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RESVERATROL IMPROVES INTRAHEPATIC ENDOTHELIAL DYSFUNCTION AND REDUCES HEPATIC FIBROSIS AND PORTAL PRESSURE IN CIRRHOTIC RATS

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Abbreviations: nitric oxide (NO), cyclooxygenase-1 (COX-1), endothelial nitric oxide synthase (eNOS), carbon tetrachloride (CCl₄), mean arterial pressure (MAP; mmHg), portal pressure (PP, mmHg), portal blood flow (PBF; ml·min⁻¹), superior mesenteric artery blood flow (SMABF; ml·min⁻¹), methoxamine (Mtx), acetylcholine (Ach), thromboxane B₂ (TXB₂), superoxide (O₂⁻), dihydroethidium
(DHE), cyclic guanosine monophosphate (cGMP), phosphorylated eNOS (P-eNOS), α smooth muscle actin (αSMA).

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Abstract:

Background and Aims: Resveratrol, a polyphenol found in a variety of fruits, exerts a wide range of beneficial effects on the endothelium, regulates multiple vasoactive substances and decreases oxidative stress, factors involved in the pathophysiology of portal hypertension. Our study aimed at evaluating the effects of resveratrol on hepatic and systemic hemodynamics, hepatic endothelial dysfunction and hepatic fibrosis in CCl₄-cirrhotic rats.

Methods: Resveratrol (10 and 20 mg/kg/day) or its vehicle were administered to cirrhotic rats for two weeks and hepatic and systemic hemodynamics were measured. Moreover, we evaluated endothelial function by dose-relaxation curves to acetylcholine, hepatic NO bioavailability and TXA₂ production. We also evaluated liver fibrosis by Sirius Red staining of liver sections, collagen-1, NF B, TGF mRNA expression, and desmin and α-smooth muscle actin (α-SMA) protein expression, as a surrogate of hepatic stellate cell activation.

Results: Resveratrol administration significantly decreased portal pressure compared to vehicle (12.1±0.9 vs 14.3±2.2 mmHg, p<0.05) without significant changes in systemic hemodynamics. Reduction in portal pressure was associated with an improved vasodilatory response to acetylcholine, with decreased TXA₂ production, increased endothelial NO and with a significant reduction in liver fibrosis. The decrease in hepatic fibrosis was associated with a reduced collagen-1, TGF , NF B mRNA expression and desmin and α-SMA protein expression.
Conclusions: Resveratrol administration reduces portal pressure, hepatic stellate cell activation and liver fibrosis, and improves hepatic endothelial dysfunction in cirrhotic rats, suggesting it may be a useful dietary supplement in the treatment of portal hypertension in patients with cirrhosis.

Keywords: oxidative stress, endothelial dysfunction, nitric oxide, thromboxane.
Introduction:

In cirrhosis, the initial factor determining the onset of portal hypertension is the increase in intrahepatic vascular resistance. This is not only due to morphological changes resulting from the chronic liver inflammation and fibrosis, but also to reversible functional alterations, including an exaggerated response of the porto-hepatic vascular bed to vasoconstrictors and a deficient response to vasodilators [1]. A decreased nitric oxide (NO) availability and an increase in cyclooxygenase-1 (COX-1)-derived prostanoids within the liver play a major role in the pathogenesis of these dynamic alterations [2-5]. Reduced NO availability has been shown to be in part due to an increase scavenging by superoxide (O$_2^-$) and different strategies aimed to reduce O$_2^-$ levels [6, 7] such as superoxide dismutase (SOD) gene transfer are able to reduce portal pressure in experimental models of cirrhosis in the rat.

Resveratrol (3,5,4’-trihydroxystilbene) is a natural polyphenolic flavonoid found in a large amount of plant species, including grapes and their derivatives, berries and nuts. It has been suggested to have important health benefits attributed to its demonstrated anti-oxidant, anti-neoplastic, anti-inflammatory and anti-platelet aggregation activities [8-11]. Specifically, in different experimental models resveratrol improves vascular dysfunction, an effect that is attributed to its ability to reduce oxidative stress, to upregulate endothelial nitric oxide synthase (eNOS) expression and activity, and to inhibit COX-1 activity [12-15].
Resveratrol has been shown to exert anti-oxidant effects in experimental models of liver injury induced by ischemia/reperfusion and ethanol by inducing the enzymatic activity of SOD and catalase [16, 17], and to attenuate fibrosis development when co-administrated with CCl₄ to rats [18]. Additionally, resveratrol reduces the hepatotoxicity induced by acetaminophen, ethanol and carbon tetrachloride (CCl₄), and prevents liver damage due to ischemia-reperfusion, irradiation and high fat diet [19]. Overall, we hypothesized that resveratrol may exert beneficial effects in the pathophysiological mechanisms involved in the development of portal hypertension in cirrhosis. Therefore, the aim of the present study was to investigate the effects of chronic administration of resveratrol in CCl₄-cirrhotic rats with portal hypertension.
Materials and Methods:

