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Hepatoprotective effect of oral application of a silymarin extract in carbon tetrachloride-induced hepatotoxicity in rats

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Abstract

Background: Silymarin derived from the milk thistle plant "Silybum marianum" is composed of four major flavonolignans. Clinical as well as experimental studies indicate hepatoprotective effects of silymarin. However, the underlying mechanisms are only incompletely understood.
The aim of this study was to assess the effect of oral administration of a defined silymarin extract in the model of acute carbon tetrachloride (CCl_4) induced liver injury.

Methods: A single dose of a silymarin extract (SE; 20 or 100 mg/kg body weight) was given to rats by oral gavage. Subsequently, rats were injected with a single dose of CCl_4 (2 ml/kg body weight).

Results: After 24h, analysis of liver to body weight ratio, serum levels of transaminases and histological analysis revealed a marked liver damage which was inhibited by SE in a dose dependent manner. CCl_4-induced expressions of pro-inflammatory and pro-fibrogenic genes were significantly reduced in SE treated rats. Molecular analysis revealed that SE reduced the expression of the pro-inflammatory chemokine MCP-1, the pro-fibrogenic cytokine TGF-beta as well as collagen I in isolated human hepatic stellate cells (HSC), which are the key effector cells of hepatic fibrosis.

Conclusion: Oral administration of the tested silymarin extract inhibited hepatocellular damage in a model of acute liver injury. Moreover, we newly found that the silymarin extract had direct effects on pro-inflammatory and pro-fibrogenic gene expression in HSCs *in vitro*. This indicates that direct effects on HSC also contribute to the *in vivo* hepatoprotective effects of silymarin, and further promote its potential as anti-fibrogenic agent also in chronic liver disease.

Keywords: Silymarin; Liver injury; CCl_4; Steatosis; Fibrosis

Background

Silymarin (*Silybum marianum*) is a herbal medicinal product constituting of a mixture of four flavonolignan isomers, namely silybin, isosilybin, silydianin and silychristin with an empirical formula $C_{25}H_{22}O_{10}$. Silybin is the major and most active component and represents about 60-70 % of silymarin, followed by silychristin (20 %), silydianin (10 %), and isosilybin (5 %). Several studies have shown silymarin as hepatoprotective herbal drug [5, 15, 18].

One of the models most frequently studied in the context of acute liver injury is the model of carbontetrachloride (CCl_4)-induced hepatotoxicity. It is known that CCl_4-induced hepatotoxicity is originated from its highly lethal reactive metabolites such as trichloromethyl (CCl_3•) and peroxy trichloromethyl (CCl_3OO•) radicals, which are generated by microsomal cytochrome P450 (CYP) in mammalian liver [19]. CCl_3• can react with a variety of biologically important cellular molecules, such as proteins, lipids, and nucleic acids. This causes impairments of liver function which can affect crucial cellular processes leading to hepatocellular injury [20, 27]. It has been shown that silymarin protected against the CCl_4-induced hepatotoxicity through scavenging free radicals and reactive oxygen species [13, 22]. Furthermore, anti-inflammatory and anti-fibrotic properties have been described (reviewed in [1]). While a few studies have investigated the effectiveness of silymarin

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in a chronic CCl_4 model upon oral application and with regards to the fibrogenic reaction [6, 16]. We for the first time studied the acute effects of orally administered milk thistle extract on early events in fibrosis development.

The activation of HSC is the key event of hepatic fibrosis. The activation of these cells occurs in response to hepatic injury in chronic liver disease. Upon HSC activation, HSC change their phenotype into myofibroblast- like cells with increased production of extracellular matrix (ECM) components such as collagen type I [8, 17]. Furthermore, activated HSC contribute to hepatic inflammation in response to acute as well as chronic liver injury by synthesizing various cytokines and chemokines such as monocyte chemoattractant protein-1 (MCP-1) [24].

The aim of this study was to evaluate the effect of a defined extract of silymarin (SE; Silimarit®) against CCl_4-induced hepatocellular damage as well as on pro-inflammatory and pro-fibrogenic gene expression. Further, we performed *in vitro* analysis in which we investigated the effects of the same extract on the gene expression of activated human HSC. We demonstrated that the application of the extract protected from the CCl_4-induced hepatic injury in a dose dependent manner. Furthermore, our data indicate that direct effects on activated HSC contributed to the hepatoprotective effects of the silymarin extract.

Methods

Chemicals

Carbon tetrachloride (CCl_4) and corn oil were obtained from Sigma Pharmaceuticals (Hamburg, Germany). For treatment of animals, Silimarit® dry extract (SE, milk thistle extract compliant with Pharmacopoea Europaea, Bionorica SE, Neumarkt, Germany) was used. For dissolution of extracts, an aqueous solution of methyl hydroxypropyl cellulose (MHPC, 0.5 %, methocel™ E4M prem) was prepared.

