Mitochondrial-targeted antioxidant mitoquinone deactivates human and rat HSC and reduces portal hypertension in cirrhotic rats

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M.V. designed the research, conceived ideas, performed experiments, and wrote the manuscript. C.I.L.-S. conceived ideas, performed experiments and analyzed data. M.P.M. provided essential reagents and revised the manuscript. J.B. critically revised the manuscript and obtained funding. V.H.-G. and J.G.-S. conceived ideas and critically revised the manuscript. J.C.G.-P. designed the research, conceived ideas, wrote the manuscript, obtained funding and directed the study. All authors edited and reviewed the final manuscript.

Abstract (248 words)

In cirrhosis, activated hepatic stellate cells (HSC) play a major role promoting the increment in the intrahepatic vascular resistance and the development of portal hypertension. We have shown that cirrhotic livers have increased reactive oxygen species (ROS), and that antioxidant therapy decreases portal pressure. Considering that part of these ROS is produced within mitochondria, our aim was to assess the effects of the oral mitochondrial-directed antioxidant mitoquinone on hepatic oxidative stress, HSC phenotype, liver fibrosis and portal hypertension.

Methods: *Ex vivo*: HSC phenotype was analyzed in human precision-cut liver slices in response to mitoquinone or vehicle. *In vitro*: HSC phenotype, proliferation and viability were assessed in LX2, and primary human and rat HSC treated with mitoquinone or vehicle. *In vivo*: CCl_4- and TAA-cirrhotic rats were treated with mitoquinone (25mg/kg/day) or its vehicle for 2 weeks. Afterwards, mitochondrial and cellular oxidative stress, systemic and hepatic hemodynamic, liver fibrosis, HSC phenotype, and liver inflammation were determined.

Results: Mitoquinone deactivated human and rat HSC, with no effects on viability but decreasing their proliferation. In CCl_4-cirrhotic rats, mitoquinone decreased hepatic oxidative stress, reduced intrahepatic vascular resistance by reducing liver fibrosis improving HSC phenotype and decreasing hepatic inflammation. These effects were associated with a significant reduction in portal pressure without changes in arterial pressure. These results were further confirmed in the TAA-cirrhotic model, suggesting that mitoquinone strongly evokes HSC deactivation, decreased liver fibrosis and reduced portal hypertension.

Summary and conclusion: We propose mitochondrial-directed antioxidants as a novel treatment against portal hypertension and cirrhosis.

Introduction

Increased intrahepatic vascular resistance is the primary factor in the development of portal hypertension, the main complication of cirrhosis. This increased resistance to portal blood flow results from both dynamic increase in the hepatic vascular tone and from architectural alterations of the liver parenchyma[1,2]. Architectural distortion of the cirrhotic liver is partly due to increased synthesis of extracellular matrix components secreted by activated hepatic stellate cells (HSC)[3]. Soluble factors such as cytokines[4] and reactive oxygen species (ROS)[5,6] play an important role in activating HSC, resulting in an enhanced collagen synthesis and deposition[7–9].

ROS are oxygen-based molecules with high chemical reactivity that include free radicals such as superoxide (O_2^-), hydroxyl (OH^-), and nonradical species such as hydrogen peroxide (H_2O_2). In health, ROS generation is counteracted by enzymatic and nonenzymatic antioxidant systems that diminish ROS levels, maintaining an adequate redox balance[10]. However, because of their ability to cause oxidative stress and promote cell damage, ROS production needs to be tightly regulated.

In liver diseases, there are increased ROS levels[7,11]. This is mainly due to an increased production that may derive from multiple cell types, including endothelial cells, vascular smooth muscle cells, infiltrating inflammatory cells and Kupffer cells among others[12]. Numerous enzyme systems can potentially produce ROS. NADPH oxidases, xantine oxidase, uncoupled nitric oxide (NO) synthase and mitochondrial enzymatic systems are those more frequently involved[13,14]. An inefficient ROS elimination also helps to maintain elevated ROS levels being superoxide dismutase (SOD) isoforms one important ROS cleaning system. Because of this, many studies have focused on the potential of antioxidants to treat chronic liver disease. In fact, studies from our laboratory further reinforced the role of antioxidants reducing O_2 content, improving intrahepatic endothelial function and reducing portal pressure in cirrhotic rats with portal hypertension[15–17].