Induction of cirrhosis by CCl₄ and resveratrol administration

In male Wistar rats (50-75 g) cirrhosis was induced by inhalation of CCl₄ three times a week, and phenobarbital (0.3 g/l) was added to the drinking water as previously described [6]. When cirrhotic rats had developed ascites, after approximately 12-15 weeks of CCl₄ inhalation, administration of CCl₄ and phenobarbital was discontinued. One week later, the animals were randomized to receive resveratrol (10 mg/kg body weight (bw); Sigma, Tres Cantos, Madrid, Spain) or its vehicle (carboxymethylcellulose 0.7%) daily by gavage for two weeks. Resveratrol or its vehicle was prepared and administered by a third person and therefore, the investigators performing the experiments were not aware of the treatment received by the rats. Experiments were initiated 1 hour after the administration of the last dose of resveratrol or vehicle. The dose of 10 mg/kg bw/day of resveratrol has been shown to reduce liver oxidative damage after bile duct ligation [20] and to decrease acute liver damage induced by acute CCl₄ intoxication [21]. To evaluate a possible dose-dependent effect of resveratrol, an additional group of rats were treated with resveratrol at 20 mg/kg bw/day (n=10) or its corresponding vehicle (n=8). The animals were kept in environmentally controlled animal facilities at the Institut d’Investigacions Biomèdiques August Pi y Sunyer (IDIBAPS). All procedures were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona.
and were conducted in accordance with European Community guidelines for the protection of animals used for experimental and other scientific purposes (EEC Directive 86/609).

**In vivo hemodynamic studies**

Rats were anesthetised with ketamine hydrochloride (100 mg/Kg; Merial Laboratories, Barcelona, Spain) plus midazolam (5mg/kg; Laboratorios Reig Jofré, Barcelona, Spain) intraperitoneally. A tracheostomy was performed and a polyethylene tube PE-240 was inserted into the trachea to ensure a patent airway. PE-50 catheters were introduced into the femoral artery to measure mean arterial pressure (MAP; mmHg) and into ileocolic vein to measure portal pressure (PP, mmHg). Perivascular ultrasonic flow probes connected to a flow meter (Transonic Systems Inc., Ithaca, NY, USA) were placed around the portal vein, as close as possible to the liver to avoid portal-collateral blood flow, in order to measure portal blood flow (PBF; ml·min⁻¹) going through the liver, and at the superior mesenteric artery to measure superior mesenteric artery blood flow (SMABF, ml·min⁻¹). Blood pressures and flows were registered on a multichannel computer-based recorder (PowerLab; AD Instruments, Colorado Springs, CO). The temperature of the animals was maintained at 37±0.5 °C and hemodynamic data were collected after a 20 minutes stabilization period.

**Evaluation of endothelial function**
After *in vivo* hemodynamic measurements, livers were quickly isolated and perfused by a flow-controlled perfusion system as previously described [22]. The perfused rat liver preparation was allowed to stabilize for 20 min before vasoactive substances were added. The intrahepatic microcirculation was preconstricted by adding the α1-adrenergic agonist methoxamine (Mtx; 10^{-4} mol/l; Sigma) to the reservoir. After 5 min, concentration–response curves to cumulative doses of acetylcholine (Ach; 10^{-7}, 10^{-6} and 10^{-5} mol/l, Sigma) were evaluated. The concentration of Ach was increased by 1 log unit every 1.5 min interval. Responses to Ach were calculated as the percentage change in portal perfusion pressure [5]. The gross appearance of the liver, stable perfusion pressure, bile production over 0.4 μL/min/g of liver and a stable buffer pH (7.4±0.3) were monitored during this period. If any viability or stability criteria were not satisfied, the experiment was discarded.

