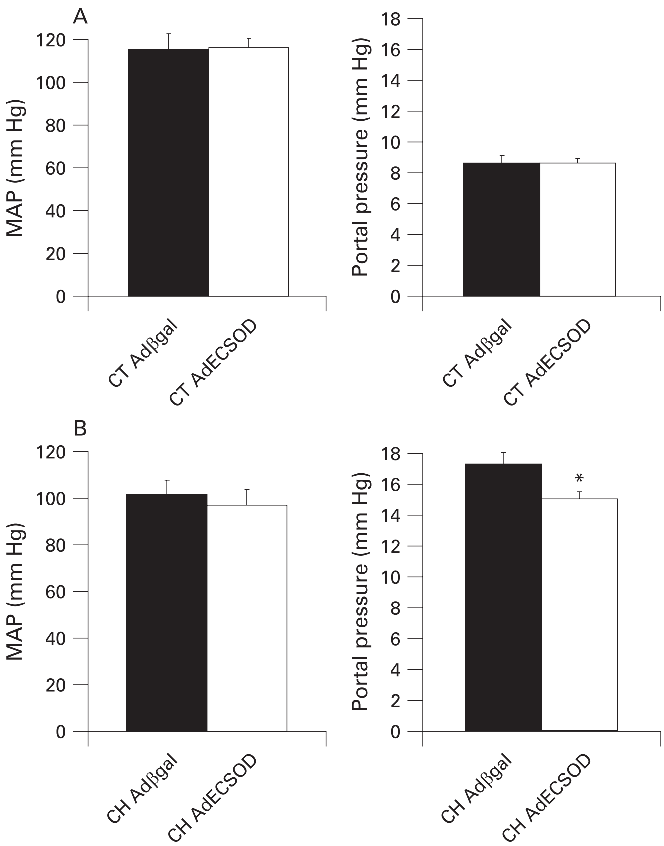



**Figure 6** Mean arterial pressure (MAP) and portal pressure (PP) in (A), control (CT) (n = 6) and (B) cirrhotic (CH) (n = 10) rats 3 days after transfection with adenoviral vectors expressing β-galactosidase (Adβgal) or adenoviral vectors expressing extracellular superoxide dismutase (AdECSOD). Results are expressed as mean (SEM). *$p<0.05$ vs Adβgal.

Adβgal cirrhotic livers exhibited a significantly higher portal perfusion pressure response to methoxamine (20.9 (SD 3 vs 10.1 (SD 3.8) mm Hg; $p<0.001$) and a lower vasodilator response to acetylcholine than Adβgal control livers (fig 7). In the control livers, AdECSOD transfection did not significantly modify the portal perfusion pressure response to methoxamine or the vasodilator response to acetylcholine (fig 7A). However, in cirrhotic livers, AdECSOD transfection reduced the portal perfusion pressure response to methoxamine (18.3 (SD 3) vs 20.9 (SD 3) mm Hg in Adβgal cirrhotic livers; $p = 0.06$) and significantly improved the vasodilatory response to acetylcholine (fig 7B).

## DISCUSSION

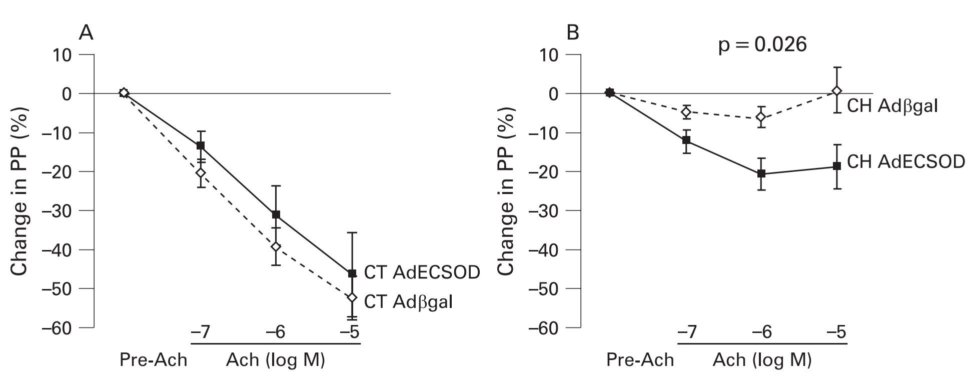
In cirrhosis, increased resistance to portal blood flow is determined by structural changes in the liver and is further aggravated by an increase in hepatic vascular tone.[1 23] This latter component, which results from the reduction of hepatic NO bioavailability[4 24] and an increased production of circulating and local vasoconstrictors,[25–28] is theoretically amenable to treatment with vasodilators.[29]

Attempts to correct the intrahepatic NO deficiency have been based on either over-expressing NOS by transfecting the liver with adenovirus encoding eNOS,[30 31] nNOS,[32] or constitutively active Akt,[33] by administration of NO donors,[34 35] or by enhancing eNOS activity by simvastatin (a 3-hydroxy-3-methyl-glutaryl-CoA (HMG–CoA) reductase inhibitor)[20 36] or by the eNOS co-factor tetrahydrobiopterin.[6] These strategies, which effectively increased NO production, have been shown to be associated, in most cases, with a slight reduction in portal pressure. This paradox may, at least in part, be due to the fact that the increased ROS levels observed in cirrhotic livers could lead to NO inactivation attenuating the efficacy of those strategies aimed at increasing NO synthesis.[8–10]

For this reason, the goal of the present study was to pursue the strategy of increasing NO bioavailability not by increasing its production but by reducing its scavenging by $O_2^-$. The local concentration of SOD is a determinant of $O_2^-$ and therefore we tested the hypothesis that, by increasing dismutation of $O_2^-$ by SOD, NO bioavailability could be increased by reducing $O_2^-$ levels.

