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Digestive and Liver Disease

Liver, Pancreas and Biliary Tract

Silibinin improves hepatic and myocardial injury in mice with nonalcoholic steatohepatitis

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ABSTRACT

Background: Nonalcoholic fatty liver disease is a chronic metabolic disorder with significant impact on cardiovascular and liver mortality.
Aims: In this study, we examined the effects of silibinin on liver and myocardium injury in an experimental model of nonalcoholic fatty liver disease.
Methods: A four-week daily dose of silibinin (20 mg/kg i.p.) was administrated to db/db mice fed a methionine–choline deficient diet. Hepatic and myocardial histology, oxidative stress and inflammatory cytokines were evaluated.
Results: Silibinin administration decreased HOMA-IR, serum ALT and markedly improved hepatic and myocardial damage. Silibinin reduced isoprostanes, 8-deoxyguanosine and nitrites/nitrates in the liver and in the heart of db/db fed the methionine–choline deficient diet, whereas glutathione levels were restored to lean mice levels in both tissues. Consistently, liver mitochondrial respiratory chain activity was significantly impaired in untreated mice and was completely restored in silibinin-treated animals. TNF-α was increased whereas IL-6 was decreased both in the liver and heart of db/db fed methionine–choline deficient diet. Silibinin reversed heart TNF-α and IL-6 expression to control mice levels. Indeed, liver JNK phosphorylation was reduced to control levels in treated animals.
Conclusions: This study demonstrates a combined effectiveness of silibinin on improving liver and myocardial injury in experimental nonalcoholic fatty liver disease.

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a chronic metabolic disorder with significant impact on cardiovascular and liver mortality [1]. NAFLD includes a wide spectrum of lesions ranging from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH) [2]. While NAFL is not generally considered a progressive liver disease, NASH may have a cirrhotic and tumorigenic evolution, causing liver-related morbidity and mortality [1]. Nevertheless, cardiovascular diseases (CVD), including coronary heart disease and non-ischemic cardiomyopathy, are the leading cause of death in patients with NAFLD [1]. The pathophysiological hallmark of NAFLD is insulin resistance (IR), and the increase in intrahepatic triglycerides (IHTG) is directly related to the impairment of insulin action in the liver, skeletal muscle, and adipose tissue of obese subjects [3–5]; recently, the Framingham Heart Study has shown that IHTG content predicts the glucose and lipid abnormalities of the metabolic syndrome independently of visceral fat [6,7]. Furthermore, liver fat content is an independent indicator of myocardial IR and impaired coronary functional capacity in diabetic patients [8], thus suggesting that NAFLD is not merely a marker, but may be actively involved in the onset and progression of CVD. On the other hand, myocardial triglyceride content is directly related to the degree of heart dysfunction both in human and rodent models [9]. Myocardial fat causes alterations in substrate utilization

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(cardiac work/myocardial oxygen consumption) that occur early in the cascade of events leading to impaired ventricular contractility [9,10]. Recently, echocardiographic features of early left ventricular dysfunction and impaired energetics, measured by cardiac ^{31}P-magnetic resonance spectroscopy, have been reported in NAFLD patients in the absence of obesity, hypertension and diabetes [11].

NAFLD pathogenesis is related to a puzzling crosstalk between liver, muscle and adipose tissue about free fatty acids (FFA) utilization, leading to an increased supply of FFA to the liver which combined with *de novo* lipogenesis determines an abnormal IHTG content [12]. The increased availability of FFA in the liver promotes FFA oxidation and increases the production of free radicals leading to lipoperoxidation, DNA and protein damage, endogenous antioxidants depletion, and mitochondrial damage [13]. Oxidative-nitrosative stress further triggers the activation of inflammatory pathways [14,15]. Similarly to the liver, cardiac lipotoxicity is associated with increasing reactive oxygen species (ROS) and reactive nitrogen species (RNS) production, which leads to DNA damage and death of myocardiocytes [16,17].

In consideration of the coexistence of liver and myocardial injury in patients with NAFLD and of the shared molecular pathways of damage, it is important to determine whether therapies aiming at improving liver histology would also be able to improve myocardial damage.

Silibinin is a polyphenolic compound contained in silymarin, a mixture of flavonolignans extracted from the seeds of milk thistle (*Silybum marianum*), used as hepatoprotective agent for millennia. Although being used in clinical practice worldwide, its therapeutic efficacy has been questioned for years. Potent scavenging properties have been demonstrated *in vitro* and *in vivo*, in different hepatic and non-hepatic cells [18,19]; and strong evidences for silibinin therapeutic efficacy have been reported in different types of experimental liver injury [20–22].

This study is aimed at assessing the efficacy of silibinin both on liver and heart injury in NAFLD and at identifying the related molecular events. In order to investigate this issue, we used db/db mice fed a methionine–choline deficient (MCD) diet, a model combining the features of the metabolic syndrome with the histological pattern of NASH [23,24]. In fact, db/db mice fed an MCD diet do partially conserve increased visceral adiposity and an insulin resistant phenotype, while developing hepatocellular injury [24].