**Effects of resveratrol on oxidative stress and hepatic SOD activity**

*Measurement of O_2^- content:*

To evaluate if resveratrol is able to reduce intrahepatic O_2^- levels, livers from cirrhotic rats treated with resveratrol (n=2) or vehicle (n=2) were promptly removed after the hemodynamic measurements and *in situ* O_2^- content was evaluated in fresh liver cryosections (10 μm) stained with the oxidative fluorescent dye dihydroethidium (DHE) (Molecular Probes, Eugene, Oregon USA), as previously described [23, 24].
SOD Activity

Total SOD activity was measured in liver homogenates obtained from CCl4-cirrhotic rats treated with resveratrol or vehicle (n=9 per group) using a commercially available immunoassay (Sigma). The assay is based on the competition reaction between the sample-containing SOD and the highly water-soluble tetrazolium salt (WST) that produces a water-soluble formazan dye upon reaction with O$_2^-$. Briefly, livers were homogenized in buffer containing 20 mM Hepes, 1 mM EDTA, 210 mM mannitol and 70 mM sucrose. After centrifugation at 1500 x g for 5 min at 4 ºC the supernatant was collected and protein concentration was quantified. SOD activity assay was performed according to manufacturer instructions.

Evaluation of NO pathway

Nitric oxide bioavailability:

Measurements of cyclic guanosine monophosphate (cGMP), a marker of NO bioavailability, were performed in liver homogenates from cirrhotic rats treated with resveratrol (n=8) or vehicle (n=8) using an enzyme immunoassay (Cayman Chemical Company, Tallin, Estonia), as previously described [7, 25].

eNOS and P-eNOS protein expression:

Total eNOS and P-eNOS protein expression were determined by western blot in liver homogenates from cirrhotic rats treated with resveratrol (n=4) or vehicle
(n=4) as previously described [7]. Antibodies against phosphorylated eNOS (Ser1176; Cell Signaling Technology, Beverly, MA) and total eNOS (BD Biosciences, San Jose, CA) were incubated for 16 h at 4°C, followed by an incubation with horseradish peroxidase-conjugated secondary antibodies (Stressgen, Victoria, BC, Canada) for 1 h at room temperature. Blots were revealed by chemiluminescence. Protein expression was determined by densitometric analysis using Science Lab 2001, Image Gauge (Fuji Photo Film Gmbh, Düsseldorf, Germany). Quantitative densitometry values of eNOS and P-eNOS were normalized to GAPDH.

**Measurement of thromboxane A₂:**

In liver-perfusion experiments, samples of the perfusate were obtained before Mtx administration and after the dose-response to Ach. The samples were stored at -80°C and thromboxane B₂ (TXB₂), the end metabolite of thromboxane A₂ (TXA₂), was quantified in duplicate using a commercially available enzyme immunoassay (Cayman) [26, 27]. TXB₂ production was expressed as absolute increment after dose-response curve to Ach over baseline before Mtx administration. [28]

**Effects of resveratrol on sinusoidal endothelial cells:**

*Isolation of sinusoidal endothelial cells*
Sinusoidal endothelial cells were isolated from control and cirrhotic rats as previously described [24]. Briefly, after collagenase perfusion of the livers and isopycnic sedimentation of the resulting dispersed cells through a two-step density gradient of Percoll, pure monolayer cultures of SEC were established by selective attachment on a substrate of rat tail collagen type I. Afterwards, cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 and studies were performed on cells from the first passage, 12 hrs after their isolation, to preserve their typical phenotype.

*Measurements of NO levels and TXA₂ production in SEC*

To determine whether resveratrol administration could increase NO bioavailability in SEC isolated from five cirrhotic rat livers (CH-SEC), CH-SEC were incubated for 24 hours at 37°C with resveratrol (50 μM) or with vehicle (ethanol). Then, nitric oxide levels were assessed with DAF-FM-DA as previously described [29]. In addition, nitrites/nitrates (NOx) production was assessed in aliquots of SEC supernatants using specific microelectrodes (Lazar Laboratories, Los Angeles, CA) according to manufacturer’s instructions.

SEC isolated from cirrhotic rats were treated with resveratrol or vehicle for 24h, and afterwards arachidonic acid (40 μM) was added for 20 minutes in order to stimulate prostanoid production. Then, the supernatant was collected and TXA₂ determined as described above.
Evaluation of hepatic fibrosis

Sirius red staining:
Livers from cirrhotic rats treated with resveratrol (n=8) or vehicle (n=9) were fixed in 10% formaldehyde, embedded in paraffin, sectioned and stained with 0.1% Sirius red, photographed, and analyzed using a microscope equipped with a digital camera [29, 30]. Eight fields from each slide were randomly selected, and the red-stained area per total area was measured using AxioVision software.

