

UNIVERSITÀ  
DEGLI STUDI  
DI PADOVA

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## RESVERATROL ADMINISTRATION IMPROVES INTRAHEPATIC ENDOTHELIAL DYSFUNCTION AND REDUCES LIVER FIBROSIS AND PORTAL PRESSURE IN CCl<sub>4</sub>-CIRRHOTIC RATS

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*A mamma e papà*

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## **Resveratrol administration improves intrahepatic endothelial dysfunction and reduces liver fibrosis and portal pressure in CCl<sub>4</sub>-cirrhotic rats**

Abbreviations: nitric oxide (NO), cyclooxygenase-1 (COX-1), endothelial nitric oxide synthase (eNOS), carbon tetrachloride (CCl<sub>4</sub>), mean arterial pressure (MAP; mmHg), portal pressure (PP, mmHg), portal blood flow (PBF; ml·min<sup>-1</sup>), superior mesenteric artery blood flow (SMABF; ml·min<sup>-1</sup>), methoxamine (Mtx), acetylcholine (ACh), thromboxane B<sub>2</sub> (TXB<sub>2</sub>), superoxide (O<sub>2</sub><sup>-</sup>), dihydroethidium (DHE), cyclic guanosine monophosphate (cGMP), phosphorylated eNOS (P-eNOS), smooth muscle actin (SMA).

## **Riassunto:**

*Introduzione e scopo dello studio:* L'ipertensione portale è una condizione fisiopatologica caratterizzata dall'aumento della pressione nel sistema portale che può causare lo sviluppo di manifestazioni cliniche severe quali le emorragie da rottura di varici esofagee e/o gastriche e da gastropatia congestizia, l'ascite, l'encefalopatia epatica e la sindrome epatorenale. L'ipertensione portale può manifestarsi in corso di diverse patologie, fra le quali la più comune nel mondo occidentale è la cirrosi epatica. Nella cirrosi il fattore iniziale alla base dello sviluppo dell'ipertensione portale è l'aumento delle resistenze vascolari intraepatiche, dovuto sia alle alterazioni morfologiche del fegato, sia alla disfunzione endoteliale nel letto vascolare porto-epatico, caratterizzata da un'esagerata risposta a sostanze vasocostrittrici e una risposta ridotta a sostanze vasodilatatrici. Il resveratrolo è un polifenolo di origine vegetale presente nell'uva e nei suoi derivati, negli arachidi, e in diverse altre piante. Tra le numerose proprietà benefiche di questa sostanza sono state studiate soprattutto quella antiossidante ed antinfiammatoria. Il resveratrolo possiede effetti benefici anche a livello endoteliale: è coinvolto nella regolazione di numerose sostanze vasoattive e riduce lo stress ossidativo, fattori entrambi coinvolti nella patogenesi della disfunzione endoteliale. Lo scopo di questo studio era quello di valutare gli effetti del resveratrolo sull'emodinamica sistemica ed epatica, la disfunzione endoteliale epatica e la fibrosi epatica nel modello animale di cirrosi epatica indotta mediante CCl<sub>4</sub>.

*Metodi:* Il resveratrolo (10 o 20 mg/kg/die) o il suo veicolo sono stati somministrati oralmente per due settimane in ratti cirrotici; successivamente sono state eseguite le seguenti misurazioni: a) *in vivo*: pressione arteriosa

media (PAM), pressione portale (PP), flusso portale (FP) e flusso arterioso mesenterico superiore (FAMS); *b) fegati isolati e perfusi*: funzione endoteliale valutata attraverso curve dose-risposta all'acetilcolina; *c) valutazione della fibrosi epatica*: colorazione Sirius Red di sezioni di fegato, espressione dell'mRNA per il collagene-1 ed espressione proteica di  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), indice dell'attivazione delle cellule stellate epatiche.

*Risultati*: La somministrazione di resveratrolo ha determinato una riduzione della PP, senza modificazioni significative della PAM, del FP e del FAMS. La riduzione della PP era associata ad un miglioramento della risposta vasodilatatrice all'acetilcolina e della fibrosi a livello epatico. Oltre al miglioramento della fibrosi valutato mediante colorazione Sirius Red, è stata osservata una riduzione dell'espressione dell'mRNA per il collagene-1 e dell'  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA).

*Conclusioni*: Nei ratti cirrotici il resveratrolo riduce la PP attraverso un miglioramento della fibrosi e della disfunzione endoteliale epatica. Il resveratrolo, dunque, potrebbe essere un supplemento ideale nel trattamento dell'ipertensione portale in pazienti con cirrosi epatica.