2. Materials and methods

2.1. Animals and treatments

All procedures fulfilled the Italian Guidelines for the Use and Care of Laboratory Animals. Six-week-old male BKS.Cg-m$^+$/$^+$ Lepr^{db}/J (db/db) obese mice and six-week-old male heterozygous db/m lean control mice were purchased from Charles River Lab (Calco, Italy). Animals were maintained in a temperature- and light-controlled facility and permitted *ad libitum* consumption of water; after two week of acclimation, db/db mice were fed a MCD diet (ICN Biomedicals, Costa Mesa, CA) for 4 weeks, whereas db/m mice were fed a MCD diet supplemented with methionine and choline (ICN Biomedicals), i.e. a standard diet (SD), for the same period. Silibinin dihydrogen succinate (Indena, Milan, Italy) was dissolved in saline (vehicle) and daily administered intraperitoneally at a dosage of 20 mg/kg of body weight. Mice were distributed in 3 groups: group I included 6 db/m mice fed a control diet and treated with vehicle (db/m + SD); group II comprised 6 db/db mice fed a MCD diet and treated with vehicle (db/db + MCD); group III included 6 db/db mice fed a MCD diet and treated with silibinin (db/db + MCD + silibinin). Treatment was administered for a 4-week period; at the end of it, animals were sacrificed after an overnight fast. Blood, liver

and heart samples were obtained and stored at −80 °C for further analysis.

2.2. Histopathology and immunohistochemistry

For conventional histopathological evaluation formalin-fixed paraffin-embedded liver and heart sections were stained with hematoxylin–eosin, using standard procedures. Liver injury was blindly evaluated according to the NAFLD activity score (NAS). Moreover, in order to investigate even modest deposition of extracellular matrix components Sirius Red staining was performed on formalin-fixed paraffin-embedded liver sections as previously described [25] with a single modification: briefly, after the usual steps to stain liver sections (2 μm thick) in 0.1% Sirius Red F3B (Sigma–Aldrich, St. Louis, MO, USA) in a picric acid solution (1.2%) (Sigma–Aldrich), sections were further rapidly exposed to Harry's hematoxylin (2 s) in order to stain nuclei. Immunohistochemistry was performed on formalin-fixed paraffin-embedded liver sections. Sections (2 μm thick) were incubated with the primary antibody anti-α-SMA (Sigma–Aldrich), final dilution 1:2000, or anti-Thr183/Tyr185 phosphorylated-JNK (Cell Signaling, MA), final dilution 1:50. Briefly, after microwave antigen retrieval, primary antibodies were labeled by using EnVision, HRP-labelled System (DAKO) antibodies directed against mouse antigen and visualized by 3′-diaminobenzidine substrate. Negative controls were performed by replacing the respective primary antibodies by isotype and concentrations matched irrelevant antibody.

2.3. Biochemical analyses

Blood glucose was measured by Accu-chek (Roche Diagnostics, Milan, Italy). Serum ALT and serum insulin were determined using a multichannel autoanalyzer (Abbott Diagnostics, Milan, Italy). Liver triglycerides content was measured using a serum/tissue triglyceride colorimetric kit (Biovision, Mountain View, CA). Liver and heart isoprostanes and 8-hydroxyguanosine (8-OHG) were determined by enzyme-linked immunosorbent assay (ELISA) test (Cayman, Ann Arbor, MI); reduced glutathione (GSH) was assessed by a GSH assay (Cayman). Liver and heart nitrite/nitrates were measured colorimetrically using Griess reagent (Merck KGaA, Darmstadt, Germany), following manufacturer's instructions. ELISA kit for TNF-α (R&D Systems, Minneapolis, MN) was used on whole myocardial tissue protein extracts, following manufacturer's instructions.

2.4. Mitochondrial respiratory chain activity assay

Mitochondrial respiratory chain (MRC) activities was determined as previously described [24]. Briefly, liver tissues (50–70 mg) were homogenized with 15 vol of 20 mmol/L KP buffer, pH 7.4, and centrifuged at $800 \times g$ for 10 min. Respiratory chain enzymes and citrate synthase activities were measured in a DU-650 spectrophotometer (Beckman Instruments, Palo Alto, CA). Incubation temperatures were 30 °C for complexes I, II, III, V, and citrate synthase, and 38 °C for complex IV. Enzyme activities were assessed in supernatants, expressed as nanomoles of substrate used per minute per milligram of protein and referred as a percentage of the specific activity of citrate synthase, to adjust for the hepatic content of mitochondria.

2.5. RNA extraction and Real Time PCR

Total RNA was extracted by homogenizing snap frozen liver samples in TRIzol reagent (Invitrogen, Milan, Italy). Quantitative real-time PCR was performed in 7900HT Fast Real-Time PCR

System Applied Biosystems (Applied Biosystems, Foster City, CA), using the EXPRESS SYBRR GreenER™ qPCR SuperMix with Premixed ROX (Invitrogen). The following primers sequences were used: α-SMA forward 5′-GCCAGTCGCTGTCAGGAACCC-3′, reverse 5′-AGCCGGCCTTACAGAGCCCA-3′; TNF-α fwd 5′-AGCCCA-CGTCGTAGCAAACCA-3′, rev 5′-GCAGGGGCTCTTGACGGCAG-3′; IL-6 fwd 5′-CCTCTCTGCAAGAGACTTCCATCCA-3′, rev 5′-AGCCTCC-GACTTGTGAAGTGGT-3′; MCP-1 fwd 5′-CCAGCACCAGCACCAGCC-AA-3′, rev 5′-TGGGGCGTTAACTGCATCTGGC-3′; IL-4 fwd 5′-AGG-TCACAGGAGAAGGGACGCC-3′, rev 5′-TGCGAAGCACCTTGGAAG-CCC-3′; IFNγ 5′-GCCAAGTTTGAGGTCAACAACCCA-3′, rev 5′-CCCACCCCGAATCAGCAGCG-3′. Reactions were performed in a 20 mL mixture containing cDNA, specific primers of each gene and the SYBRR GreenER™ qPCR SuperMix. Amplification conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The specific PCR products were detected by the fluorescence of SYBR Green, the double stranded DNA binding dye. The relative mRNA expression level was calculated by the threshold cycle (Ct) value of each PCR product and normalized with that of GAPDH by using comparative $2\triangle\triangle$Ct method.