Immunohistochemistry of SMA and Desmin.
Immunostaining of paraffin-embedded liver sections was performed with mouse anti-smooth muscle actin (SMA) antibody (1:1000; Sigma) and mouse anti-desmin antibody (1:50; DAKO, Denmark) or, as a negative control, with phosphate-buffered saline. Bound antibodies were visualized using with Dako Real Envision Detection System Peroxidase/DAB+, and slides were then counterstained with hematoxylin.

SMA and desmin relative volume was determined by point-counting morphometry on immunoperoxidase-stained sections, using a point grid to obtain the number of intercepts over SMA and desmin positive cells over the tissue. Fourteen fields were counted in each liver (n=6 per group). All measurements were performed by two 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. Results are normalized to vehicle.
Fibrosis markers expression

Hepatic protein expression of SMA was determined by Western blot in hepatic samples using a mouse antibody against SMA (Sigma).

Collagen I, SMA, TGF, NF-B, MMP2, MMP9, TIMP1 and TIMP2 gene expression

Hepatic gene expression of all genes was assessed by real time quantitative reverse transcriptase-polymerase chain reaction using pre-designed gene expression assays obtained from Applied Biosystems (AB, Foster City, CA) according to the manufacturer’s protocol and reported relative to endogenous control GAPDH. All PCR reactions were performed in duplicate and using nuclease-free water as no template control.

Effects of resveratrol on SMA and procollagen I gene expression, and Bad protein expression in hepatic stellate cells

To study the effect of resveratrol treatment on HSC, we used the immortalized humane stellate cell line LX2 (kindly provided by Dr Bataller), which has been previously well characterised [31]. LX2 were seeded onto p35 plates at density of 150,000 per plate in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin and cultured overnight. Resveratrol was added at concentration of 10 or 50 M to subconfluent cultures of LX2 for 24 hours.
SMA and procollagen I gene expression were determined by real time PCR using predesigned gene expression assays obtained from Applied Biosystems according to the manufacturer’s protocol and reported relative to endogenous control 18S as indicated above.

Protein expression of Bad, a proapoptotic member of the Bcl-2 gene family which is involved in initiating apoptosis, was determined by Western blot using a rabbit antibody against Bad (Cell Signaling Technology).

Effects of resveratrol on CD68 expression in cirrhotic rat livers
Livers were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin (H&E). For CD68, that is typically expressed by infiltrating inflammatory cells [32], immunostaining of paraffin-embedded liver sections was performed with a mouse anti-CD68 antibody diluted (1:100; Serotec, Kidlington, UK) as described above. The number of CD68-positive cells was quantified using AxioVision software.

Statistical analysis
Statistics were performed using the SPSS 19.0 (IBM) 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, or Mann–Whitney test when assumptions of
normality could not be verified. The ANOVA test for repeated measurements was used if appropriate. Significance was set at the 0.05 level.
Results:

Effect of resveratrol on hepatic and systemic hemodynamics in cirrhotic rats

Cirrhotic rats receiving long term treatment with resveratrol (10 mg/kg bw/day) had a significantly lower PP than cirrhotic rats treated with vehicle (12.1±0.9 vs 14.3±2.2 mmHg; p=0.02), corresponding to a PP reduction of 15%. No significant changes in PBF, SMABF, MAP or HR were observed (Table 1). Intrahepatic vascular resistance was lower in resveratrol-treated rats, although the difference did not reach statistical significance.

Effect of resveratrol on endothelial function in cirrhotic rat livers

To further characterize the effects of resveratrol on liver vasculature, livers from cirrhotic rats treated with resveratrol or vehicle were isolated and perfused. As expected, livers from cirrhotic rats treated with vehicle exhibited endothelial dysfunction, as shown by the reduced vasorelaxation in response to 10^{-7} M Ach and by a paradoxical vasoconstriction at the 10^{-6} M and 10^{-5} M dose. Resveratrol administration improved hepatic vasorelaxation, indicating an amelioration of endothelial dysfunction (Figure 1A).