**Abstract:**

*Background and Aims:* In the Western countries, liver cirrhosis is the main cause of portal hypertension, which is defined as a clinical syndrome characterized by an increased hydrostatic pressure within the portal venous system. The importance of this syndrome is defined by the frequency and severity of its complications: gastrointestinal bleeding from ruptured gastroesophageal varices, ascites, hepatorenal syndrome and hepatic encephalopathy. In cirrhosis, the initial factor determining the onset of portal hypertension is the increase in intrahepatic vascular resistance, which is due to the morphological changes of the liver, but also to endothelial dysfunction in the porto-hepatic vascular bed, characterized by an exaggerated response to vasoconstrictors and a deficient response to vasodilators. Resveratrol, a polyphenol found in a variety of fruits, possesses a wide range of beneficial properties, e.g. anti-oxidant and anti-inflammatory activity. In the endothelium resveratrol regulates several vasoactive substances and decreases oxidative stress, both factors involved in the pathophysiology of endothelial dysfunction. This study aimed at evaluating the effects of resveratrol on hepatic and systemic hemodynamics, hepatic endothelial dysfunction and hepatic fibrosis in CCl<sub>4</sub>-cirrhotic (CH) rats.

*Methods:* Resveratrol (10 or 20mg/kg/day) or its vehicle were administered to CH rats orally for two weeks; then the following measurements were done: *a) in vivo:* mean arterial pressure (MAP), portal pressure (PP), portal blood flow (PBF) and superior mesenteric artery blood flow (SMABF); *b) isolated perfused livers:* endothelial function assessed by dose-relaxation curves to acetylcholine; *c) assessment of fibrosis:* Sirius Red staining of liver sections, collagen-1

mRNA expression and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) protein expression, as a surrogate of hepatic stellate cell activation.

*Results:* Resveratrol administration significantly decreased PP without significant changes in MAP, PBF and SMABF. Reduction in PP was associated with an improved vasodilatory response to acetylcholine and with a significant reduction in liver fibrosis. The decrease in hepatic fibrosis was associated with a reduced collagen-1 mRNA expression and  $\alpha$ -SMA protein expression.

*Conclusions:* In cirrhotic rats, resveratrol administration reduces portal pressure by improving liver fibrosis and endothelial dysfunction, suggesting that it may be an ideal supplement in the treatment of portal hypertension in patients with cirrhosis.

## **Introduction:**

### *Physiopathology of portal hypertension in liver cirrhosis:*

Liver cirrhosis is characterized by the replacement of normal tissue with fibrous tissue leading to progressive loss of the organ function. In the Western countries, liver cirrhosis is the main cause of portal hypertension, which is defined as a clinical syndrome characterized by an increased hydrostatic pressure within the portal venous system (normal values: 1-5 mmHg). The importance of this syndrome is defined by the frequency and severity of its complications: gastrointestinal bleeding from ruptured gastroesophageal varices, ascites, hepatorenal syndrome and hepatic encephalopathy. These complications represent the primary cause of death and the main indication for liver transplantation in patients with cirrhosis (1). Portal pressure is the result of the relationship between the blood flow volume entering the portal system and the resistance to portal blood flow. The mathematical expression of this relationship is given by the Ohm's formula:  $P=Q \cdot R$ , where P represents change in pressure along the vessel, Q represents blood flow and R resistance to the flow. In cirrhosis, portal hypertension results from an increase both in intrahepatic resistance and in splanchnic blood flow. The initial factor determining the onset of portal hypertension is the increase in intrahepatic vascular resistance, which is mainly due to the progressive deposition of collagen in the hepatic acini. Collagen deposition in the Disse's space narrows the sinusoidal lumen, causing an increase in resistance to portal flow. The further transformation of collagen into fibrotic tissue, together with regeneration of hepatocytes, leads to the derangement in hepatic vascular structure. The

compression of centrolobular venules by regenerating nodules, granulomas, or portal inflammation are major further mechanisms responsible for the increase in hepatic resistance. Stellate or Ito cells, located in the Disse's space, are pivotal for the deposition of extracellular matrix and collagen. Following liver damage, these cells undergo a phenotypic transformation into myofibroblastic-like cells, which are able to contract and to secrete collagen (2). The increase in intrahepatic vascular resistance is due not only to these morphological changes, 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, 3). Smooth muscle cells are present in the wall of hepatic and portal veins and venules, and myofibroblasts, derived from stellate cells, are present in the cirrhotic liver, around the sinusoids and hepatic venules (4). The imbalance between the endothelial production of vasodilators and the response to vasoconstrictors is defined as endothelial dysfunction. In cirrhosis a decreased nitric oxide (NO) bioavailability and an increase in cyclooxygenase-1 (COX-1)-derived prostanoids within the liver play a major role in the pathogenesis of endothelial dysfunction (5-8). NO has a very short half-life (20–30 s) and freely diffuses through cellular membrane. It mainly acts by activation of guanylate cyclase with production of cGMP and subsequent relaxation of smooth muscle cells. Endothelial NO synthase (eNOS) is responsible for most of the vascular NO produced in a reaction where L-arginine is oxidized to L-citrulline and NO (9). In cirrhotic liver the bioavailability of NO is decreased, even though eNOS protein levels are not changed. This may be partly explained by a decrease in the activity of eNOS, which is due to a reduced eNOS phosphorylation, an increased caveolin expression and a decreased

intrahepatic availability of the eNOS cofactor, tetrahydrobiopterin (10). Also an increased hepatic production of COX-derived prostanoids may play a role in the dynamic intrahepatic changes which occur in cirrhosis. In the sinusoidal endothelial cells of cirrhotic liver, an upregulation of COX-1 leads to an increase in thromboxane A<sub>2</sub> production, which may be responsible for the hyper-response to alpha-adrenergic stimuli and contribute to the increase in intrahepatic resistance (11). It has been shown that the impaired vasorelaxation in cirrhotic livers is completely prevented by COX-1 inhibition (7).