2.6. Statistical analysis

Statistics were aided by GraphPad Prism (GraphPad, San Diego, CA). All results were expressed as mean ± standard error of the mean. One-way ANOVA with Bonferroni Post Hoc analysis were used for parametric data. Kruskal–Wallis was used for non-parametric data. P values less than 0.05 were considered significant.

3. Results

3.1. Silibinin decreases insulin resistance and serum ALT

All db/db mice weighted more than their lean controls at week 8, before starting the MCD diet (Table 1). After 4 weeks of MCD diet, at week 12, body weight was still higher in vehicle-treated animals; silibinin treatment did not significantly decrease body weight (Table 1) and did not modify food intake (data not shown). Vehicle db/db fed MCD diet had higher serum ALT when compared to lean animals; in silibinin-treated mice, ALT levels were 3-fold decreased (Table 1). Untreated db/db fed MCD diet were insulin resistant, as shown by HOMA-IR; silibinin decreased fasting glucose and insulin, completely reversing insulin resistance (Table 1).

3.2. Silibinin improves hepatic and myocardial injury

As expected, none of the lean mice presented histological features of NAFLD (Fig. 1). Liver sections of vehicle db/db fed MCD diet mice showed marked steatosis with an azonal pattern (Fig. 1), mild lobular inflammation and diffuse hepatocytes ballooning. In silibinin-treated animals steatosis was markedly reduced in grade (Fig. 1) and had a prevalent zone 3 pattern. Biochemical analysis confirmed that silibinin induced a marked decrease of liver triglycerides content in db/db fed MCD diet (Table 1). Ballooning degeneration was observed in the liver sections of all db/db fed MCD diet but was less pronounced in those treated with silibinin. Moreover, lobular inflammation was almost absent in silibinin-treated mice. Overall, NAS was significantly decreased in silibinin-treated animals (Fig. 1). No significant deposition of extracellular matrix components, as assessed by Sirius Red stain, was detected in vehicle-treated db/db mice after four weeks of MCD diet as well as in db/db mice that received silibinin (Fig. 2). This

scenario was also consistent with the lack of any significant differences detected for α-SMA immunohistochemistry and α-SMA gene expression among the groups (Fig. 2).

Similarly, the myocardium of db/db fed MCD diet showed diffuse vacuolar degeneration at hematoxylin–eosin staining, consistent with intracellular accumulation of lipids (Fig. 1); myocardiocytes with abnormal size and altered nuclear morphology were also observed. Consistently with liver findings, silibinin treatment markedly improved myocardial injury and reversed the morphological abnormalities in most myocardiocytes (Fig. 1).

3.3. Silibinin counteracts liver and heart oxidative stress and inflammation

In comparison to lean controls, isoprostanes and 8-OHG, markers of lipoperoxidation and DNA damage, were markedly increased both in liver and heart of vehicle db/db fed MCD diet (Figs. 3 and 4). Silibinin treatment significantly decreased liver isoprostanes and 8-OHG (Fig. 3); strikingly, heart isoprostanes and 8-OHG were decreased to the levels of lean controls (Fig. 4). The main scavenger GSH was decreased by 50% both in livers and hearts of vehicle db/db fed MCD diet as compared to control animals, whereas treatment with silibinin restored GSH content to lean mice levels (Figs. 3 and 4). Likewise oxidative stress, also nitrosative stress, as assessed by nitrite/nitrates levels, was significantly increased in the liver and heart of db/db fed MCD diet and was restored to lean controls levels by silibinin (Figs. 3 and 4). Consistently, the activity of the five complexes of MRC was reduced by 50% in vehicle db/db fed MCD diet and was completely restored by silibinin administration (Table 1).

Gene expression of TNF-α was slightly increased in the liver of db/db fed MCD diet as compared with lean animals, whereas IL-6 was reduced; silibinin administration reversed gene expression of both (Fig. 3). MCP-1, IL-4 and IFN-γ gene expression was not significantly modified in any group (data not shown). Similarly, heart TNF-α protein expression was increased and IL-6 markedly decreased in the vehicle group. Silibinin was able to restore heart TNF-α and IL-6 to lean animals levels (Fig. 4). In agreement with the improvement of insulin resistance and of redox and inflammatory *status*, we observed a decrease of immunohistochemical staining for phosphorylated JNK isoforms in MCD fed db/db mice treated with silibinin versus mice only receiving the steatogenic diet (Fig. 5).

4. Discussion

In the current study, we explored the effect of a 4-week daily administration of silibinin both in the liver and in the myocardium of db/db mice fed a MCD diet. This animal model displayed histological features of progressive NAFLD and accumulation of lipid droplets in the myocardium, in keeping with analogous findings of myocardial steatosis in patients with obesity and diabetes [9]. The antisteatotic effect of silibinin in the heart was particularly impressive because it was largely unexpected and completely reversed myocardial damage. To our knowledge, this is the first demonstration of the effectiveness on myocardial damage of a compound used for liver protection in NASH.