Effect of resveratrol on O$_2^-$ levels in cirrhotic rat livers

Resveratrol administration produced a significant decrease in O$_2^-$ levels, as shown by DHE fluorescence, demonstrating its antioxidant effect. To evaluate whether part of this effect was due to an upregulation of the reduced SOD activity of cirrhotic rat livers, SOD activity in vehicle and resveratrol-treated
cirrhotic rats was measured. No differences in SOD activity were observed between both groups (Figure 1B).

*Effect of resveratrol on TXB₂ production*

Resveratrol resulted in a significant decrease in hepatic TXB₂ production compared to vehicle (0.15±0.05 vs 1±0.6 normalized arbitrary units; 85% reduction; p<0.01) (Figure 1C).

*Effect of resveratrol on NO pathway*

No significant differences in eNOS expression and eNOS phosphorylation at Ser 1176 were observed among livers from cirrhotic rats treated with resveratrol or vehicle. Moreover, no significant differences in cGMP content (a marker of NO bioavailability) were observed (Figure 1D).

*Effects of resveratrol in sinusoidal endothelial cells*

Resveratrol administration promoted a significant increase in NO in CH-SEC (Figure 2A). On the other hand, resveratrol significantly attenuated the increase in TXA₂ levels induced by AA administration in CH-SEC (Figure 2B).

*Effect of resveratrol on hepatic fibrosis and hepatic stellate cells*

As expected, cirrhotic rats had a marked architectural distortion with abundant fibrosis. Rats receiving resveratrol exhibited a significant reduction in hepatic fibrosis, as proved by a decreased fibrosis area on Sirius Red stained liver sections (Figure 3A). This was associated with a significant reduction in
collagen I, and in the profibrogenic factors NF B and TGF mRNA expression (Figure 3B Top). Moreover, a marked decrease in ϴSMA, a surrogate marker of hepatic stellate cell activation, gene (Figure 3B) and protein (Figure 3A and 3C) expression was observed together with a profound decline in desmin expression (Figure 3A), thus suggesting that the decrease in HSC activation may be mostly due to the consequence of apoptosis of activated HSC. On the other hand, no differences in MMP2, MMP9, TIMP1 and TIMP2 were observed among both groups (Figure 3B Bottom).

To further explore the role of resveratrol on hepatic stellate cells, we performed in vitro experiments with LX2. As shown in figure 4, resveratrol produced a significant reduction in procollagen I and ϴSMA gene expression in LX2 (Figure 4A) and an increase in Bad expression (Figure 4B).

All together, these results support that resveratrol markedly reduces hepatic stellate cell activation and hepatic fibrosis.

Effects of resveratrol on CD68 expression in cirrhotic rats

Using histological analyses as well as immunohistochemistry for CD68, we found that resveratrol attenuate the inflammatory infiltration (34% decrease) in cirrhotic rat livers (Supplementary Figure 1).

Effects of 20 mg/kg bw/day of resveratrol

Cirrhotic rats treated with double dose of resveratrol also showed a significantly lower PP than cirrhotic rats treated with vehicle (11.6±2.2 vs 14.7±1.8 mmHg;
p<0.05), similar to that observed in rats treated with the 10 mg/kg bw/day dose. Treatment with 20 mg/kg bw/day of resveratrol also did not reduce MAP (90±15 mmHg vs. 91±20 mmHg in rats treated with vehicle) (Table 2).

Rats receiving 20 mg/kg bw/day of resveratrol exhibited a marked and similar reduction in collagen deposition, collagen I mRNA expression and αSMA protein expression with those receiving the 10 mg/kg bw/day dose (Supplementary Figure 2).
**Discussion:**

In cirrhosis, an increase in hepatic vascular resistance to portal blood flow is the primary factor in the development of portal hypertension [3]. Therefore, it is of great interest to develop therapeutic strategies aimed at decreasing portal pressure by reducing hepatic vascular resistance.

Resveratrol is a natural substance with many biologic functions; among others, it induces antioxidant enzymes, increases NO bioavailability and inhibits the production of inflammatory factors [33].

The main finding of the present study is that in established cirrhotic rats with severe portal hypertension, the oral administration of resveratrol for two weeks reduces portal pressure, without affecting portal blood flow, indicating an effective reduction in hepatic vascular resistance. This beneficial effect on portal pressure was confirmed when a double dose of resveratrol was used. Remarkably, the beneficial effect of resveratrol on portal pressure occurred in the absence of deleterious effects on systemic hemodynamics, as shown by the absence of significant changes in mean arterial pressure, heart rate and superior mesenteric artery blood flow.