Mitochondrial respiratory chain is one of the key sites where ROS are produced[18]. Hence, there is considerable interest in developing strategies to target molecules with therapeutic potential for mitochondria.

The best characterized mitochondria-targeted antioxidant to date is mitoquinone, which have shown beneficial effects in pre-clinical models of liver diseases such as steatosis[19] or ischemia-reperfusion injury[20] and, importantly, promoted decreased hepatic necroinflammation in hepatitis C patients[21].

We hypothesize that mitoquinone may be a potent and a new therapeutic strategy to decrease mitochondrial O_2^-, reduce liver fibrosis and improve portal hypertension.

Materials and Methods

1- Ex vivo procedures

Preparation and treatment of human precision cut liver slices (hPCLS)

Human precision cut liver slices (hPCLS) were obtained using remnant peritumoral histological normal liver tissues obtained after partial hepatectomy. In all cases surgery was recommended to excise tumour metastasis from colon carcinoma and patients signed the informed consent (n=3 patients). Any of these patients had previously received adjuvant quimiotherapy that is known to promote liver changes. The Ethics Committee of the Hospital Clinic of Barcelona approved the experimental protocol. Tissues were prepared with a surgical biopsy punch and sectioned to 250 µm slices using a Vibratome VT1000S (Leica Microsystems, Wetzlar). Samples were washed in PBS, soaked in 4% agarose solution (Ultrapure LMP Agarose, Invitrogen) for 20 min, and then orientated, mounted and immobilised using cyanoacrylate glue. Tissue slices were placed on organotypic tissue culture plate inserts for up to 24 h (Millicel®-CM, Millipore). Liver slices were incubated in William's E Medium (Gibco, Paisley) supplemented with penicillin/streptomycin and L-glutamine in 6-well plates at 37 °C/ 5% CO_2/ 80% O_2. Duplicate wells were used per donor with 3 liver slices per well. After 1 hour the media was replaced with fresh William's E Medium in the presence or absence of mitoquinone (3 nM and 10 nM) for 24h. hPCLS were lysed for mRNA expression analysis.

2- In vitro procedures

Hepatic stellate cells (HSC) treatments and characterization

a) LX2 cells (kindly provided by Dr. Bataller) were seeded onto 12-well plates at density of 20.000 per well in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin and cultured overnight.

b) Human primary HSC (hHSC) were isolated from the above mentioned histological normal human liver biopsies, as previously described[22]. Briefly, livers were perfused for 10 min at a flow rate of 20 ml/min at 37ºC with Gey's balanced salt solution (GBSS) with 0.6% heparin an then 30 min at a flow rate of 5 ml/min at a 37ºC with 0.02% collagenase A, 0.005% DNAse, and 0.16% pronase in GBSS. The resultant digested liver was excised and *in vitro* digestion was performed at 37ºC 10 min with 0.013% collagenase, 0.01% DNAse, and 0.004% pronase in GBSS. Cells were passed through nylon filters (100 µm) and centrifuged at 50 g for 5 min and the dispersed cells were fractionated by density gradient centrifugation using Histodenz (Sigma). Cells were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% Fetal Bovine Serum, 1% L-glutamine, 1% penicillin/streptomycin and 1% amphotericin B and cultured overnight. hHSC were cultured until passage 3 in IMDM for *in vitro* activation. Mitoquinone (10nM) or vehicle (PBS) was added for 24h.

c) Primary HSC (rHSC) from cirrhotic rats were isolated and cultured as described above. For *in vitro* treatments, mitoquinone (1, 3 and 10nM) or vehicle (PBS) was added to subconfluent cultures of HSC for 24 hours.