The initiation and perpetuation of cell injury in NAFLD is associated with the increase of free radicals and the depletion of endogenous anti-oxidant defense both in human and rodents [26,27]. Several data suggest that lipotoxicity plays a crucial role also in the pathogenesis of cardiomyopathy underlying non-ischemic chronic heart failure, a leading cause of death in patients with obesity and/or diabetes [9,16]. In this NAFLD model, we

Table 1
Biometric and biochemical parameters in the three experimental groups.

|	db/m + SD	db/db + MCD	db/db + MCD + silibinin
8-Weeks weight (g)	24.5 ± 0.5	38.0 ± 2.2[*]	37.5 ± 2.7[*]
12-Weeks weight (g)	26.8 ± 1.2	36.4 ± 1.8[*]	34.8 ± 1.9[*]
Serum ALT (IU/L)	38.4 ± 12.0	454.2 ± 80.4[*]	180.5 ± 66.5[*,**]
Blood glucose (mg/dL)	68.2 ± 7.6	181.8 ± 40.2[*]	103.7 ± 16.8[*,**]
Serum insulin (mU/mL)	6.8 ± 1.2	8.3 ± 0.9[*]	6.5 ± 1.1[**]
HOMA-IR	1.1 ± 0.2	3.7 ± 0.7[*]	1.7 ± 0.5[*,**]
Liver triglycerides (mg/g protein)	3.8 ± 0.8	13.6 ± 1.3[*]	8.3 ± 2.4[*,**]
Liver MRC complex I (%)	100 ± 5.4	56.4 ± 4.6[*]	90.2 ± 5.2[**]
Liver MRC complex II (%)	100 ± 4.8	52.2 ± 4.8[*]	88.4 ± 4.3[**]
Liver MRC complex III (%)	100 ± 3.2	54.4 ± 3.6[*]	94.4 ± 6.8[**]
Liver MRC complex IV (%)	100 ± 4.8	56.8 ± 4.2[*]	96.1 ± 5.2[**]
Liver MRC complex V (%)	100 ± 5.2	58.6 ± 3.3[*]	94.3 ± 6.2[**]

SD, standard diet; MCD, methionine–choline deficient; MRC, mitochondrial respiratory chain.

[*] $P < 0.05$ vs db/m + SD.

