Original Article

The mitochondria-targeted antioxidant MitoQ attenuates liver fibrosis in mice

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Abstract: Oxidative stress plays an essential role in liver fibrosis. This study investigated whether MitoQ, an orally active mitochondrial antioxidant, decreases liver fibrosis. Mice were injected with corn oil or carbon tetrachloride (CCl4, 1:3 dilution in corn oil; 1 µl/g, ip) once every 3 days for up to 6 weeks. 4-Hydroxynonenal adducts increased markedly after CCl4 treatment, indicating oxidative stress. MitoQ attenuated oxidative stress after CCl4. Collagen 1α1 mRNA and hydroxyproline increased markedly after CCl4 treatment, indicating increased collagen formation and deposition. CCl4 caused overt pericentral fibrosis as revealed by both the sirius red staining and second harmonic generation microscopy. MitoQ blunted fibrosis after CCl4 treatment. Profibrotic transforming growth factor-β1 (TGF-β1) mRNA and expression of smooth muscle α-actin, an indicator of hepatic stellate cell (HSC) activation, increased markedly after CCl4 treatment. Smad 2/3, the major mediator of TGF-β fibrogenic effects, was also activated after CCl4 treatment. MitoQ reduced necrosis, apoptosis and inflammation after CCl4 treatment. In cultured HSCs, MitoQ decreased oxidative stress, inhibited HSC activation, TGF-β1 expression, Smad2/3 activation, and extracellular signal-regulated protein kinase activation. Taken together, these data indicate that mitochondrial reactive oxygen species play an important role in liver fibrosis and that mitochondria-targeted antioxidants are promising potential therapies for prevention and treatment of liver fibrosis.

Keywords: Antioxidant, hepatic stellate cell, liver fibrosis, mitochondria, MitoQ, oxidative stress

Introduction

Liver fibrosis/cirrhosis affects more than 100 million people worldwide and represents one of the most common causes of death in adults [1, 2]. Moreover, cirrhosis markedly increases the risk of hepatocellular carcinoma (HCC), and about 75% of HCC occurs on the basis of liver fibrosis. Despite extensive studies, the mechanisms of fibrosis are not well understood, and effective therapies are lacking [2, 3]. Liver fibrosis/cirrhosis ultimately leads to end-stage liver disease, which requires liver transplantation. However, this resource is limited [4], and many patients die while waiting for a transplant. Therefore, the ideal approach to management of patients with chronic liver disease would be to understand the mechanisms of fibrosis in order to develop mechanism-based, effective therapies to inhibit the progression of and/or reverse fibrosis/cirrhosis.

Liver fibrosis represents a wound healing response to chronic liver injury. Liver injury stimulates a multicellular response involving multiple resident hepatic cells. In particular, hepatic stellate cells (HSCs) play a key role with their activation leading to formation and deposition of collagen-rich extracellular matrix (ECM) [1, 2, 5, 6]. Multiple other cell types, including injured hepatocytes, activated Kupffer cells, stimulated cholangiocytes, and various infiltrating cells (e.g. leukocytes and platelets), appear to fuel the fibrotic process by producing cytokines,
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chemokines, growth factors, miRNAs, reactive oxygen species (ROS) and/or damage-associated molecular pattern molecules (DAMPs) [1, 2, 7].

Clinical and experimental studies suggest that oxidative stress plays an important role in the development of fibrosis [8-10]. Oxidative stress is common in different types of chronic liver injury [11-13]. ROS not only induce hepatocyte damage/death but also stimulate/amplify inflammatory and profibrotic responses [8-13]. Our previous study showed that antioxidant green tea polyphenols decreased cholestatic liver fibrosis in rats [14]. Interestingly, over-expression of mitochondrial superoxide dismutase-2 (SOD2, which degrades superoxide radicals) attenuated liver injury and fibrosis much better than over-expression of cytosolic SOD1, suggesting mitochondrial oxidative stress plays a crucial role in development of liver fibrosis [15].