Cell phenotype was determined by mRNA analysis. Cell viability was analysed using trypan blue staining, and proliferation calculated as the ration between initial/final cells[23]

Results from LX2 cells were derived from n=3-5 independent experiments. Results using primary HSC were derived from triplicates from three independent isolations.

3- In vivo experiments

Induction of cirrhosis by carbon tetrachloride (CCl_4) and by thioacetamide (TAA)

a) CCl_4: Male Wistar rats weighting 50-75 g were induced to cirrhosis by inhalation of CCl_4 three times a week. Phenobarbital (0.3 g/l) was added to the drinking water as

previously described[24,25]. A high yield of micronodular cirrhosis was obtained after approximately 14-18 weeks of CCl_4 inhalation. When the cirrhotic rats developed ascites, administration of CCl_4 and phenobarbital was stopped.

b) TAA: Male Sprague Dawley rats (150-200g) were induced to cirrhosis by intraperitoneal injection of TAA (Sigma-Aldrich) dissolved with normal saline, twice a week at a dose of 250 mg/kg for 12 weeks.

The animals were kept in environmentally controlled animal facilities at the Institut d'Investigacions Biomèdiques August Pi i Sunyer. All experiments 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).

Mitoquinone treatment

Cirrhotic rats were randomized to receive Mitoquinone (MIT: MS010 25 mg/kg/day; 20-25% w/w of mitoquinone and 75-80% w/w β-cyclodextrin) or its vehicle, DecylTPP (Veh), administered orally by *gavage* once a day for 2 weeks. Treatment started one week after the recognition of ascites in CCl_4 model and immediately after stopping TAA, hence drugs were administered to animals with fully developed cirrhosis. Experiments were performed 1 hour after the last dose of mitoquinone or vehicle. Treatments were prepared by a third person and experimental studies were realized blindly. The dose of mitoquinone used has been previously shown to have antioxidant properties[19].

***In vivo* hemodynamic study & biochemical analysis**

Rats were anesthetized with ketamine (100 mg/kg body weight, Imalgene 1000; Merial) plus midazolam (5 mg/kg body weight, Laboratorio Reig Jofré S.A.) intraperitoneally, fastened to a surgical board and maintained at a constant

temperature of 37 ± 0.5ºC (continuously monitored during the entire experiment). A tracheotomy and cannulation with a PE-240 catheter (Portex) was performed in order to maintain adequate respiration during the anaesthesia. Indwelling catheters made of polyethylene tubing (PE-50; Portex) were placed into the femoral artery to measure mean arterial pressure (MAP: mmHg) and to the ileocolic vein to measure portal pressure (PP: mmHg)[26]. Portal blood flow (PBF: ml/min) was measured with a non-constrictive peri-vascular ultrasonic transit-time flow probe (2PR, 2-mm diameter, Transonic Systems Inc.) placed around the portal vein just before its entrance in the liver avoiding the measurement of most portal-collateral blood flow. 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 (AD Instruments). Intrahepatic vascular resistance (IHVR) was calculated as PP/PBF and corrected by liver weight. Hemodynamic data were collected after a 20-min stabilization period.

At the end of the *in vivo* hemodynamic study, serum samples from cirrhotic rats were collected to subsequently evaluate alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirrubin and albumin, all by standard protocols.

Evaluation of endothelial function

In CCl_4-cirrhotic rats, after the *in vivo* hemodynamic measurements, livers were quickly isolated and perfused by a flow-controlled perfusion system as previously described[24]. 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 α_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; 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[27]. 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.

Indocyanine green (ICG) clearance

In a subgroup of TAA-cirrhotic rats (n=7 per group), after the *in vivo* hemodynamic measurements, ICG (0.5 mg/kg) was injected through the femoral vein. Arterial blood samples (0.3ml) were taken at baseline and 2, 4 and 15 min after the injection and centrifuged at 10,000g for 10 min. ICG absorbance was determined spectrophotometrically at a wavelength of 805 nm. Measured ICG absorbance was converted into the corresponding plasma concentration using a standard curve. ICG plasma disappearance rate was defined as the percentage decrease in ICG-plasma concentration per minute (%/min)[28].