[**] $P < 0.05$ vs db/db + MCD.
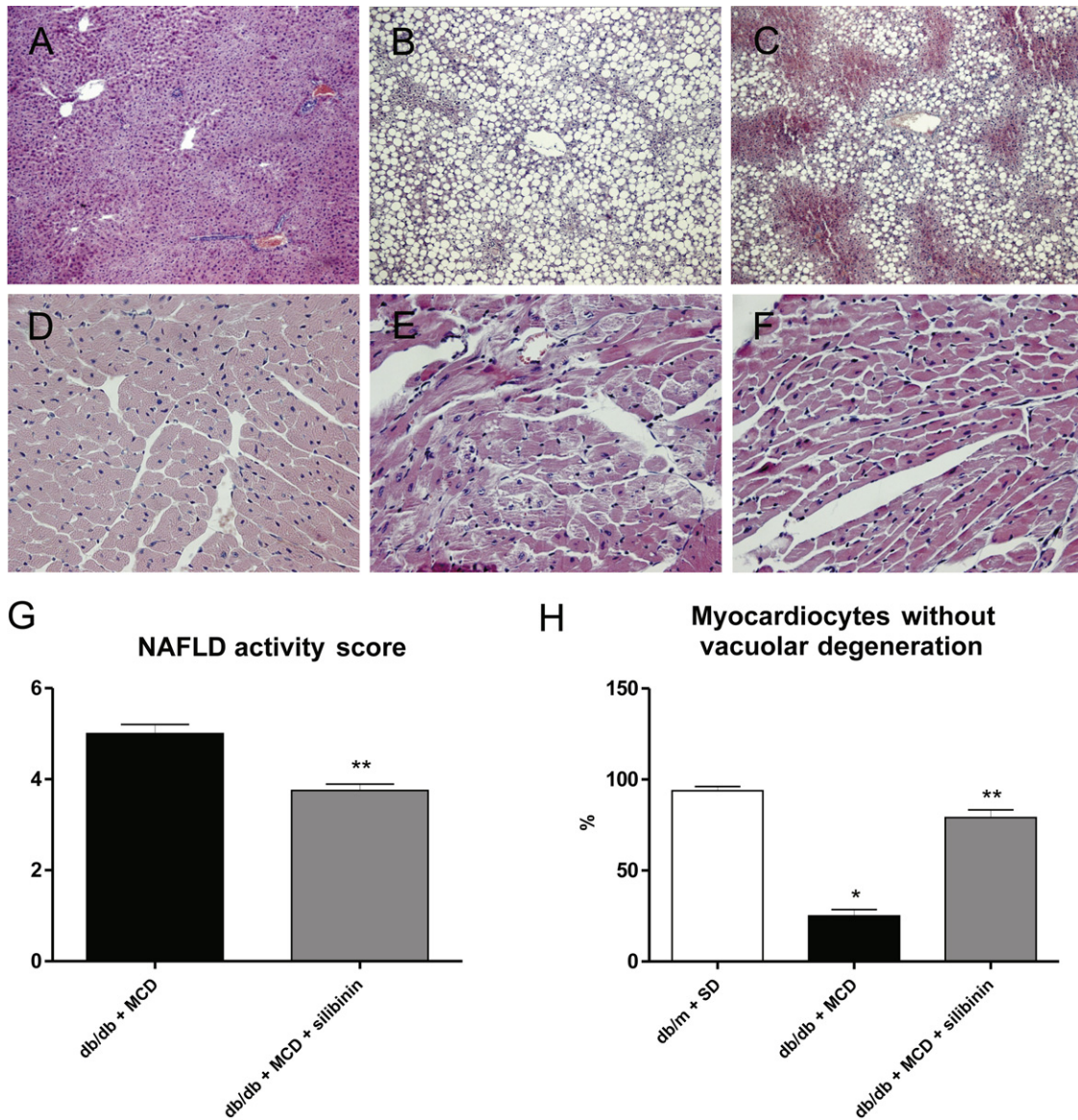
Fig. 1. Effects of silibinin on liver and heart injury. (A) Hematoxylin–eosin stained liver sections of vehicle db/m mice showed normal morphology. (B) Liver sections of db/db mice fed a methionine–choline deficient (MCD) diet revealed severe azonal steatosis, diffuse hepatocyte ballooning and scattered inflammatory foci. (C) Liver sections of silibinin-treated mice evidenced marked decrease of steatosis, reduced ballooning and absence of inflammatory cells. (D) Hematoxylin–eosin stained sections of myocardial tissue demonstrated normal appearance. (E) Myocardial sections of vehicle db/db mice fed MCD diet showed diffuse vacuolar degeneration. (F) Myocardial sections of silibinin-treated animals demonstrated absence of fat accumulation and regular myocardiocytes morphology. (G) Overall, nonalcoholic fatty liver disease (NAFLD) activity score was significantly decreased in animals treated with silibinin. (H) Myocardiocytes morphology was preserved in the silibinin group. *$P < 0.05$ vs db/m + SD, **$P < 0.05$ vs db/db + MCD [magnification: 10× (A–C); 40× (D–F)].
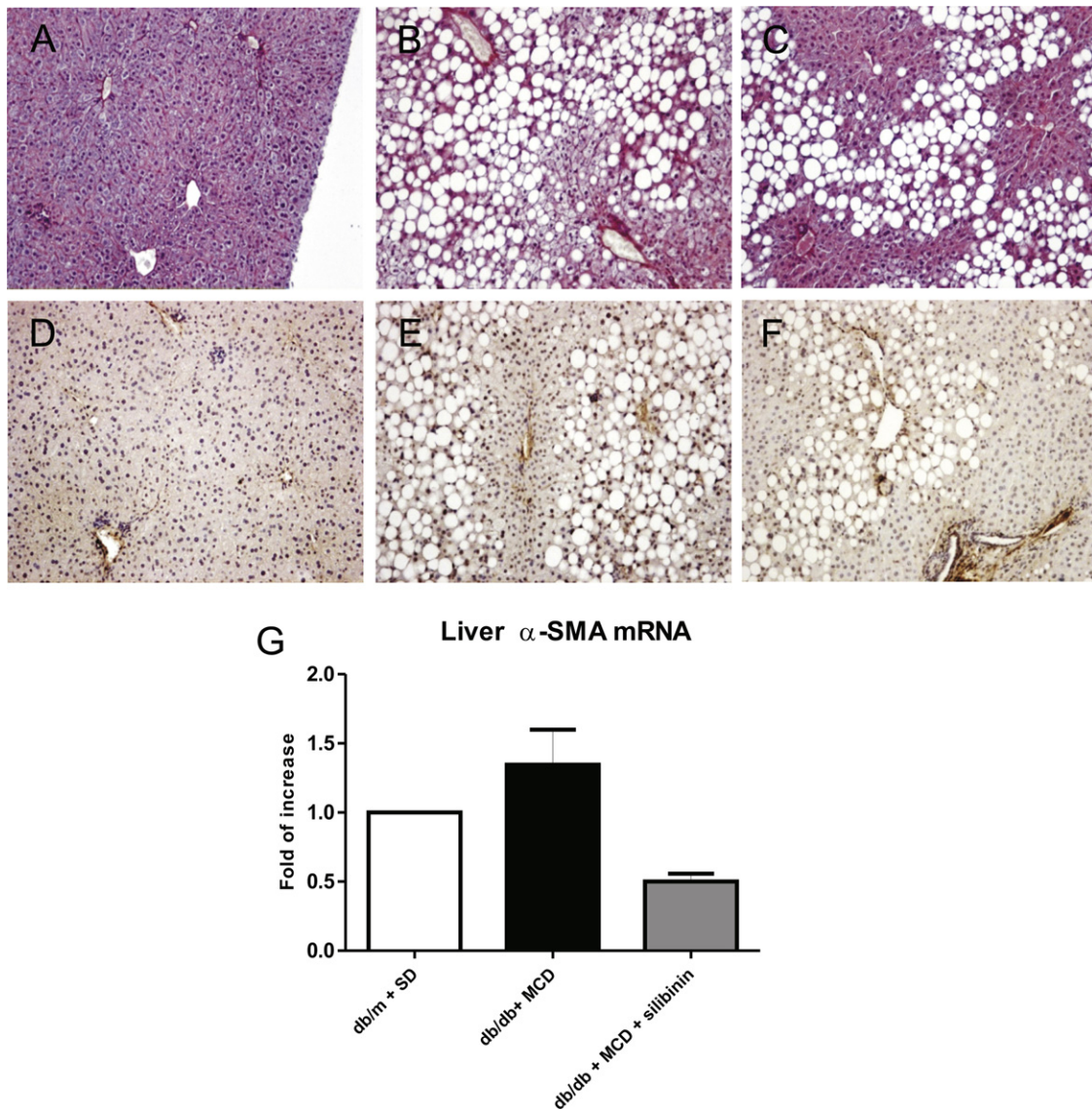
Fig. 2. Effects of silibinin on liver fibrosis. Sirius Red staining was performed on liver sections from (A) vehicle db/m mice, (B) vehicle db/db mice fed a methionine–choline deficient (MCD) diet or (C) silibinin-treated db/db mice receiving MCD diet. No apparent differences in deposition of extracellular matrix components was detected, a scenario also confirmed by the absence of significant changes in activation of profibrogenic myofibroblasts, as detected by (D–F) α-SMA immunohistochemistry and (G) α-SMA gene expression [magnification: $10\times$ (A–F)].