We used the gene transfer approach, using recombinant replication-deficient adenovirus vectors carrying the human ECSOD gene, in order to reduce $O_2^-$ levels because gene transfer causes stable expression of protective enzymes and proteins.[37] Human ECSOD protein was used because it is tetrameric and glycosylated[38] and thus it has a longer plasma half-life than intracellular CuZnSOD. In addition, ECSOD contains a positively charged heparin-binding domain, which aids the binding of ECSOD to heparin sulfate proteoglycans on the cell surface and in the extracellular matrix. ECSOD in these extracellular locations is highly effective in preventing the scavenging of NO when it diffuses from sinusoidal endothelial cells to hepatic stellate cells.[39] One possible concern of our approach could be the accumulation of hydrogen peroxide, which can act as a vasoconstrictor.[40] However, the results of the present study support the hypothesis that, once produced, hydrogen peroxide is rapidly decomposed to water and oxygen by the action of catalase, an enzyme mostly produced in the liver and which constitutes 0.5–1% of the total protein in this organ.[41]

**Figure 7** Vasorelaxation to acetylcholine (Ach) in (A) control (CT, n = 6) or (B) cirrhotic (CH, n = 10) rat livers 3 days after adenoviral infection with adenoviral vectors expressing extracellular superoxide dismutase (AdECSOD) or adenoviral vectors expressing β-galactosidase (Adβgal). Results are expressed as mean (SEM). PP, portal pressure.



Cirrhotic rat livers showed an increased $O_2^-$ content in comparison to control rat livers. Superoxide levels were similar in cirrhotic rats livers treated with vehicle or transfected with Adβgal, discarding a significant effect of adenoviral transfection increasing hepatic $O_2^-$ per se.

As shown by immunohistochemical and western blot analysis, intravenous administration of AdECSOD resulted in effective transgene expression either in control or cirrhotic rat livers. This resulted in a marked reduction in $O_2^-$ and enhanced NO bioavailability in cirrhotic livers which were then associated with a reduction in intrahepatic nitrotyrosinated proteins, a well recognised marker of the reaction of $O_2^-$ with NO. For the first time, these findings support, in vivo, the concept that reducing increased hepatic $O_2^-$ levels in cirrhosis can improve hepatic NO bioavailability, which has recently been demonstrated in vitro.[8]

In accordance with the working hypothesis, increasing NO bioavailability by AdECSOD transfection improved the impaired vasorelaxation to the endothelium-dependent vasodilator acetylcholine observed in cirrhotic rat livers and, more importantly, promoted a significant reduction of portal pressure in vivo. Reduction in portal pressure was probably due to the improvement of the hepatic vascular bed to vasodilatory stimuli and the attenuation of the hyper-response to vasoconstrictors. However, we can not completely discard the suggestion that a possible reduction in portal blood flow could also contribute. It is important to note that AdECSOD transfection did not modify MAP, and this may be the result of a relatively targeted effect of SOD on the liver microcirculation. This would be a theoretical advantage over other non-selective strategies aimed at increasing NO bioavailability, which may produce deleterious effects worsening the hyperdynamic syndrome found in cirrhosis.[42] This possibility seems reasonable because previous studies in rodents have shown that approximately 90% of the adenoviral vector is localised in the liver, both in animals with normal liver function[43] and in $CCl_4$-induced cirrhotic rats. Although transduction efficiency is reduced in cirrhosis, expression is nevertheless of high magnitude,[44 45] and the liver is still the main adenoviral target.[46]

Reduction in portal pressure averaged 13.3% on magnitude. This is similar to that observed in other studies aimed at reducing portal pressure in cirrhotic rats through other strategies, such as the administration of non-selective beta-blockers.[47]

In conclusion, this study provides evidence, for the first time in vivo, that decreasing hepatic $O_2^-$ levels by increasing SOD activity (ie, an antioxidant treatment) may represent an effective strategy to improve NO bioavailability within the liver and therefore, strongly supports the possibility that antioxidant therapy might be an attractive proposition to treat portal hypertension in cirrhosis.

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**Competing interests:** None.

**Ethics approval:** All experiments were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona, and were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, NIH publication 86–23, revised 1985).

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# Editor's quiz: GI snapshot

## ANSWER

*From the question on page 67*

Skin biopsy confirmed the diagnosis of Sweet's syndrome (SS; inflammatory infiltrate with numerous neutrophils). Azathioprine was stopped and corticotherapy led to a spectacular clinical improvement after 2 days (neutrophils $= 7 \times 10^9$/l and complete healing of cutaneous lesions). The patient was discharged on prednisolone at a dose of 60 mg/day for 2 weeks, tapered during 3 weeks. As SS could be associated with inflammatory bowel disease, azathioprine was reintroduced 1 month later followed by a clinical relapse of the skin lesions, leading to its definitive withdrawal with favourable outcome.

SS is an acute, febrile neutrophilic dermatosis that includes the following diagnostic criteria: abrupt onset of painful, erythematous papules and plaques, histopathological evidence of a dense neutrophilic infiltrate without leucocytoclastic vasculitis and an excellent response to glucocorticoid. The main aetiologies of SS are idiopathic, inflammatory or neoplastic diseases.[1] Some drugs could induce SS (granulocyte colony-stimulating factor, vaccine, all *trans* retinoic acid). A few cases of azathioprine-induced SS have been reported in the literature.[1–4]

The prompt recognition of this association is important to allow the withdrawal of azathioprine and the initiation of corticotherapy to cure the condition.

**Patient consent:** Obtained.

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