Mitochondria are a major source of ROS in cells [16, 17]. Mitochondrial ROS production mediates pathological processes in many diseases and in aging [18]. Therefore, in recent years increasing efforts have focused on development of mitochondria-targeted antioxidants. MitoQ (10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)decyltriphenyl-, methanesulfonate) is a derivative of the potent antioxidant ubiquinone conjugated to triphenylphosphonium (TPP), which enables MitoQ to enter and accumulate within mitochondria [19]. As such, MitoQ is more effective in preventing mitochondrial oxidative damage compared to untargeted antioxidants. MitoQ has been found effective in vitro, in animals, and in humans in attenuating cell/tissue damage in many situations, including Parkinson’s disease, aging, colitis, metabolic syndrome, hepatitis C, and cardiac dysfunction [20-25]. Since mitochondrial ROS may be crucial in development of liver fibrosis, we explored whether decreased mitochondrial oxidative stress by MitoQ ameliorates liver fibrosis in vivo and directly inhibits HSC activation in vitro.

Methods

In vivo liver fibrosis model and MitoQ treatment

Liver fibrosis was induced in vivo by carbon tetrachloride (CCL4) treatment, one of the most widely used experimental liver fibrosis models [26, 27]. Male C57BL/6J mice (8-9 weeks, Jackson Laboratory, Bar Harbor, Maine) were allowed access to drinking water containing 500 µM MitoQ or the inactive comparison compound (decyTPP, both from the MRC Mitochondrial Biology Unit, Cambridge, U.K.) ad libitum. Three days after starting MitoQ, mice were injected with CCl4 (Sigma, St. Louis, MO; 1:3 dilution in corn oil; 1 µl of the dilution/g, i.p.) or an equal volume of corn oil once every 3 days for up to 6 weeks [26, 27]. MitoQ was given throughout the CCl4 treatment period. All animals received humane care in compliance with institutional guidelines. Animal protocols were approved by the Institutional Animal Care and Use Committee.

Alanine aminotransferase (ALT) measurement

After 5 and 6 weeks of CCl4 treatment, mice were anesthetized with pentobarbital (80 mg/kg, i.p.), and blood was collected from the inferior vena cava. Serum alanine transaminase (ALT) was measured using a kit from Pointe Scientific (Canton, MI).

Histology and immunohistochemical staining

Livers were harvested under pentobarbital anesthesia after rinsing with ~2 mL normal saline. Liver tissue was fixed and processed for paraffin sections, as described elsewhere [28]. In liver sections stained with hematoxylin and eosin (H&E), histological images were captured under a microscope (Zeiss Axiovert 100 microscope, Thornwood, NY) using a 20x objective lens.

Apoptosis was detected on liver slides by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) using an In Situ Cell Death Detection Kit according to the manufacturer’s protocol [29]. TUNEL-positive and negative cells were counted in a blinded manner in 10 randomly selected fields using a 40x objective lens.

Liver fibrosis was analyzed on liver slides by 2 different methods. Some liver slides were stained with 0.1% sirius red (Polysciences Inc., Warrington, PA) and fast green FCF (Sigma-Aldrich, St. Louis, MO) to reveal liver fibrosis, and light microscopic images were captured using a 10x objective lens [14].
Liver fibrosis was also revealed by second harmonic generation (SHG) microscopy of liver sections. When intense laser light passes through a material with a non-linear, noncentrosymmetric molecular structure (e.g., collagen and muscle myosin), 2 photons with the same frequency in the laser light can interact with the nonlinear material to generate a new photon possessing twice the energy and hence half the wavelength of the original photons [30]. Imaging of the SHG emission allows visualization of collagen fibers without the use of stains or fluorophores, which avoids non-specific staining that occurs frequently in injured tissue using other methods. SHG imaging was performed on de-paraffinized, unstained slides using a Zeiss LSM 510 NLO laser scanning confocal/multiphoton microscope (Thornwood, NY) and a 25x 0.8 NA water-immersion objective lens. Two-photon excitation was performed with 900-nm light from a Coherent Chameleon Ultra laser. The emission wavelength was 450 nm.