observed a parallel fat accumulation in the liver and heart, in association with oxidative stress. The importance of oxidative stress in NASH pathogenesis is underscored by recent findings showing the effectiveness of vitamin E in preventing liver injury progression in patients [28]. In our animal model, silibinin was able to decrease both isoprostanes, which are sensitive markers of lipoperoxidation [29], and 8-OHG, a marker of DNA damage, which is increased in NAFLD patients in relation to degree of liver injury [30] and in patients with obesity/diabetes cardiomyopathy [17].

The used dose is higher than in the commercially available oral formulations of silymarin/silibinin, but the safety for comparable dosage of silibinin has been showed both in healthy volunteers [31] and in patients with chronic liver disease [32,33]. Previous findings reported the efficacy of a silibinin/vitamin E oral formulation on surrogate serum markers of liver injury in NAFLD patients [34] and the inhibitory effect of silibinin on human hepatic stellate cells activation *in vitro* [35]. The antioxidant action of silibinin in our animal model was further confirmed by its effect on GSH levels. GSH levels

are decreased in NASH patients [25,26] and in mice treated with a MCD diet [26]. Restoring mitochondrial GSH to normal levels by the administration of GSH precursors prevents the establishment of inflammation in the MCD diet model [36]. A marked decrease of MRC activity have been evidenced in patients with NAFLD [37], and liver mitochondrial dysfunction