While in the liver with normal resistance and compliance an increase in flow does not modify portal pressure, in cirrhosis, where the outflow portal resistance is enhanced, an increase in portal flow is responsible for an increase in portal pressure. In patients with portal hypertension, total splanchnic inflow is increased (10). These data have been confirmed also in experimental models of portal hypertension (12, 13). The efficacy of non selective beta-blockers in patients with portal hypertension is due to the fact that they increase splanchnic resistance and decrease cardiac output, so decreasing portal inflow (14). The increase in portal inflow occurring in cirrhosis is caused mainly by an arterial vasodilatation in the splanchnic territory, due to an increase in endogenous circulating agents with vasodilating effects, such as glucagon, prostacyclin, histamine, intestinal vasoactive peptide, substance P, colecystokinin, estrogens, ammonia, endotoxins, adenosine, biliary acids, NO, alpha-calcitonin gene-related peptide, adrenomedullin, vascular endothelial growth factor and CO (10).

The current options for the treatment of chronic liver disease have shown limited therapeutic benefits and are associated with serious complications.

Therefore, we are in need for exploring novel and alternative approaches for the treatment of cirrhosis and portal hypertension.

*Oxidative stress and intrahepatic vascular resistance:*

Reactive oxygen species (ROS) are oxygen-based molecules with high chemical reactivity that include free radicals (species with one or more unpaired electrons) such as superoxide ( $O_2^-$ ) and hydroxyl ( $OH^\cdot$ ), and non-radical species such as hydrogen peroxide ( $H_2O_2$ ). Increased oxidative stress has been suggested to play a pathophysiological role in the progression of the disease in patients with alcoholic liver diseases (15), nonalcoholic steatohepatitis (16), and chronic hepatitis C (17), as well as in most types of experimental liver fibrogenesis (18). ROS cause hepatocyte necrosis and apoptosis by promoting mitochondrial permeability transition, affect directly the HSC and myofibroblasts behaviour and up-regulate the expression of critical fibrosis-associated genes via activation of signal transduction pathways and transcription factors, including JNK, activator protein-1, and NF $\kappa$ B (19). Therefore, in cirrhosis any pharmacological intervention using antioxidants to decrease ROS may attenuate liver fibrosis and, as a consequence, ameliorate portal hypertension. On the other hand, the beneficial effects of antioxidants on portal pressure could also occur through a modulation of the vascular tone in the liver, the dynamic component of intrahepatic resistance. In fact, it is known that NO is capable of reacting with  $O_2^-$ , leading to peroxynitrite ( $ONOO^-$ ) formation with an ongoing decrease in NO bioavailability (20). Antioxidants may improve intrahepatic endothelial function by reducing NO scavenging.

### Resveratrol:

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 is a phytoalexin, produced in response to pathogenic attack and environmental stress (21). Resveratrol has been suggested to be implicated in the health benefits of red wine, as it may explain the lower incidence of myocardial infarction in France, the so called "French paradox". Preclinical and clinical studies have demonstrated the therapeutic usefulness of resveratrol in chronic illnesses such as arthritis, diabetes, neoplastic and neurodegenerative diseases (22, 23). Extensive research in animal models has attributed several pharmacological effects to resveratrol: anti-oxidant, anti-neoplastic, modulator of lipidic metabolism, as well as anti-inflammatory and anti-platelet activity (24-27).

In the liver, resveratrol increases hepatic glutathione (GSH) content, scavenges free radicals, inhibits the production of the inflammatory cascade inducer NF- $\kappa$ B, and decreases the expression of several pro-inflammatory cytokines (28). Resveratrol reduces the hepatotoxicity induced by acetaminophen, ethanol and carbone tetrachloride (CCl<sub>4</sub>), and prevents liver damage due to ischemia-reperfusion, irradiation and high fat diet (28). More specifically, in the animal model of CCl<sub>4</sub>-induced hepatotoxicity, resveratrol has been shown to decrease the levels of malondialdehyde, an important marker of lipid peroxidation, and increase the production of GSH (29). In rats, chronic oral administration of grape juice containing resveratrol decreased CCl<sub>4</sub>-induced protein oxidation and lipid peroxidation by inducing the enzymatic activity of superoxide dismutase and catalase (30). In this animal model resveratrol inhibited the production of