Our results further suggest that the reduction in hepatic resistance induced by resveratrol was the result of both amelioration of the liver architectural abnormalities and an improvement in endothelial dysfunction. Indeed, resveratrol significantly reduced liver fibrosis, as shown by the reduction in fibrosis area on Sirius Red stained liver sections and the decrease in collagen I
mRNA expression, an effect that was not observed in cirrhotic rats treated with vehicle, demonstrating that this was due to an enhancement by resveratrol of the regression of fibrosis that occurs after ceasing CCl₄ administration. Our results showing a decline in both desmin and SMA expression in liver tissue suggests that a decrease in HSC activation may be mostly due to the consequence of apoptosis of activated HSC. This suggestion is further supported by our findings in LX2 cells showing that resveratrol significantly decreases collagen I and SMA gene expression in association with an increase in Bad protein expression, a marker of apoptosis induction.

It has been well documented that a reduction of oxidative stress can deactivate mechanisms leading to liver fibrosis [34]. Therefore, we could speculate that in our setting the antifibrotic effect of resveratrol may be due, at least partly, to its anti-oxidant activity [35]. Indeed, it has been shown that resveratrol inhibits the activation of NF-kappaB, which promotes the transcription of several cytokines including the pro-fibrogenic TGF-β [18, 36, 37]. In that regard, we have also demonstrated that resveratrol treatment produces a significant reduction in the NF-κB and TGF gene expression, suggesting that resveratrol is able to reduce fibrosis by reducing profibrogenic stimuli in cirrhotic rat livers. Whatever the mechanism, these effects on liver fibrosis were not boosted by higher doses of resveratrol, since similar reductions in fibrosis were observed with 10 and 20 mg/kg bw/day.
Cirrhotic livers of rats treated with resveratrol had a reduction in $O_2^-$ levels. Resveratrol itself, as a polyphenolic compound, has been shown to scavenge hydroxyl, $O_2^-$ and other radicals [38, 39]. Moreover, resveratrol have also indirect antioxidant effects by upregulating different endogenous cellular antioxidant systems, such as SOD, catalase, glutathione peroxidase, ... and by inhibition of enzymatic systems involved in ROS formation, like NAPDH oxidase [40, 41]. Although decreased scavenging of $O_2^-$ by diminished SOD activity has been reported as one of the causes of the increased oxidative stress in cirrhotic livers [24], we did not find any effect of resveratrol treatment on SOD activity. Therefore, we think that the observed is a direct antioxidant effect of resveratrol although we can not discard that other mechanisms different from SOD activity increase could be implicated.

Resveratrol administration also improved endothelial dysfunction. We have previously demonstrated that reduced NO bioavailability and a COX-1-dependent increase in TXA$_2$ are the main factors mediating the endothelial dysfunction of cirrhotic rat livers [5, 42, 43]. Resveratrol has been shown to act as a peroxidase-mediated inactivator of COX-1 [15] and, in agreement with that, we found that resveratrol markedly inhibited TXA$_2$ production in whole tissue. We also observed that resveratrol could attenuate the increase in TXA$_2$ levels induced by AA administration in SEC-CH, confirming the role of COX-1-derived prostanoids in the improvement of endothelial dysfunction by resveratrol. In addition, resveratrol treatment promoted a significant increase in
NO bioavailability in endothelial cells isolated from cirrhotic livers. Taken together, these results suggest that resveratrol may improve endothelial dysfunction by increasing endothelial NO and reducing endothelial TXA₂. Besides, it is highly likely that the improvement in liver fibrosis produced by resveratrol itself may play a major role in the recovery of endothelial function. Furthermore, we herein show that resveratrol reduces the number of CD68-positive cells (34% decrease) in cirrhotic livers. This finding is in agreement with a published study showing that resveratrol may attenuate inflammatory infiltration [37], an effect that may represent an additional benefit of resveratrol treatment.