seems to be an early pathogenetic step that precedes fatty liver in rats [38]. Mitochondria are a target of oxidative stress, but also the main source of free radicals and the impairment in MRC activity is directly responsible for the increase in cellular ROS in a vicious cycle [13]. Our results are consistent with previous findings on MRC restoration by silibinin in a rodent model of iron overload [20] and with recent data in another model of NASH [39].
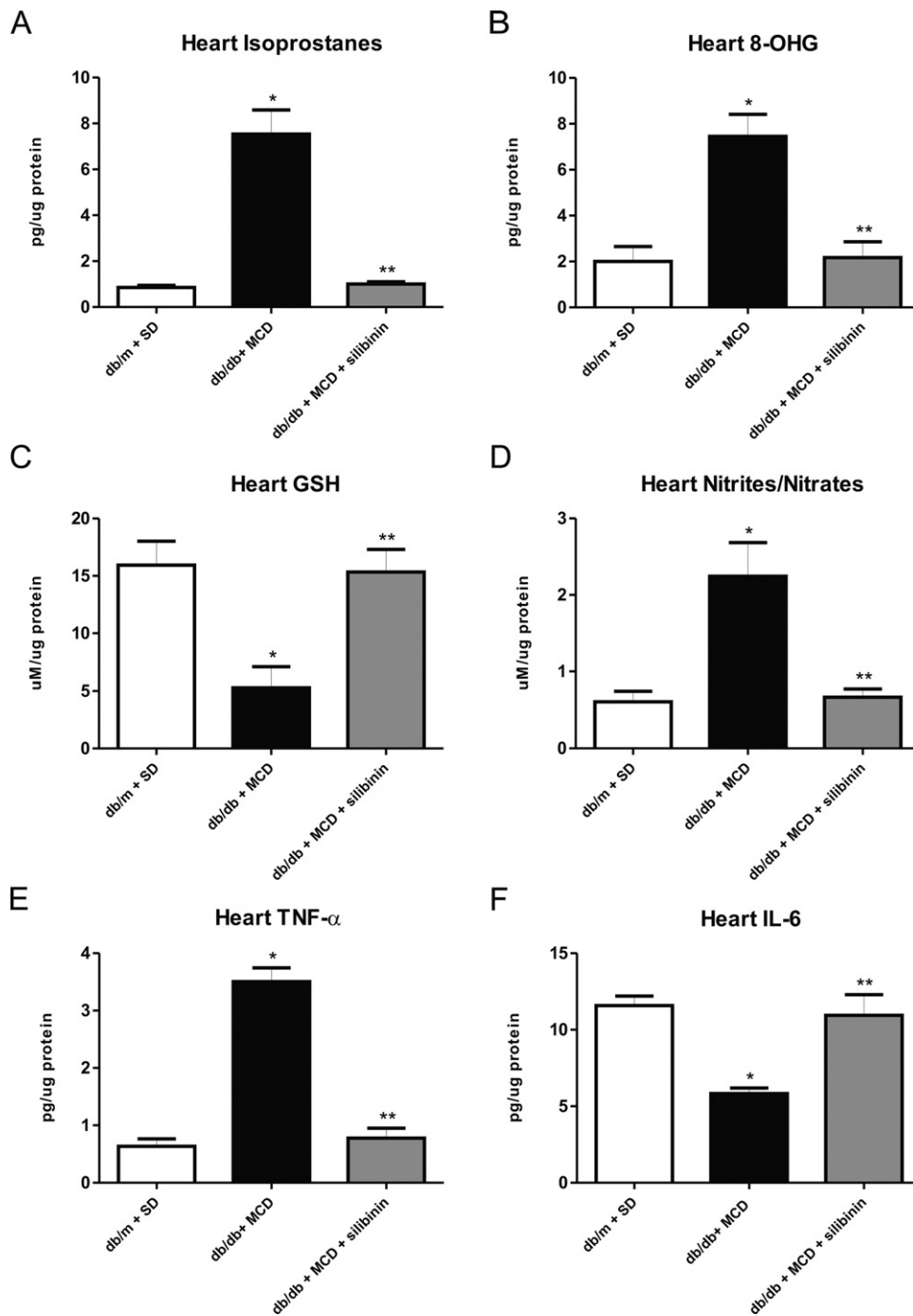
Beyond the effects of silibinin on oxidative stress in hepatic and myocardial tissue, silibinin also displayed a significant effect on inflammatory cytokines levels. The cellular redox *status* is one of the main stimuli for TNF-α mediated inflammation [40]. Interestingly, we observed a stronger increase of TNF-α levels in
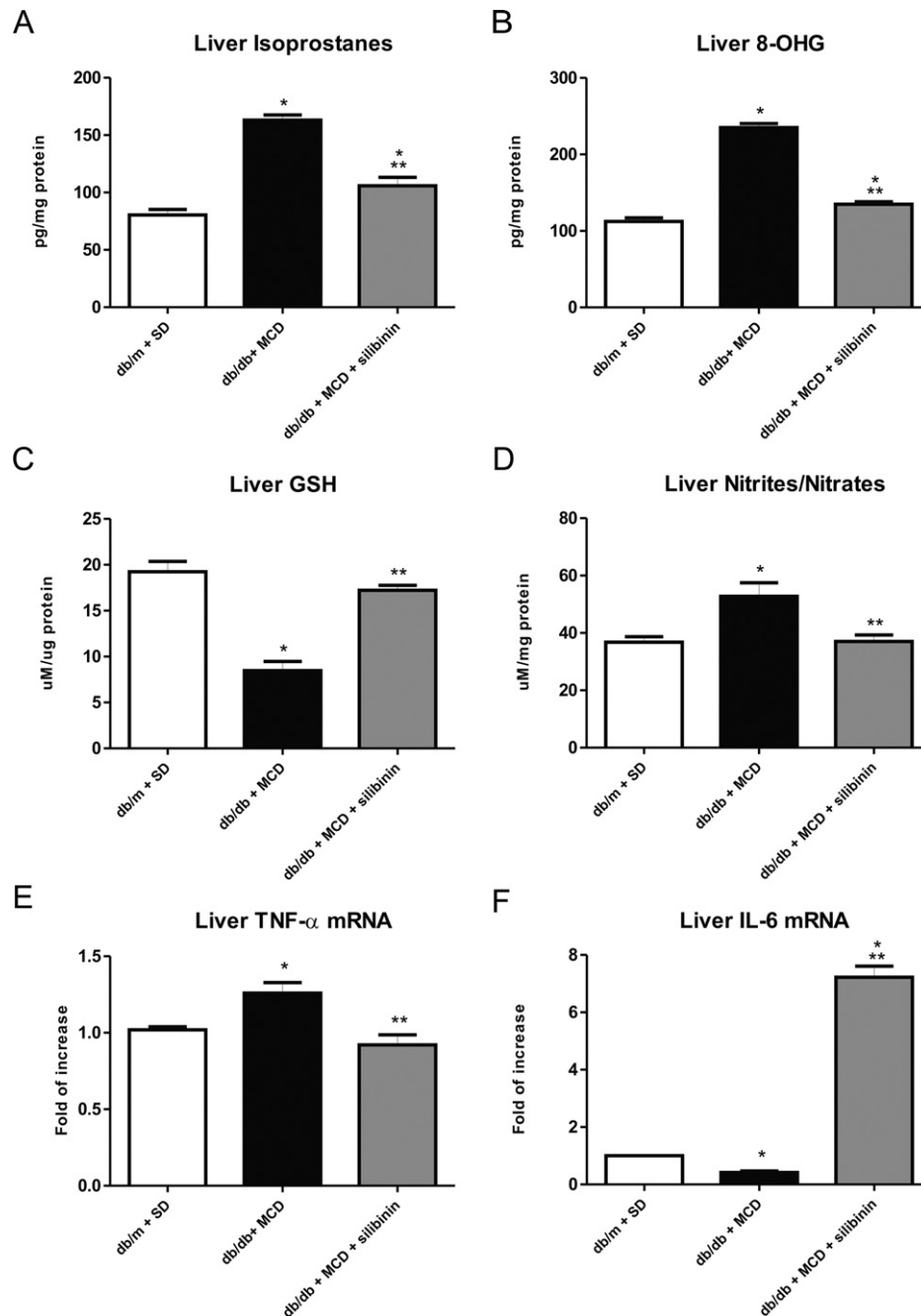
Fig. 3. Effects of silibinin on liver oxidative stress and inflammatory cytokines. (A) Isoprostanes and (B) 8-deoxyguanosine (8-OHG) were markedly increased in vehicle db/db fed a methionine–choline deficient (MCD) diet and significantly decreased by silibinin. (C) GSH levels was completely restored by silibinin administration. (D) Nitrite/nitrates were increased in vehicle db/db fed MCD diet whereas silibinin restored them to the levels of lean mice. (E) TNF-α gene expression was reversed by silibinin treatment. (F) IL-6 was significantly decreased in vehicle db/db fed MCD diet whereas increased by silibinin administration. *$P < 0.05$ vs db/m + SD, **$P < 0.05$ vs db/db + MCD.