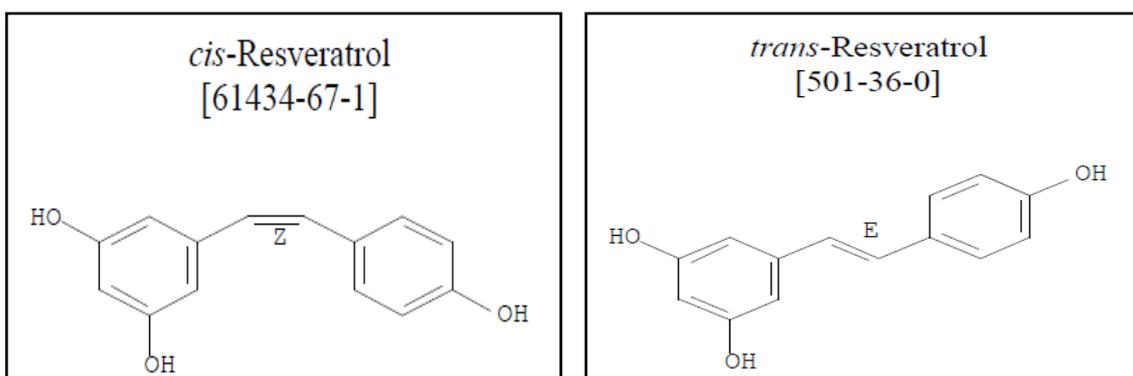
transforming growth factor- $\beta$  (TGF-  $\beta$ ) and decreased the levels of bilirubin and collagen (31). These data were confirmed in a recent study by Vitaglione et al. (32), who showed that oral administration of resveratrol for 2 weeks prevented CCl<sub>4</sub>-induced lipid peroxidation in rats.

The beneficial effects of resveratrol on vascular function may be partly due to an upregulation of endothelial nitric oxide synthase (eNOS) expression and activity, as it has been demonstrated in endothelial cell culture studies after acute and chronic resveratrol exposure (33-35). Moreover, it has been shown that resveratrol inactivates COX-1 through a potent inhibition of both catalytic activities of the enzyme, (36).

Overall, it is plausible to hypothesize that resveratrol exerts beneficial effects in the pathophysiological mechanisms involved in the development of portal hypertension in cirrhosis.

### *Chemical Structure*

The chemical formula of Resveratrol possesses two phenolic rings, which permit two structural conformations: the *cis* conformation and the *trans* conformation, with the latter present in wine in much higher concentrations (24). Several studies have shown that the *trans* conformation is the active form of Resveratrol and is stable for months if completely protected from light (37, 38).



### *Solubility*

Resveratrol in the solid state is whitish and highly soluble in ethanol and DMSO but poorly soluble in water (*Toxicological Summary for trans-Resveratrol [501-36-0]*).

### *Metabolism and Bioavailability*

An *in vitro* study investigating the absorption and metabolism of Resveratrol by using an isolated preparation of lumenally and vascularly perfused rat small intestine showed that the vascular uptake of lumenally administered Resveratrol was 20.5%. The majority of the absorbed Resveratrol was conjugated to yield Resveratrol glucuronide (16.8%), which was also the main luminal metabolite. Lesser amounts of Resveratrol sulfate, 3.0% and 0.3%, were found on the luminal and vascular side, respectively, while only minute amounts of Resveratrol and Resveratrol conjugates (1.9%) were found in the intestinal tissue (39). Another study performed to analyze the absorption and metabolism of Resveratrol in the rat jejunum showed that the major compound detected was the glucuronide conjugate of Resveratrol (96.5% of the amount absorbed) indicating its susceptibility to glucuronidation (40). Accordingly, it has been shown in cells that the most abundant metabolic forms of Resveratrol are glucuronidated and sulfated (41). Therefore, these *in vitro* studies confirm that Resveratrol is well absorbed and metabolized in the small intestine.

An *in vivo* study in rats showed that after oral administration of red wine Resveratrol absorption is fast and the maximum concentration in liver and

kidney is detectable after 60 minutes. Resveratrol is mainly excreted by the kidney (42). Another study showed that 77-80% of Resveratrol is absorbed in the intestine and the maximum blood concentration is reached 15 minutes after its administration. Nevertheless, after 24 hours less than 1% of the dose could be detected in liver, kidney, heart and spleen (43). In mice, 1.5 hours after the administration of <sup>14</sup>C-marked Resveratrol, radioactivity diminished in the blood and increased in the urine. Radioactivity accumulated mainly in liver and stomach, while it gradually decreased in the kidney. The glucuronidated and sulfated forms of Resveratrol were identified in these organs (44). All these studies show that Resveratrol is well absorbed in rats and mice.

Studies have also been carried out in humans. The absorption of a dietary relevant 25 mg oral dose was at least 70%, with a plasma half-life of about 9 hours. Most of Resveratrol was recovered in urine (53.4-84.9%) and a lower but variable proportion in feces (0,3-38.1%). The results were similar after intravenous administration of Resveratrol (45).

### *Toxicity*

Cases of toxicity have been observed in rats only after the administration of high concentrations. To evaluate the potential toxicity, rats were administered high doses (300, 1000, 3000 mg/kg/day) of Resveratrol by gavage for 28 days. The adverse events included dehydration, labored breathing, hunched posture and diarrhea. White blood cell counts were significantly increased in both sexes in the 3000 mg/kg/day dose group and in the males in the 1000 mg/kg/day dose group. In this study no adverse effects were observed in animals treated with

300 mg/kg/day oral dose of Resveratrol (46). Oral administration of Resveratrol to male rats for 28 days at a dose of 20 mg/kg/day did not cause any toxic effect (47).