In conclusion, our data show that chronic resveratrol administration to cirrhotic rats reduces portal pressure both by causing a regression of liver fibrosis and by correcting hepatic endothelial dysfunction, without affecting systemic hemodynamics. Due to these properties and its low toxicity, resveratrol, which is widely available, may be a useful supplement in the treatment of patients with cirrhosis and portal hypertension.
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Figures legends:

Fig. 1: Effect of resveratrol on endothelial dysfunction. A) Endothelium-dependent vasorelaxation to acetylcholine (Ach) in isolated and perfused livers from CCl₄- cirrhotic rats. Animals were treated with 10 mg/kg bw/day of resveratrol (n=6) or vehicle (n=7). Results are expressed as the percentage change of PP in response to Ach and presented as mean±SEM. Resveratrol administration significantly improved the impaired vasodilatory response to Ach in CCl₄- cirrhotic rat livers. B) Effect of resveratrol on superoxide levels in CCl₄- cirrhotic rats. Top: Representative confocal microscopy images of in situ intrahepatic detection of O₂⁻ with dihydroethidium in fresh liver sections from cirrhotic rats treated with resveratrol (10 mg/kg bw/day; n=2) or vehicle (n=2). Bottom: Left: DHE fluorescence intensity analysis showed a marked and significant reduction in intrahepatic O₂⁻ in cirrhotic rats treated with resveratrol. Values are represented as arbitrary units (AU) normalized to vehicle livers ± SEM. Right: Total SOD activity determined in liver homogenates from cirrhotic rats treated with resveratrol or vehicle. C) TXB₂ production in resveratrol (n=6) or vehicle-treated (n=7) CCl₄-cirrhtoic rat livers. Values represent mean±SEM. D) Effect of resveratrol on NO pathway. Top: Representative western blot image of P-eNOS and total eNOS in CCl₄-cirrhotic rat livers treated with vehicle or resveratrol. Bottom: Densitometry analysis of eNOS and P-eNOS (n=4 per group). Values represent arbitrary units normalized to GAPDH/vehicle livers. Intrahepatic cGMP levels in cirrhotic rats chronically treated with resveratrol or
vehicle. Values represent arbitrary units normalized to vehicle livers. No significant differences in cGMP levels were observed (n=8 per group).

**Fig. 2: Effects of resveratrol in sinusoidal endothelial cells.** A) Fluorescent detection of intracellular nitric oxide (NO) in sinusoidal endothelial cells (SEC) isolated from cirrhotic rat livers (CH-SEC). Fluorescence intensity of DAF-FM-DA in arbitrary units was normalized by the total number of cells. The data shown are from 4888 individual resveratrol- and 4081 vehicle-treated CH-SEC obtained from three different experiments. **Bottom:** NOx production in SEC supernatants (n=12 per group). Resveratrol promoted a significant increase in NO bioavailability. *p<0.01 vs vehicle-treated CH-SEC. B) Thromboxane (TXA$_2$) production by SEC from cirrhotic rats stimulated with AA in the presence or absence of resveratrol (n=12 per group). In SEC-CH, resveratrol treatment produced a significant decrease in TXA$_2$ (-80%).

**Fig. 3: Effect of resveratrol (10 mg/kg bw/day) on hepatic fibrosis in CCl$_4$-cirrhotic rats.** A) **Top:** Representative histological images (staining with Sirius red) and immunohistochemistry (SMA and desmin staining) of livers from resveratrol- or vehicle-treated CCl$_4$-cirrhotic rats. (original magnification 10x for Sirius Red and 20X for SMA and desmin). **Bottom:** Quantification of liver fibrosis (Sirius red staining area per total area, and SMA and desmin relative volume) in cirrhotic rats treated with resveratrol or vehicle. Values represent
arbitrary units normalized to vehicle livers. **B) SMA, Collagen I, NF B, TGF, MMP2, MMP9, TIMP1 and TIMP2 mRNA expression levels in livers from cirrhotic rats treated with resveratrol or vehicle. Values are normalized to vehicle-treated livers expression. C) Top** Representative Western blot analysis for SMA in livers from vehicle (n=7) or resveratrol-treated (n=7) CCl4-cirrhotic rats. **Bottom:** Densitometry quantification of α-SMA in cirrhotic livers treated with resveratrol and vehicle. Values represent arbitrary units normalized to vehicle livers.

**Fig. 4. Effects of resveratrol on hepatic stellate cells in vitro. A)** Relative SMA and collagen I mRNA levels in LX2 treated with resveratrol (n=12) or vehicle (n=12) normalized to an endogenous reference gene (18S). Values (mean±SEM) are normalized to vehicle-treated LX2 cells. **B)** Top: Representative Western blot analysis for Bad in LX2 treated with resveratrol (n=7) or vehicle (n=7). **Bottom:** Densitometry quantification of Bad in cirrhotic livers treated with resveratrol and vehicle. Values represent arbitrary units normalized to vehicle livers.