the heart, which indicates that myocardial tissue is a main target of inflammation at the early stage of the natural history in this NAFLD model. Noteworthy, TNF-α and oxidative stress plays a sinergic role for the progression of heart failure [41] and the vicious cycle inflammation-oxidative stress represents a main target for preventing myocardial damage [41]. Consistently with the observed improvement of mitochondrial function [42], silibinin administration decreased also TNF-α gene expression in the liver. Surprisingly, IL-6 expression was decreased in the liver and in the myocardium of db/db mice fed MCD diet. However, numerous experimental and clinical evidences suggest that IL-6 is anti-inflammatory and

anti-atherogenic cytokine [43], i.e. IL-6 increases in some pathological conditions as a compensatory pathway. In the liver, IL-6 administration in db/db mice and in mice fed HFD decreases fatty liver and insulin resistance [44] and ameliorates mitochondria lipid disturbance in hepatocytes isolated from steatotic animals fed a choline deficient diet [45]. In the heart, it has been demonstrated that animal lacking IL-6 display an accumulation of lipids, particularly FFA and ceramides [46], and therefore it is conceivable that the decrease of IL-6 in the heart and liver of db/db mice fed a MCD diet significantly contributes to lipotoxicity. MCP-1 levels were unchanged, confirming that this cytokine does not play a

Fig. 4. Effects of silibinin on heart oxidative stress and inflammatory cytokines. (A) Isoprostanes and (B) 8-deoxyguanosine (8-OHG) were markedly increased in vehicle db/db fed a methionine–choline deficient (MCD) diet and decreased to the levels of lean controls by silibinin. (C) GSH levels were also restored by silibinin treatment. (D) Nitrite/nitrates were significantly increased in vehicle db/db fed MCD diet whereas silibinin returned them to the levels of lean mice. (E) TNF-α protein levels were strongly increased in the myocardium of vehicle db/db fed MCD diet and decreased to the levels of lean animals by silibinin. (F) IL-6 levels were decreased in the heart of vehicle db/db fed MCD diet and were restored to the levels of lean animals by silibinin administration. $^*P < 0.05$ vs db/m + SD, $^{**}P < 0.05$ vs db/db + MCD.

relevant role in liver steatosis and inflammation induced by MCD diet [47], whereas phosphorylation of JNK, which is associated with insulin resistance and inflammation [48], was significantly decreased.

In summary, this study suggests a combined effectiveness of silibinin on preventing hepatic and myocardial injury in experimental NAFLD. These effects are mediated by improvement of insulin resistance, reduction of oxidative stress, and restore of inflammatory signalling, key events in the pathogenesis of NASH. Our findings provide a rationale for clinical studies on the use of silibinin in the management of liver and cardiovascular damage in patients with NAFLD.
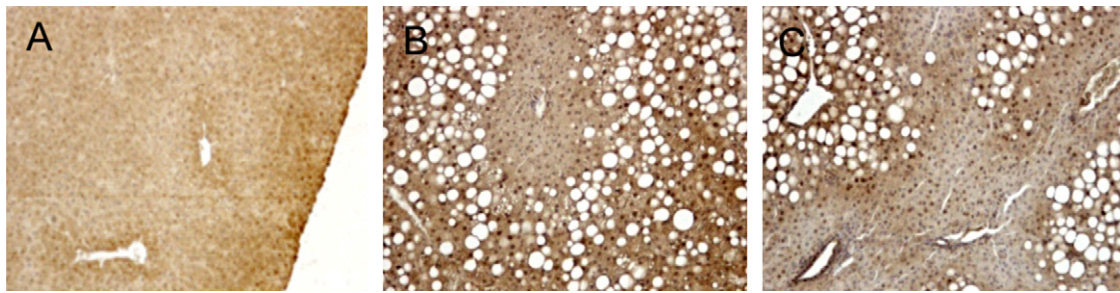
Fig. 5. Effects of silibinin on liver JNK phosphorylation. Immunohistochemistry for p-JNK was performed on liver sections from (A) vehicle db/m, (B) vehicle db/db fed a methionine–choline deficient (MCD) diet and (C) silibinin-treated db/db fed MCD diet. In agreement with the improvement of insulin resistance, redox and inflammatory *status* we observed a decrease of immune-positivity for phosphorylated JNK in silibinin-treated vs vehicle db/db mice fed MCD diet.

Conflict of interest

None declared.

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