Measurement of hydroxyproline in liver tissue

About 100 mg of frozen liver tissue was hydrolyzed in 1 ml of 2 N NaOH at 120°C in a heating block for 20 minutes. The hydrolysates were centrifuged at 10,000 rpm for 10 min at room temperature. The supernatant was mixed gently with 450 µl chloramine-T reagent (Sigma-Aldrich, St. Louis, MO) and kept at room temperature for 25 minutes. Finally, 500 µl of Ehrlich’s aldehyde reagent (Mallinckrodt Baker Inc., Phillipsburg, NJ) containing 5% (w/v) p-dimethylaminobenzaldehyde in n-propanol/perchloric acid (2:1, v/v) was added to each sample, and the chromophore was developed by incubating the samples at 65°C for 20 minutes. Absorbance was measured at 550 nm using a SpectraMax M2 spectrophotometer/micropla-
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**Immunoblotting**

Some liver tissue was snap-frozen in liquid nitrogen during liver harvesting and kept at -80°C until use. Proteins of interest in liver tissue and HSC extracts were then detected by immunoblotting, as described previously [28]. The membranes were blotted with primary antibodies specific for cleaved caspase-3 (CC3) and actin (Cell Signaling Technology, Danvers, MA), 4-hydroxynonenal adducts (4-HNE, Alpha Diagnostic, San Antonio, TX), transforming growth factor-β1 (TGF-β1, Abcam, Cambridge, MA), collagen-I (Abcam, Cambridge, MA), Smad2/3 and phospho-Smad2/3, extracellular signal-regulated protein kinase 1/2 (ERK1/2) and phospho-ERK1/2 (Santa Cruz Biotech., Santa Cruz, CA), and myeloperoxidase (MPO), smooth muscle α-actin (α-SMA, DAKO, Carpinteria, CA) at 1:1000 to 1:3000 overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies of appropriate species were used to detect proteins of interest.

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Figure 2. MitoQ inhibits apoptosis and inflammation in the liver after CCl₄ treatment in vivo. Mice were treated as in Figure 1, and livers were collected at 5 and 6 weeks. A: Representative immunoblot images of cleaved caspase-3 (CC3) and myeloperoxidase (MPO); B: Quantification of CC3 immunoblot images by densitometry; C: TUNEL-positive hepatocytes were counted in 10 random fields per slide as percentage of total; D: Quantification of MPO immunoblot images by densitometry. Values are means ± SEM. a, p<0.05 vs control; b, p<0.05 vs the corresponding CCl₄ group (n = 4/group).
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Detection of collagen 1α1 mRNA by quantitative real time PCR (qPCR)

Total RNA was isolated with Trizol (Invitrogen, Grand Island, NY) from liver tissue, and qPCR detection of collagen 1α1 mRNA was performed, as described elsewhere [28, 33]. The abundance of mRNAs was normalized against hypoxanthine phosphoribosyltransferase (HPRT) housekeeping gene using the ΔΔCt method.

Statistical analysis

Groups were compared using ANOVA plus Student-Newman-Keuls posthoc test. Data
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Results

MitoQ attenuates liver injury and inflammation after CCl₄ treatment in vivo

Liver injury and subsequent inflammation may contribute to development of liver fibrosis. ALT release, an indicator of liver injury, increased from ~30 U/L after vehicle treatment (corn oil and decylTPP for 6 weeks) to 410-470 U/L after 5 and 6 weeks of CCl₄ treatment (Figure 1A). Administration of MitoQ decreased ALT after CCl₄ to 154-182 U/L (Figure 1A).

No pathological changes were observed in liver tissue in the control group (corn oil and decylTPP treatment for 6 weeks, the same for all in vivo experiments) (Figure 1B), but cell swelling, fatty infiltration, cell death and leukocyte infiltration became overt in livers after CCl₄ treatment, primarily in the pericentral regions (Figure 1C). MitoQ markedly decreased these pathological changes (Figure 1D).

Caspase-3 mediates apoptosis. Cleaved caspase-3 (CC3) increased after 5 and 6 weeks of CCl₄ treatment (Figure 2A and 2B). TUNEL-positive cells also increased, indicating apoptosis (Figure 2C). MitoQ markedly decreased caspase-3 activation and TUNEL staining (Figure 2A-C).

Myeloperoxidase (MPO), a marker of neutrophil infiltration, was essentially undetectable in the control group but increased 15-19-fold after 5 and 6 weeks of CCl₄ treatment (Figure 2A and 2D). In the presence of MitoQ, MPO increased only 6-7-fold after CCl₄ treatment.