The aim of the present study was to investigate the effects of chronic administration of resveratrol in CCl<sub>4</sub>-cirrhotic rats with portal hypertension.

## **Materials and Methods:**

### **Induction of cirrhosis by CCl<sub>4</sub> and resveratrol administration**

In male Wistar rats (50-75 g) cirrhosis was induced by inhalation of CCl<sub>4</sub> three times a week, and phenobarbital (0,3 g/l) was added to the drinking water as previously described (48). CCl<sub>4</sub> is an extensively used hepatotoxic agent in preclinical animal studies. When cirrhotic rats had developed ascites, after approximately 12-15 weeks of CCl<sub>4</sub> inhalation, administration of CCl<sub>4</sub> and phenobarbital was discontinued. One week later, animals were randomized and daily treated with resveratrol (10 mg/kg body weight (bw); Sigma, Tres Cantos, Madrid, Spain; n=10) or its vehicle (carboxymethylcellulose 0,7%; n=10) 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. The used dose of 10 mg/kg bw/day resveratrol has been shown to reduce liver oxidative damage after bile duct ligation (49) and to decrease acute liver damage induced by acute CCl<sub>4</sub> intoxication (29). Afterwards, an additional group of rats was treated with resveratrol at 20 mg/kg bw/day (n=10) or the corresponding vehicle (n=8) to evaluate if the observed effects of resveratrol on portal pressure and liver fibrosis are dose-dependent. The animals were kept in environmentally controlled animal facilities at the Institut d'Investigacions Biomèdiques August Pi I 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 the 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, to measure only portal blood flow (PBF; ml·min<sup>-1</sup>) going through the liver, and at the superior mesenteric artery to measure superior mesenteric artery blood flow (SMABF; ml·min<sup>-1</sup>). 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.

### **Biochemical analysis**

At the end of the *in vivo* hemodynamic study, serum samples were collected from femoral artery to subsequently evaluate: alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin (BR) and albumin by current standard protocols.

### **Evaluation of endothelial function**

After *in vivo* hemodynamic measurements, livers were quickly isolated and perfused by a flow-controlled perfusion system as previously described (48). The perfused rat liver preparation was allowed to stabilize for 20 min before vasoactive substances were added. The intrahepatic microcirculation was precontracted by adding the  $\alpha_1$ -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 per cent change in portal perfusion pressure (7). The gross appearance of the liver, stable perfusion pressure, bile production over  $0.4 \mu\text{L}/\text{min}/\text{g}$  of liver and a stable buffer pH ( $7.4 \pm 0.3$ ) were monitored during this period. If any viability criteria were not satisfied, the experiment was discarded.

### **Molecular studies**

#### **Measurement of superoxide ( $\text{O}_2^-$ ) content:**

To evaluate if resveratrol is able to reduce intrahepatic  $\text{O}_2^-$  levels, livers from cirrhotic rats treated with resveratrol ( $n=2$ ) or vehicle ( $n=2$ ) were promptly removed after the hemodynamic measurements and *in situ*  $\text{O}_2^-$  content was evaluated in fresh liver cryosections ( $10 \mu\text{m}$ ) stained with the oxidative fluorescent dye dihydroethidium (DHE) (Molecular Probes, Eugene, Oregon USA), as previously described (50).

### 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 placebo (n=8) using an enzyme immunoassay (Cayman), as previously described (51).

#### *eNOS and P-eNOS protein expression:*

Total eNOS and P-eNOS protein expression were determined by western blot in liver homogenates from CH rats treated with resveratrol (n=7) or vehicle (n=7) as previously described (51). 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 B<sub>2</sub>:

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 B<sub>2</sub> (TXB<sub>2</sub>), the end metabolite of thromboxane A<sub>2</sub>, was quantified in duplicate using a commercially available enzyme immunoassay (Cayman Chemical Company, Tallin, Estonia) (52). TXB<sub>2</sub>

production was expressed as absolute increment after dose-response curve to Ach over baseline before Mtx administration.

### **Evaluation of intrahepatic fibrosis**

#### *Quantification of intrahepatic fibrosis*

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 (53). Eight fields from each slide were randomly selected, and the red-stained area per total area was measured using AxioVision software.

#### *Protein expression of $\alpha$ SMA*

Hepatic protein expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) was determined by Western blot in hepatic samples using a mouse antibody against  $\alpha$ SMA (Sigma).

#### *Collagen I gene expression*

Hepatic gene expression of collagen I was assessed by real time quantitative reverse transcriptase-polymerase chain reaction using predesigned gene expression assays obtained from Applied Biosystems (AB, Foster City, CA) according to the manufacturer's protocol and reported relative to endogenous control 18S. All PCR reactions were performed in duplicate and using nuclease-free water as no template control.