Measurement of the cellular and mitochondrial superoxide (O_2^-) content in liver tissue

In situ O_2^- levels were evaluated in fresh cryosections (10 µm) obtained from cirrhotic rats treated with mitoquinone (n=3) or vehicle (n=3) using the oxidative fluorescent dye dihydroethidium (DHE; Molecular Probes) or MitoSox (Life Technologies) for cellular and mitochondrial oxidative stress, respectively. Six fields from each slide at 20x were randomly selected. Fluorescent images were obtained with a fluorescent microscope (Olympus) and quantitative analysis was performed with Image J 1.33 software (National Institutes of Health)[29].

Evaluation of hepatic fibrosis

Sirius Red staining

Livers from cirrhotic rats were fixed in 10% formalin, embedded in paraffin, sectioned and stained with 0.1% Sirius red, photographed using a microscope equipped with a

digital camera, and analyzed. Fifteen fields from each slide at 20x were randomly selected, and the red-stained area per total area was measured using AxioVision software[30]. Values are expressed as the mean of 15 fields taken from vehicle and mitoquinone-treated rats.

Hepatic hydroxyproline content

Hydroxyproline content was assessed with Hydroxyproline Colorimetric Assay Kit (Biovision) following manufacturer's instructions. Briefly, liver tissues were homogenized in dH_2O. HCl 12N was added to the homogenized and samples were incubated at 120ºC for 3 hours. Kit reagents were added and absorbance at 560 nm was read in plate reader and expressed as µg of hydroxyproline/mg liver.

Immunohistochemistry

Immunostaining of paraffin-embedded liver sections was performed with anti-α-SMA (Sigma), anti-desmin (Dako), anti-CD68 (Serotec), and anti-CD163 (Bio Rad) all 1/100 or phosphate-buffered saline as a negative control. Bound antibodies were visualized using with Dako Real Envision Detection System Peroxidase/DAB+, and slides were counterstained with hematoxylin. For quantification, a point grid was placed over the slide-pictures and the number of positive cells that collocate with the dots of the grid was counted. This value is expressed as a percentage of the total grid dots of the slide[29]. Counting was performed by 2 researchers blindly. Fifteen slide-pictures were counted in each liver.

Western Blot

Expression of α-SMA, desmin and nitrotyrosinated proteins was determined by western blot in hepatic samples as previously described[31]. Briefly, snap-frozen liver samples were used and processed by sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE) gels and nitrocellulose membranes. The following antibodies were used: α-SMA (Sigma), desmin (Dako) and nitrotyrosine (Sigma) all 1/1000. Thereafter, the membranes were incubated with the corresponding secondary peroxidase-coubled antibody (Santa Cruz). Blots were developed with enhanced chemiluminiscence (ECL, Amersham). Intensities of the digital bands were evaluated densitometrically using Multi Gauge V.2.1 (Fujifilm). Ponceau S staining assured equal protein loading. GAPDH (Santa Cruz) served as endogenous control.

Isolation of total RNA and Real time RT-PCR

Total RNA was isolated from liver tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and from cells lisates using RNAeasy Mini Kit (Qiagen) according to manufacturer's protocols. Hepatic mRNA expression of *α-sma*, *col1α1*, *col1α2*, *pdgfrβ* and *hif1α* was analyzed by real-time PCR using predesigned gene expression assays and reported relative to endogenous control GAPDH. All PCR reactions were performed in duplicate, using nuclease-free water as no template control.

Statistical analysis

Statistics were performed using the SPSS 18.0 for Windows statistical package (SPSS Inc., Chicago. IL, USA). All results are expressed as mean ± S.E.M. Comparisons between groups were performed using the Student's *t*-test, one sample *t*-test or the non-parametric test (Mann-Whitney) when adequate. Differences between the groups were assessed by one-way ANOVA. Results were considered significant when $p<0.05$.