MitoQ decreases liver fibrosis after CCl₄ treatment in vivo

As expected, sirius red staining revealed fibrosis in liver sections (Figure 3, left panels). In the livers of control mice, sirius red stained only large portal and central structures and not the liver parenchyma (Figure 3 and data not shown). By contrast, sirius red staining increased markedly after CCl₄ treatment, particularly in pericentral regions. MitoQ treatment substantially decreased this CCl₄-induced fibrosis (Figure 3, left panels). Detection of collagen by SHG imaging confirmed the marked increase of fibrosis after CCl₄ which was substantially decreased by MitoQ (Figure 3 right panels).

Hydroxyproline, an indicator of collagen deposition, increased from 127 µg/g liver to 295 and 329 µg/g liver after 5 and 6 weeks of CCl₄ treatment, respectively (Figure 4A). With MitoQ treatment, hydroxyproline increased to only 193 and 232 µg/g liver after 5 and 6 weeks of CCl₄ (Figure 4A). Collagen 1α1 mRNA also increased ~3.6-fold after 5 and 6 weeks of CCl₄ treatment, signifying increased collagen expression (Figure 4B). By contrast in mice given MitoQ, collagen 1α1 mRNA only increased ~1.6-fold after CCl₄ (Figure 4B).

MitoQ inhibits hepatic stellate cell activation and TGF-β/Smad signaling in the liver after CCl₄ treatment in vivo

Expression of smooth muscle α-actin (α-SMA), an indicator of HSC activation, increased 4- and
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5.8-fold, respectively, after 5 and 6 weeks of CCl₄ treatment. MitoQ blunted these increases in α-SMA after CCl₄ (Figure 5A and 5B). Pro-fibrogenic cytokine TGF-β1 mRNA increased 5- and 6.8-fold, respectively, after 5 and 6 weeks of CCl₄ treatment in the absence of MitoQ but increased only 2.3- and 3-fold with MitoQ treatment (Figure 5C). Smad2/3 mediates TGF-β fibrogenic effects. Although total Smad2/3 expression was not altered after CCl₄ treatment, phospho-Smad2/3 increased 3.8- and 4.8-fold, respectively, after 5 and 6 weeks of CCl₄ treatment, indicating Smad2/3 activation. In the presence of MitoQ, phospho-Smad2/3 in-

Figure 5. MitoQ inhibits stellate cell activation and TGF-β/Smad signaling in the liver after CCl₄ treatment in vivo. Mice were treated as in Figure 1, and livers were collected at 5 and 6 weeks. A: Representative immunoblot images of smooth muscle α-actin (α-SMA), Smad2/3, phospho-smad2/3 (pSmad2/3), and β-actin. B: Quantification of α-SMA immunoblot images by densitometry. C: Quantification of transforming growth factor-β1 (TGFβ1) mRNA by qPCR. D: Quantification of pSmad2/3 immunoblot images by densitometry. Values are means ± SEM. a, p<0.05 vs control; b, p<0.05 vs the corresponding CCl₄ group (n = 4/group).

Figure 6. MitoQ inhibits oxidative stress in the liver after CCl₄ treatment in vivo. Mice were treated as in Figure 1, and livers were collected at 5 and 6 weeks. Shown are representative immunoblot images of 4-hydroxynonenal adducts (4-HNE) and β-actin (n = 4/group).
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creased only 2.5- and 2.1-fold, respectively (Figure 5D).

**MitoQ decreases hepatic oxidative stress after CCl₄ treatment in vivo**

4-Hydroxynonenal (4-HNE) is a product of lipid peroxidation and a widely used marker of oxidative stress. Multiple weak bands of 4-HNE adducts were observed in the livers of control mice (Figure 6). 4-HNE adducts increased substantially after 5 and 6 weeks of CCl₄ treatment. MitoQ blunted the production of these 4-HNE adducts (Figure 6).