### **Statistical analysis**

Statistics were performed using the SPSS 19.0 for Windows statistical package. All results are expressed as mean ( $\pm$ 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:**

All rats had macroscopic cirrhosis and signs of portal hypertension, as shown by the presence of ascites. No rats died during treatment. None of the two tested doses of resveratrol modified body, liver and spleen weight of rats. Moreover, there were no significant differences in the biochemical parameters evaluated (AST, ALT, bilirubin and albumin) between the two groups (Table 1).

### Protocol 1: CCl<sub>4</sub>-cirrhotic rats treated with 10 mg/kg bw/day of resveratrol

#### *Effect of resveratrol on superoxide levels in cirrhotic rat livers*

In livers from cirrhotic rats, resveratrol administration produced a marked and significant decrease in O<sub>2</sub><sup>-</sup> levels as shown by DHE fluorescence, clearly showing its potency as an antioxidant (Figure 1).

#### *Effect of resveratrol on hepatic and systemic hemodynamics in cirrhotic rats*

Cirrhotic rats receiving long term treatment with resveratrol had a significantly lower PP than cirrhotic rats treated with vehicle (12.1±0.9 mmHg in Resveratrol vs 14.3±2.2 mmHg in vehicle; p<0.05), corresponding to a PP reduction of 15%. No changes in PBF, SMABF or systemic hemodynamic parameters (MAP, HR) were observed (Table 2).

#### *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<sup>-7</sup> M Ach and the paradoxical vasoconstriction at the 10<sup>-6</sup> M and 10<sup>-5</sup> M dose. Resveratrol

administration improved the endothelial dysfunction of cirrhotic rat livers, as shown by the observed vasorelaxation in response to Ach (Figure 2).

#### *Effect of resveratrol on the NO pathway*

No significant difference in eNOS expression was observed among livers from cirrhotic rats treated with vehicle or resveratrol (Figure 3A). Livers from rats treated with resveratrol exhibited a reduction in eNOS phosphorylation at Ser 1176 (Figure 3A). In addition, cGMP content (a marker of NO bioavailability) was not significantly different among both groups of cirrhotic rat livers ( $6.1 \pm 2.7$  pmol/ml in resveratrol vs  $5.9 \pm 1.4$  pmol/ml in vehicle) (Figure 3B). These data suggest that, in cirrhotic livers of rats treated with resveratrol, a decrease in oxidative species scavenging NO may balance its decreased production due to reduced eNOS activity.

#### *Effect of resveratrol on COX activity*

In the perfusate of cirrhotic livers resveratrol administration resulted in a significant decrease in TXB<sub>2</sub> production ( $96,4 \pm 108$  pg/ml) compared to vehicle ( $33,3 \pm 15,7$  pg/ml) (Figure 4).

#### *Effect of resveratrol on intrahepatic fibrosis*

As expected, cirrhotic rats exhibited a marked architectural distortion with abundant fibrosis revealed by extensive collagen staining. Importantly, rats receiving resveratrol exhibited a significant reduction in hepatic fibrosis, as proved by a decreased fibrosis area on Sirius Red stained liver sections (Figure 5A). This was associated with a significant reduction in collagen I mRNA expression (Figure 5B) and with a marked decrease in  $\alpha$ -SMA expression, a surrogate marker of hepatic stellate cells activation (Figure 5C).

## Protocol 2: CCl<sub>4</sub>-cirrhotic rats treated with 20 mg/kg bw/day of resveratrol

### *Effect of resveratrol on hepatic and systemic hemodynamics in cirrhotic rats*

Cirrhotic rats treated with this dose of resveratrol showed a decrease in PP (22%) compared to cirrhotic rats treated with vehicle (11.6±2.2 mmHg in resveratrol vs 14.7±1.8 mmHg in vehicle: p<0.05), greater than the one observed in the rats treated with the lower dose. In addition, no significant difference in MAP was observed (data not shown).

### *Effect of resveratrol on intrahepatic fibrosis*

Similarly to what happened with 10 mg of resveratrol/kg bw/day, rats receiving 20 mg of resveratrol/kg bw/day exhibited a 38% reduction in collagen deposition as shown by the analysis of Sirius Red stained liver sections. Collagen I mRNA expression and  $\alpha$ -SMA protein expression were also reduced in livers from rats receiving resveratrol in comparison with those treated with vehicle (45% and 55%, respectively).

**Discussion:**

High oxidative stress within the cirrhotic liver plays a major role in increasing hepatic vascular resistance, the primary factor in the development of portal hypertension (6). Oxidative stress induces vascular occlusion and fibrosis in the liver and activates hepatic stellate cells, which display contractile properties and produce extracellular matrix and collagen. These architectural changes determine an increase in intrahepatic resistance and consequently PP. Moreover, due to oxidative stress, the endothelium loses its normal vasodilatory and anti-thrombotic properties and becomes vasoconstrictive and pro-thrombotic. These dynamic and reversible components further increase the resistance in the liver (54). Many potential treatments are in development for reducing PP. Agents that reduce oxidative stress and inflammation may lower resistance to blood flow within the liver. Different approaches have been carried out to reduce hepatic oxidative stress in cirrhotic animal models. A previous study showed that tempol, a superoxide dismutase mimetic, was able to reduce hepatic vascular resistance and PP in CCl<sub>4</sub>-cirrhotic rats, but it also determined a significant decrease in MAP (55). In the same animal model, another study showed that superoxide dismutase gene transfer reduced PP by improving endothelial dysfunction (56). Regardless, the use of both strategies in the clinical practise is far from being implemented.