**Supplementary Fig. 1:** Effects of resveratrol on intrahepatic inflammation in CCl4-cirrhotic rats. **Top** Photomicrographs of histology (H&E stain) and immunohistochemistry (CD68 immunostaining) in livers from resveratrol (n=6) or vehicle-treated (n=6) CCl4-cirrhotic rats, showing that resveratrol markedly
attenuated the inflammatory infiltratation of cirrhotic livers. *Bottom*) Quantification of the number of CD68-positive cells in livers from vehicle or resveratrol-cirrhotic rats.

**Supplementary Fig. 2: Effect of resveratrol (20 mg/kg bw/day) on hepatic fibrosis in CCl₄-cirrhotic rats.** A) *Top:* Representative histological images of livers stained with Sirius red from resveratrol- or vehicle-treated CCl₄-cirrhotic rats. (original magnification 10x). *Bottom:* Quantification of liver fibrosis (Sirius red staining area per total area) in cirrhotic rats treated with resveratrol or vehicle. Values represent arbitrary units normalized to vehicle livers. B) Collagen I mRNA expression levels in livers from cirrhotic rats treated with resveratrol or vehicle. Values are normalized to vehicle-treated livers expression. C) *Top* Representative Western blot analysis for SMA in livers from resveratrol (n=7) or vehicle-treated (n=7) CCl₄-cirrhotic rats. *Bottom:* Densitometry quantification of α-SMA in cirrhotic livers treated with resveratrol and vehicle. Values represent arbitrary units normalized to vehicle livers.
Reference List


[40] Li H, Xia N, Forstermann U. Cardiovascular effects and molecular targets of resveratrol. Nitric Oxide 2012;26:102-110.


Figure 1
Figure 2

- A: Bar graph showing normalized NO levels under Vehicle and Resveratrol treatments.
- B: Bar graph showing TXA₂ production (pg/ml) under AA and Resveratrol treatments.
Figure 3

A) Sirius Red staining images showing Vehicle and Resveratrol treatments. Images are labeled 10X and 20X magnification.

B) Bar graphs showing normalized α-SMA, COLL-1, NFKB, and TGFB mRNA levels for Vehicle (Veh) and Resveratrol (RSV). Bars with asterisks indicate significant differences (p<0.05).

C) Western blot images showing α-SMA protein levels for Vehicle and Resveratrol treatments. GAPDH is used as a loading control. The bar graph below indicates normalized α-SMA protein levels with a p-value of p<0.05.
Figure 4
Table 1: Effects of resveratrol (10 mg/kg bw/day) administration on hepatic and systemic hemodynamics in cirrhotic rats. Results are expressed as mean ± SD.

Portal pressure (PP); mean arterial pressure (MAP); portal blood flow (PBF); superior mesenteric artery blood flow (SMABF); heart rate (HR).

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<th>Parameter</th>
<th>Vehicle n=8</th>
<th>Resveratrol n=8</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td>PP (mmHg)</td>
<td>14.3 ± 2.2</td>
<td>12.1 ± 0.9*</td>
<td>0.02</td>
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<tr>
<td>MAP (mmHg)</td>
<td>94 ± 18</td>
<td>94 ± 16</td>
<td>0.98</td>
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<tr>
<td>PBF (ml·min⁻¹)</td>
<td>12.9 ± 4.2</td>
<td>12.9 ± 3.1</td>
<td>0.99</td>
</tr>
<tr>
<td>SMABF (ml·min⁻¹)</td>
<td>11.9 ± 1.9</td>
<td>11.3 ± 2.1</td>
<td>0.57</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>313 ± 56</td>
<td>326 ± 40</td>
<td>0.59</td>
</tr>
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</table>
**Table 2**: Effects of resveratrol (20 mg/kg bw/day) administration on hepatic and systemic hemodynamics in cirrhotic rats. Results are expressed as mean±SD.

Portal pressure (PP), mean arterial pressure (MAP), heart rate (HR).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle n=8</th>
<th>Resveratrol n=9</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP (mmHg)</td>
<td>14,7 ± 1,7</td>
<td>11,6 ± 2,1*</td>
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<tr>
<td>MAP (mmHg)</td>
<td>91 ± 21</td>
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<td>HR (beats/min)</td>
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<td>347 ± 40</td>
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