**MitoQ inhibits hepatic stellate cell activation in vitro**

We next investigated the effects of MitoQ on cultured HSCs. Rat HSCs were employed, since

![Figure 7. MitoQ inhibits stellate cell activation in vitro. HSCs were isolated from normal rats and cultured in 20% serum-containing medium. After 2 days, serum was decreased to 0.5%. MitoQ (0.5-2 μM) or an equal volume of DMSO (Control) was added at days 2 and 4, and cells were harvested at 6 days. A: Representative immunoblot images of smooth muscle α-actin (α-SMA), collagen-I, and β-actin. B: Quantification of α-SMA immunoblot images by densitometry. C: Quantification of collagen-I immunoblot images by densitometry. D: Representative images of cultured HSCs at 6 days. Values are means ± SEM. a, p<0.05 vs corresponding controls; b, p<0.05 vs 0.5 μM MitoQ. c, p<0.05 vs 1 μM MitoQ (n = 3/group).](image-url)
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MitoQ inhibits oxidative stress, TGF-β expression and canonical signaling in cultured hepatic stellate cells

In the lysates of control HSCs, multiple strong 4-HNE adduct bands were observed after 6 days of culture, indicating lipid peroxidation from formation of ROS (Figure 8). MitoQ decreased 4-HNE adduct formation in a concentration-dependent manner (Figure 8). Production of the profibrogenic cytokine, TGF-β1, was also reduced by 36%, 51% and 86%, respectively, by 0.5, 1 and 2 µM MitoQ compared to control cells after 6 days of culture (Figure 9A and 9B). Total Smad2/3 expression was not changed by MitoQ, but phospho-Smad2/3 decreased by 21%, 41% and 87%, respectively, with 0.5, 1 and 2 µM MitoQ compared to control HSCs (Figure 9A and 9C). Since ERK activation also mediates the fibrogenic effects of TGF-β, we evaluated total and phospho-ERK1/2 in 6-day cultured HSCs. Total ERK1/2 expression was not altered by MitoQ, but phospho-ERK1/2 decreased by 31%, 55% and 85%, respectively, with 0.5, 1 and 2 µM MitoQ (Figure 9A and 9D).

Discussion

Despite extensive studies, effective therapies for fibrosis/cirrhosis are still lacking. Chronic liver injury leads to damage/death of hepatocytes and persistent inflammation. A complex network of profibrogenic, proinflammatory and proliferative mediators are produced during liver injury by neighboring and infiltrating cells, which leads to HSC activation and production of ECM [1, 2, 7]. No doubt, the most effective anti-fibrotic therapies are those targeting the primary stimuli of fibrogenesis, e.g., inhibition of viral hepatitis [34, 35] and iron depletion in patients with hemochromatosis [36]. Blockade of common profibrogenic and proinflammatory pathways, inhibition of HSC activation, enhancement of apoptosis, inactivation or senescence of HSCs, and/or stimulation of ECM degradation are also potential therapeutic targets.

Many previous studies have shown that ROS are important mediators of liver injury and fibrosis [8]. For example, ROS attack macromolecules (lipids, proteins, DNA), inhibit mitochondrial function, damage cell membranes, and induce necrosis and apoptosis, which may subsequently lead to initiation of fibrogenesis [9, 10, 37]. ROS also amplify the inflammatory response. Damage of hepatocytes by ROS causes release of inflammasomes and other damage-associated molecular pattern molecules (DAMPs, e.g. HMGB1), which are potent inflammatory mediators [38, 39]. ROS cause nuclear factor-kB activation that subsequently leads to formation of proinflammatory cytokines/chemokines (e.g., TNFα, interleukin-1, macrophage inflammatory protein 1&2, CXC chemokine-10) and adhesion molecules which attract leukocytes [40, 41]. Infiltrating leukocytes are activated to produce more ROS, causing a vicious cycle.

ROS also stimulate the production/activation of profibrotic and proliferative mediators (e.g.,
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TGF-β, connective tissue growth factor, platelet-derived growth factor) in Kupffer cells, cholangiocytes, endothelial cells, and infiltrating platelets and inflammatory cells [8, 11, 12, 43, 42]. Moreover, oxidative stress directly activates HSCs [44, 45]. Antioxidants inhibit upregulation of tissue metalloproteinase inhibitor 1 after bile duct ligation, a molecule that inhibits metalloproteinases which are responsible for ECM degradation [46]. Since ROS play important roles in many aspects of pathogenesis of liver fibrosis, inhibiting ROS formation or accelerating their degradation are promising therapeutic targets for prevention or treatment of fibrosis. Indeed, vitamin E has been shown to prevent progression of fibrosis in non-alcoholic steatohepatitis patients [30].