Resveratrol is a natural substance with potent antioxidant activity, which is attracting increased attention due to its health benefits, especially in common aging-related diseases such as cancer, type 2 diabetes and cardiovascular disease (57). Among other actions, resveratrol induces antioxidant enzymes, suppresses the expression of antiapoptotic genes, inhibits the production of

angiogenic and inflammatory factors and modulates cell-cycle regulatory genes (57). Due to the low toxicity and rare minor side effects reported, resveratrol is widely available in the pharmacies as a supplement.

The main finding of this study is that oral administration of resveratrol for two weeks caused a reduction in PP in cirrhotic rats, without affecting PBF and SMABF, suggesting that this decrease in PP was due to a reduction in intrahepatic resistance. Remarkably, in cirrhotic rats receiving the higher dose of resveratrol (20 mg/kg bw/day), PP reduction was greater than the one observed with 10 mg/kg bw/day (22% and 15%, respectively). It is worth remembering that this reduction is similar to the 20% target reduction that in patients with cirrhosis has been associated with an improvement in prognosis (58), and that this is the most pronounced reduction in PP observed using antioxidant strategies in cirrhotic rats (55, 56). The results suggest that this reduction in intrahepatic resistance/PP is due to both an improvement of endothelial dysfunction and an amelioration of architectural abnormalities of the cirrhotic liver.

First of all, I corroborated the efficacy of resveratrol at reducing the increased superoxide levels of cirrhotic rat livers. This result is consistent with previous reports showing that, in liver cirrhosis, resveratrol exerts its anti-oxidant properties by inducing superoxide dismutase and catalase activity (59, 60). Moreover, previous studies have demonstrated that resveratrol prevents hepatic injury. Indeed, resveratrol provides protection against liver damage induced by several hepatotoxins, such as acetaminophen, ethanol and CCl<sub>4</sub> (28). In rats, resveratrol has been shown to prevent CCl<sub>4</sub>-induced fibrosis by inhibiting the activation of NF-kappaB, which promotes the transcription of several cytokines

including the pro-fibrogenic TGF-  $\beta$  (31). Accordingly, I found that resveratrol significantly decreased hepatic stellate cells activation and liver fibrosis, as shown by a decreased fibrosis area on Sirius Red stained liver sections and the reduction in  $\alpha$ -SMA protein and collagen I mRNA expression. These effects were not boosted by higher doses of resveratrol, since we found similar results with 10 and 20 mg/kg bw /day.

Resveratrol administration also improves endothelial dysfunction. It has been demonstrated that in cirrhotic rat livers a COX-1-dependent increase in TXA<sub>2</sub> is the main factor mediating the endothelial dysfunction, which can be corrected by COX-1 inhibition (7, 8). In that regard, resveratrol has been shown to act as a peroxidase-mediated inactivator of COX-1 (36). In agreement with that, in our study we found that resveratrol inhibited TXA<sub>2</sub> production in cirrhotic livers. Based on these results, it is reasonable to presume that the protective effect of resveratrol on the increase in PP and the development of endothelial dysfunction were mediated by a reduction in TXB<sub>2</sub> production. However, the improvement itself in liver fibrosis may have contributed to recover endothelial function.

In cirrhosis, hepatic endothelial dysfunction has also been attributed to a diminished intrahepatic NO availability (5). Some studies reported that acute and chronic resveratrol administration enhances endothelial NO bioavailability through an increase in eNOS expression and activity (33-35). However, contrary to our initial hypothesis, resveratrol did not increase NO bioavailability in cirrhotic rat livers despite the reduction in intrahepatic superoxide levels. This may be explained by the fact that, in resveratrol-treated cirrhotic rat livers, eNOS activation was reduced. It is well-known that oxidative stress is a potent

inducer of eNOS expression and activity (61). Accordingly, the reduction in intrahepatic superoxide levels observed in resveratrol-treated rats may be the mechanism causing the lower eNOS activity and, as a consequence, the lack of the expected increase in NO bioavailability. Consistent with our data, it has been shown that in aorta of spontaneously hypertensive rats resveratrol improves endothelium-dependent relaxation without modifying eNOS levels (62).

In conclusion, our data show that a chronic resveratrol administration to cirrhotic rats reduces PP, mainly due to a regression of liver fibrosis and to a correction of endothelial dysfunction, without affecting systemic hemodynamics. Due to these properties, resveratrol may constitute an ideal supplement as an adjunctive therapy for cirrhotic patients with portal hypertension.