RESULTS

Mitoquinone deactivates rat and human HSC

To ascertain whether mitoquinone may promote HSC deactivation, three different approaches were analyzed. First, the effects of mitoquinone were characterized in hPCLS, which represents an ex-vivo model system capable of mimicking the complex natural environment found in the human liver[32]. We exposed human liver slices to mitoquinone or its vehicle for 24h, observing that the expression of the HSC activation markers, *α-SMA*, *col1α1* and *pdgfrβ* were decreased upon mitoquinone treatment, indicating that mitoquinone is able to dose dependently deactivate HSC of human livers (Fig 1A).

Secondly, and to further confirm that mitoquinone promotes human HSC deactivation, we analyzed HSC activation markers in LX2, an immortalized human hepatic stellate cell line[33] and in primary human HSC activated *in vitro*. HSC activation markers *α-SMA, col1α1* and *pdgfrβ* were markedly and dose dependently decreased in mitoquinone-treated human HSC (Fig 1B-C). Furthermore, mitoquinone treatment decreased LX2 proliferation without affecting cell viability (Supplementary Fig 1A).

Finally, and aimed to validate that mitoquinone also deactivates HSC in preclinical models of cirrhosis, we tested its effects in HSC isolated from cirrhotic rats and subsequently treated *in vitro* with mitoquinone or vehicle and, importantly, in HSC freshly isolated from mitoquinone or vehicle-treated cirrhotic rats. In both cases, HSC became deactivated as demonstrated by significant lower expressions of *α-SMA*, *col1α1* and *pdgfrβ* in comparison to the vehicle group (Fig 1E). Importantly, mitoquinone did not affect rat HSC viability when treated in vitro (Supplementary Fig 1B), thus confirming the results obtained using LX2.

Mitoquinone decreases O_2^- levels in cirrhotic rat livers

Oral mitoquinone administration produced a significant decrease in mitochondrial O_2^- levels, as shown by mitosox fluorescence (Fig 2A). Similarly, DHE fluorescence showed decreased levels of cytoplasmatic superoxide levels in mitoquinone-treated rats indicating that mitoquinone also decreases cytoplasmatic oxidative stress (Fig 2B). Moreover, nitrotyrosinated proteins were analysed as a surrogate marker of oxidative stress. Mitoquinone-treated rats had less nitrotyrosinated protein levels (Fig 2C). Finally, we assessed the expression of *hif1α*, a factor which expression is induced by oxidative stress, observing that its expression was decreased by mitoquinone (Fig 2D). Altogether strongly suggesting an important antioxidant effect of this compound in cirrhotic rat livers.

Mitoquinone improves IHVR and decreases PP in two different experimental models of cirrhosis in the rat

Mitoquinone-treated CCl_4-cirrhotic rats had a significant lower PP than vehicle-treated animals (MIT: 10.8 ± 0.6 *vs.* Veh: 13.6 ± 0.9 mmHg; -21%; $p<0.05$), without significant changes in PBF, thus suggesting an improvement in IHVR (MIT: 10.3 ± 1.3 *vs.* Veh: 15.6 ± 1.9 $mmHg \cdot g \cdot ml \cdot min^{-1}$; -33%; $p<0.05$) (Table 1).

Similar results were obtained in TAA-cirrhotic rats (Table 2): Mitoquinone-treated rats had a lower PP (MIT: 12.2 ± 2.4 *vs.* Veh: 14.3 ± 3.0 mmHg; -15%; $p=0.06$) without changes in PBF. This suggests a decrease in IHVR (MIT: 12.0 ± 4.9 *vs.* Veh: 13.3 ± 5.2 $mmHg \cdot g \cdot ml \cdot min^{-1}$; -12%) although this difference did not reach statistical significance.

Importantly, no changes in MAP or HR were observed in any model suggesting that mitoquinone treatment does not affect systemic hemodynamic.

Biochemical analysis showed no significant differences between mitoquinone and vehicle-treated rats in CCl_4-cirrhotic rats (Table 1) or TAA-cirrhotic rats (Table 2). Furthermore, the ICG clearance (evaluated in the TAA model), was a 33% higher in

cirrhotic rats treated with mitoquinone than in vehicle. However, the difference was not statistically significant (Table 2).