While there are many different sources of ROS formation in cells, such as NADPH oxidase, xanthine oxidase, cytochrome P450 and peroxisomes, mitochondria are recognized as a major source of ROS in numerous pathophysiological settings. During mitochondrial respiration, some electrons escape the electron transport chain prematurely to form superoxide at Complexes I and III [47]. Production of ROS from mitochondria increases markedly in many pathological conditions, including CCl4 intoxication [47, 48]. Mitochondria are also a target of ROS, resulting in induction of the mitochondrial permeability transition, mitochondrial membrane potential collapse, failure of oxidative phosphorylation, and oncotic cell death [49]. Mitochondrial swelling causes release of pro-apoptot-

Figure 9. MitoQ inhibits TGF-β, Smad and ERK signaling in cultured stellate cells. HSCs were isolated from normal rats and cultured as described in Figure 7. After 6 days, cell lysates were collected to detect transforming growth factor-β1 (TGF-β1), Smad2/3, phospho-Smad2/3 (p-Smad2/3), extracellular signal-regulated protein kinase 1/2 (ERK1/2), phospho-ERK1/2 (p-ERK1/2) and β-actin. A: Representative immunoblot images. B: Quantification of TGF-β1 immunoblot images by densitometry. C: Quantification of p-Smad2/3 immunoblot images by densitometry. D: Quantification of p-ERK1/2 immunoblot images by densitometry. Values are means ± SEM. a, p<0.05 vs corresponding controls; b, p<0.05 vs 0.5 µM MitoQ; c, p<0.05 vs 1 µM MitoQ (n = 3/group).
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ic factors such as cytochrome c, leading to apoptosis [49], and apoptotic bodies from hepatocytes can cause HSC activation [50].

Previous studies also show that compared to nuclear DNA, mitochondrial DNA is more sensitive to oxidative damage due to the lack of histone protection and the proximity to the major sites of ROS production [51]. Emerging evidence shows that mitochondrial dysfunction leads to inflammatory reactions by increasing the formation and activation of the inflammatory signaling platform NLRP3-inflammasomes [52, 53]. Mitochondrial damage causes release of mitochondrial DNA, which is known to induce inflammation [13, 54, 55]. Mitochondrial oxidative stress increases formation/activation of profibrogenic TGF-β [56, 57]. Mitochondrial uncoupling, increased consumption of oxygen, and subsequent liver hypoxia can induce hypoxia inducible factor-1α [58]. Inflammation, TGF-β and hypoxia inducible factor-1α all promote liver fibrosis [59-61]. Together, mitochondrial damage/dysfunction may be a critical step in liver injury, inflammation and fibrosis.

Previously, we showed that overexpression of mitochondrial SOD protected against cholestatic liver injury and fibrosis to a much greater extent than overexpression of cytosolic SOD, suggesting a mitochondrial targeted antioxidant may have greater benefit compared to untargeted antioxidants [15]. In this study, we explored the effects of MitoQ, a mitochondria-targeted antioxidant [19], on liver fibrosis in vivo. We demonstrated that MitoQ treatment in vivo decreased oxidative stress (4-HNE), inhibited formation of the profibrogenic cytokine TGF-β, blocked downstream signaling pathways of TGF-β (Smad activation) and suppressed liver fibrosis (sirius red staining, SHG, hydroxyproline, collagen synthesis) after exposure to CCl₄. Moreover, MitoQ also decreased hepatocellular injury/death (ALT, necrosis, apoptosis) and reduced subsequent inflammation (MPO), which may contribute to the attenuation of liver fibrosis by MitoQ. HSC activation is an essential step in liver fibrosis. MitoQ not only inhibited HSC activation in vivo but also suppressed spontaneous HSC activation in culture. Therefore in addition to protecting against hepatocellular injury and thus inhibiting subsequent inflammatory and fibrogenic responses, MitoQ also decreased liver fibrosis by direct inhibition of HSC activation.

Together, this study demonstrates that mitochondrial oxidative stress plays an essential role in liver fibrosis after CCl₄, and demonstrates that the mitochondria-targeted antioxidant MitoQ is an effective therapeutic strategy against liver fibrosis. Moreover, MitoQ is orally active and can be safely administered over the long-term [62]. Therefore, MitoQ is suitable for clinical application and may be a promising drug for prevention and/or treatment of liver fibrosis in humans.

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