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**Tables:****Table 1:** Effects of resveratrol administration (10 mg/kg bw/day) on metabolic parameters

<b>Parameter</b>	<b>Vehicle (n=5)</b>	<b>Resveratrol (n=6)</b>
AST (U/L)	175 ± 95	202 ± 89
ALT (U/L)	68 ± 21	90 ± 25
Bilirubin (mg/dl)	0,36 ± 0,30	0,18 ± 0,26
Albumin (g/l)	27 ± 5	30 ± 5

Biochemical parameters in cirrhotic rats treated with resveratrol or vehicle.

Results are expressed as mean±SD.

**Table 2:** Effects of resveratrol (10 mg/kg bw/day) administration on hepatic and systemic hemodynamics in cirrhotic rats.

<b>Parameter</b>	<b>Vehicle n=8</b>	<b>Resveratrol n=8</b>
PP (mmHg)	14,27 ± 2,21	12,14 ± 0,95*
MAP (mmHg)	94,12 ± 18,31	94,35 ± 17,89
PBF (ml·min <sup>-1</sup> )	12,88 ± 4,17	12,88 ± 3,07
SMABF (ml·min <sup>-1</sup> )	11,87 ± 1,95	11,26 ± 2,11
IVR (mmHg/ml·min <sup>-1</sup> )	1,02 ± 0,11	1,24 ± 0,12
HR (beats/min)	313 ± 56	326 ± 40

Results are expressed as mean±SD. \*= p<0,05 vs. vehicle

### Figures legends:

#### Figure 1: **Effect of resveratrol on superoxide levels in CCl<sub>4</sub>-cirrhotic rats.**

*Top:* Representative confocal microscopy images of *in situ* intrahepatic detection of O<sub>2</sub><sup>-</sup> with dihydroethidium in fresh liver sections from cirrhotic rats treated with vehicle or 10 mg/kg bw/day of resveratrol (both n=2). *Bottom:* DHE fluorescence intensity analysis showed a marked and significant reduction in intrahepatic O<sub>2</sub><sup>-</sup> in cirrhotic rats treated with resveratrol. Values are represented as arbitrary units (AU) normalized to vehicle livers ± SEM; \*p<0.01 vs vehicle.

#### Figure 2: **Effect of resveratrol administration on endothelial function in CCl<sub>4</sub>-cirrhotic rats.**

Endothelium-dependent vasorelaxation to acetylcholine (Ach) in isolated and perfused livers from CCl<sub>4</sub>-cirrhotic rats chronically treated with 10 mg/kg bw/day of resveratrol (n=6) or vehicle (n=7). Results are expressed as *per cent* change of PP in response to Ach and presented as mean±SEM. Resveratrol administration significantly improved the impaired vasodilatory response to Ach in CCl<sub>4</sub>-cirrhotic rat livers. PP, portal perfusion pressure.

#### Figure 3: **Effect of resveratrol administration on NO pathway**

A) *Top:* Representative western blot image of P-eNOS and total eNOS in CCl<sub>4</sub>-cirrhotic rat livers treated with vehicle (n=7) or resveratrol (n=7). B) *Bottom:* Densitometry analysis of eNOS (A) and P-eNOS/eNOS ratio (B) in livers from cirrhotic rats treated with vehicle or 10 mg/kg bw/day of resveratrol (n=7 per group). Values represent arbitrary units normalized to GAPDH /vehicle livers. C) Intrahepatic cGMP levels in cirrhotic rats chronically treated with resveratrol or

vehicle. Values represent arbitrary units normalized to vehicle livers (C). cGMP levels were significantly increased in resveratrol rat livers (n=8 per group).

\*p<0.05

Figure 4: **Effect of resveratrol administration on COX activity.** Normalized values for TXB<sub>2</sub> hepatic production in cirrhotic rats treated chronically with vehicle or 10 mg/kg bw/day of resveratrol (n=7 in both groups). \*p<0,05 vs vehicle.

Figure 5: **Effect of resveratrol administration on hepatic fibrosis in CCl<sub>4</sub>-cirrhotic rats.** **A)** *Top:* Representative histological images of livers stained with Sirius red from resveratrol- or vehicle-treated CCl<sub>4</sub>-cirrhotic rats. (original magnification 50x). *Bottom:* Quantification of liver fibrosis (Sirius red staining area per total area) in cirrhotic rats treated with resveratrol (10 mg/kg bw/day) or vehicle. Values represent arbitrary units normalized to vehicle livers; \*p<0.005 vs vehicle. **B)** Collagen I mRNA expression levels in livers from cirrhotic rats treated with resveratrol (10 mg/kg bw/day) or vehicle. Values are normalized to vehicle-treated livers expression; \*p<0.05 vs vehicle. **C)** *Top* Representative Western blot analysis for  $\alpha$ SMA in livers from vehicle (n=7) or resveratrol-treated (n=7) CCl<sub>4</sub>-cirrhotic rats. *Bottom:* Densitometry quantification of  $\alpha$ -SMA in cirrhotic livers treated with 10 mg/kg bw/day (A) of resveratrol and vehicle. Values represent arbitrary units normalized to vehicle livers; \*p<0,05 vs vehicle.