Mitoquinone does not modify hepatic vascular tone in cirrhotic rat livers

There were no differences in baseline portal perfusion pressure between vehicle and mitoquinone-treated CCl_4-cirrhotic rats. A slight non significant improvement in the the dose response vasodilatation to Ach was observed (Supplementary Fig 2).

Mitoquinone induces liver fibrosis regression

As expected, CCl_4 and TAA cirrhotic rats had marked architectural distortion with abundant fibrosis. Rats receiving mitoquinone exhibited a significant reduction in hepatic fibrosis proved by decreased collagen deposition demonstrated by Sirius Red staining of liver sections (-36% in CCl_4-cirrhotic rats & -23% in TAA; Fig 3A), reduced hepatic hydroxyproline content (-20% in CCl_4-cirrhotic animals; Fig 3B) and reduced mRNA expression of collagen (*col1α1:* -68% in CCl_4 & –48% in TAA; Fig 3C).

Mitoquinone deactivates HSC in vivo

Improvement in collagen synthesis and deposition due to mitoquinone treatment was associated with lower mRNA expression of *pdgfrβ* (-54% in CCl_4 & -30% in TAA; Fig 3C) and *α-SMA* (-12% in CCl_4-cirrhotic rats & -32% in TAA-cirrhotic rats; Fig 3C). α-SMA protein expression was also decreased (-18% in CCl_4-cirrhotic rats & -42% in TAA-cirrhotic; Fig 3D). Analysis of Desmin, a marker of hepatic stellate cell presence, showed no differences between groups (Fig 3E), which was in accordance with the *in vitro* experiments using LX2 and rHSC.

Effect of mitoquinone in liver inflammation

Mitoquinone-treated CCl_4-cirrhotic rats had a significantly decreased number of CD68 positive cells compared with the vehicle-treated rats, with no differences in

hepatic CD163 expression (Fig 5A). Furthermore, mitoquinone decreased the pro-inflammatory markers *TNF-α* (-27%), *iNOS* (-74%), *IL-6* (-60%) and *IL-1β* (-57%) in CCl_4-cirrhotic rats (Fig 5B).

DISCUSSION

In chronic liver disease, increment in IHVR is the primary factor contributing to develop portal hypertension, being activated HSC the main cells involved in extracellular matrix deposition[34,35], and therefore increasing IHVR. Considering that elevated oxidative stress in the cirrhotic liver is one of the major mechanisms leading to HSC activation and that mitochondria are central sites where ROS are produced[18], the present study evaluated the effects of the mitochondrial-targeted antioxidant mitoquinone on HSC phenotype, liver fibrosis and portal hypertension in pre-clinical models of cirrhosis.

The results of the current study clearly show that oral mitoquinone administration is able to reduce liver superoxide levels, deactivate HSC and reduce fibrosis and portal hypertension in cirrhosis.

As previously shown by our and other groups, liver cirrhosis is associated with increased superoxide levels. Mitoquinone, as it was previously shown with other antioxidant approaches such as tempol[17] and resveratrol[16], is able to markedly and significantly reduce liver superoxide levels. As expected from the targeted mitochondrial characteristics of the drug, this antioxidant effect was mainly, but not limited, at the mitochondria level. Contrary to previous antioxidant approaches that required parenteral administration, mitoquinone has the clear advantage of being orally administered.

The antioxidant effect of mitoquinone was associated with both *in vitro* and *in vivo* HSC deactivation. Indeed, mitoquinone significantly reduced the expression of several markers of HSC activation in human precision cut liver slices, the best ex vivo model to study gene regulation in human liver, and also in primary human HSC activated in vitro and in LX2 immortalized human HSC. These effects were observed with a reduction in HSC proliferation rate but not in HSC viability. These beneficial effects *ex vivo* and *in vitro* of mitoquinone improving human HSC phenotype were

also confirmed when analyzing HSC from cirrhotic animals. Indeed, improvement in HSC phenotype was also observed in cells isolated from mitoquinone-treated cirrhotic rats, and in cirrhotic HSC treated with mitoquinone *in vitro*.

To further analyze the therapeutic capability of mitoquinone in cirrhosis, we evaluated mitoquinone effects in two murine models of advanced cirrhosis: CCl_4 and TAA.

The *in vivo* data clearly demonstrated that mitoquinone is able to reduce portal pressure (PP) in these two models of established cirrhosis and portal hypertension in the rat. The fact that mitoquinone did not produce significant changes differences in portal blood flow strongly suggests that reduction in PP was mainly due to reduced IHVR. IHVR results from both hepatic vascular tone and architectural alterations of the liver parenchyma. The results of our study showing a consistent reduction in liver fibrosis by mitoquinone treatment in the 2 different models of cirrhosis suggests that this is the main mechanisms to reduce portal pressure. Although some improvement was observed in the endothelium-dependent vasorelaxation to acetylcholine, this was not significant suggesting that improvement in hepatic vascular tone is not playing a major role in reducing portal pressure. However, we cannot discard that after a longer treatment period an amelioration in the endothelial phenotype may also be observed.

It has been documented that oxidative stress activate HSC leading to increased liver fibrosis[36]. Therefore, we speculate that mitoquinone exerts its antifibrotic effects by reducing oxidative stress, thereby leading to HSC deactivation. Furthermore, as ROS levels are directly linked to inflammation, we analyzed the expression of CD68 and CD163, as markers of liver inflammation & macrophage phenotype. Mitoquinone treatment significantly decreased the number of CD68 positive cells within the liver parenchyma and decreased pro-inflammatory markers, with no changes in CD163, suggesting that mitoquinone may have also an effect decreasing liver inflammation and/or promoting macrophages polarization towards a M2 phenotype.

Importantly, mitoquinone treatment did not cause apparent deleterious effects in cirrhotic animals. No differences in liver enzymes but a trend to improve ICG clearance were observed comparing both groups and no reduction in mean arterial pressure was observed..

In conclusion, this study shows for the first time that mitoquinone treatment improves portal hypertension and liver cirrhosis by promoting fibrosis regression, leading to a reduction of PP without worsening systemic hemodynamic. Moreover, mitoquinone was able to deactivate HSC in human liver tissue. These results further support explore the use of mitochondrial-targeted antioxidants for patients with cirrhosis and portal hypertension.

Tables

	Vehicle n=9	Mitoquinone n=9	p value
MAP (mmHg)	95 ± 4	104 ± 10	.42
HR (bpm)	339 ± 12	343 ± 16	.72
PP (mm Hg)	13.6 ± 0.9	10.8 ± 0.6	*.03*
PBF (ml/min)	12.1 ± 1.8	10.1 ± 0.7	.59
IHVR (mmHg·g·min·ml^{-1})	15.6 ± 1.9	10.3 ± 1.3	*.04*
AST (U/L)	153 ± 26	121 ± 12	.28
ALT (U/L)	76 ± 10	74 ± 9	.86
Albumin (g/L)	29.2 ± 0.8	28.5 ± 1.2	.66
Direct bilirubin (mg/dl)	0.1 ± 0.04	0.03 ± 0.01	.17
Total bilirubin (mg /dl)	0.1 ± 0.05	0.03 ± 0.01	.18

Table 1. Effects of mitoquinone on hepatic and systemic hemodynamic, and biochemical parameters in CCl_4-cirrhotic rats. MAP, mean arterial pressure; HR, heart rate; PP, portal pressure; PBF, portal blood flow; IHVR, intrahepatic vascular resistance; AST, aspartate aminotransferase; ALT, alanine aminotransferase. Values represent mean ± SEM.

	Vehicle n=12	Mitoquinone n=13	p value
MAP (mmHg)	123 ± 22	113 ± 15	.22
HR (bpm)	410 ± 46	388 ± 60	.32
PP (mm Hg)	14.2 ± 3.0	12.2 ± 2.4	.06
PBF (ml/min)	17.8 ± 4.6	18.6 ± 8.1	.78
IHVR (mmHg·g·min·ml^{-1})	13.2 ± 5.2	12.0 ± 4.9	.64
Clearance ICG (%/min)	16.2 ± 0.1	21.7 ± 0.3	.23
AST (U/L)	93 ± 47	91 ± 28	.87
ALT (U/L)	37 ± 10	43 ± 15	.25
Albumin (g/L)	28 ± 3	26 ± 4	.16
Direct bilirubin (mg/dl)	0.04 ± 0.05	0.02 ± 0.04	.23
Total bilirubin (mg /dl)	0.05 ± 0.05	0.02 ± 0.04	.1

Table 2. Effects of mitoquinone on hepatic and systemic hemodynamic, and biochemical parameters in TAA-cirrhotic rats. MAP, mean arterial pressure; HR, heart rate; PP, portal pressure; PBF, portal blood flow; IHVR, intrahepatic vascular resistance; ICG, Indocyanine Green; AST, aspartate aminotransferase; ALT, alanine aminotransferase. Values represent mean ± SEM.

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Figure Legends

Figure 1. Mitoquinone inactivates both human and rat HSC. *In vitro*, dose-dependent inactivation of human HSC caused by mitoquinone, assessed in human precision cut liver slices (hPCLS) **(A)**, LX2 **(B)** and primary human HSC (hHSC) activated *in vitro* **(C)**. Analysis of HSC phenotype in cells isolated from CCl_4-cirrhotic rats (rHSC) treated *in vitro* with mitoquinone or vehicle **(D)**, and HSC isolated from vehicle and mitoquinone-treated CCl_4-cirrhotic rats (rHSC) **(E)**. Values represent mean ± SEM. $*p$ <0.05 *vs.* vehicle. #p<0.1 *vs*. vehicle.

Figure 2. Mitoquinone reduces oxidative stress in cirrhotic rat livers. Mitochondrial superoxide content measured with mitosox staining in CCl_4 and TAA-cirrhotic rats **(A)** and cellular superoxide content measured with dihydroethidium staining in CCl_4 and TAA-cirrhotic rats **(B)** in livers from vehicle and mitoquinone-treated cirrhotic rats. **(C)** Peroxynitrite levels measured as nitrotyrosinated proteins in livers from CCl_4-cirrhotic rats treated with mitoquinone or vehicle. **(D)** mRNA expression of the surrogate marker for oxidative stress *hif1α* in livers from CCl_4-cirrhotic rats treated with mitoquinone or vehicle. Values represent mean ± SEM. $*p$ <0.05 *vs.* vehicle. #p<0.1 *vs*. vehicle.

Figure 3. Mitoquinone promotes regression of hepatic fibrosis and HSC deactivation in cirrhotic rats. Representative images and liver fibrosis quantification by Sirius Red in livers from CCl_4 and TAA-cirrhotic rats **(A)**, hydroxyproline content from CCl_4-cirrhotic rats **(B)**. Expression of the HSC phenotype markers *α-SMA*, *col1α1*, and *pdgfrβ* in CCl_4 and TAA-cirrhotic rats **(C)**. *α-SMA* protein expression in livers in CCl_4 and TAA-cirrhotic rats **(D)**. Desmin protein expression evaluated by immunohistochemistry and Western blot in CCl_4-cirrhotic rats **(E)**. Values represent mean ± SEM. $*p$ <0.05 *vs.* vehicle. #p<0.1 *vs*. vehicle.

Figure 4. Mitoquinone promotes Kupffer Cell polarization towards a restorative phenotype. Immunostaining of vehicle and mitoquinone-treated CCl_4-cirrhotic livers for CD68 (M1 marker) and the M2 marker CD163 (M2 marker) **(A)**. mRNA expression of pro-inflammatory markers *TNFα*, *iNOS*, *IL-6* and *IL-1β* in livers described in A **(B)**. Values represent mean ± SEM. $*p$ <0.05 *vs.* vehicle.

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