Isolation and stimulation of activated hepatic stellate cells

Hepatic stellate cells (HSC) were isolated from liver specimens using a modified 2-step EGTA/collagenase perfusion procedure [28]. Subsequently, HSC were purified by arabinogalactan gradient ultracentrifugation [11]. HSC were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % FCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a 95 % air/5 % CO2-humidified atmosphere. For induction of *in vitro* activation, freshly isolated HSC were seeded on uncoated plastic dishes for two weeks [11].

Animals and model of acute liver injury

Sprague-Dawley rats were obtained from Charles River Laboratories (Sulzfeld, Germany). Animals were housed in the animal facilities at the University of Regensburg and fed with a standard diet. For *in vivo* application SE was suspended in 0.5 % solution of MHPC and given to six weeks old male rats at a single dose of 20 or 100 mg/kg body weight (BW) by oral gavage (n = 4-5/group). Control rats (n = 5) received solvent MHPC by oral gavage. After 3 h, rats were injected i.p with a single dose of carbon tetrachloride (CCl_4) (1 ml/kg BW.; 1:1, v/v in corn oil) or an equal volume of the solvent (corn oil). 24 h after CCl_4 injection, rats were sacrificed by heart puncture under deep ketamine/xylazine (2:1) anesthesia and liver tissue and blood samples were collected for further analysis. Livers and blood from HPMC/corn oil treated animals served as controls.

Histology

For histological analysis liver tissue specimens were fixed for 24 h in 4 % formalin at room temperature, dehydrated by graded ethanol and embedded in paraffin. Tissue sections (thickness 5 μm) were deparaffinized with xylene and stained with hematoxylin and eosin (H&E). Hepatic staining for Ki67 (a marker of regeneration) was examined using a standard immunohistochemical procedure as described [3]. The number of ballooning cells or Ki67-positive hepatocytes was counted in 4 randomly selected areas on each section.

Quantitative real-time-PCR analysis

RNA isolation from hepatic tissues or cultured cells and reverse transcription were performed as described [10]. Quantitative real-time-PCR was performed applying LightCycler technology (Roche) [10] using specific sets of QuantiTect Primer Assays according to the manufacturer's instructions (Qiagen, Hilden, Germany). Amplification of cDNA derived from 18s rRNA (for: 5'-TGATTAAGTCCCTGCCCTTTGT; rev: 5'-GATCCG AGGGCCTCACTAAAC) was used for normalization.

Analysis of hepatic collagen levels

Liver specimens were subjected to analysis of acid-pepsin soluble collagen using the Sircol Collagen Assay (Biocolor, Carrickfergus, UK) according to the manufacturer's instructions.

Quantification MCP-1 protein levels

MCP-1 protein levels in cell culture supernatant were analyzed using the Quantikine Human MCP-1 Immunoassay (R&D systems, Wiesbaden, Germany) according to manufacturer's instructions.

Statistical analysis

Values are presented as mean ± SEM. Comparison between groups was made using the Student's unpaired *t*-test. A p value <0.05 was considered statistically significant. All calculations were performed using the statistical computer package GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, USA).

Results

Effect of the silymarin extract (SE) on hepatocellular damage in the model of acute CCl_4-induced liver injury

24 h after CCl_4-induced toxic liver injury was initiated, the liver to body weight ratio was significantly elevated in CCl_4-treated rats while this increase was abrogated in SE- treated groups (Fig. 1a). The increase of liver to body weight ratio in CCl_4-treated rats was mainly caused by a significant loss in the body weight which was less prominent in SE-treated rats (data not shown). Also macroscopically, livers of CCl_4-treated rats appeared larger and with a pale and irregular surface indicative of severe hepatocellular damage; representative images are depicted in Fig. 1b. Histopathological analysis confirmed large areas of necrotic tissue and a significant number of ballooning hepatocytes in the livers of CCl_4-treated rats which was less prominent in SE-treated animals (Fig. 1c). CCl_4-induced macroscopic and histopathological changes were attenuated in SE-treated animals (Fig. 1b and c). In normal liver, almost no hepatic cells showed Ki67 staining, a marker of cellular proliferation, while Ki67 immunosignals were markedly enhanced in response to CCl_4-treatment reflecting repair of considerable liver damage (Fig. 1d). SE-treated rats showed significantly less Ki67 positive liver cells indicating reduced hepatocellular damage (Fig. 1d). Hepatocellular injury in the CCl_4 groups was also reflected by a marked increase of serum transaminases compared to the control group, but this increase was less pronounced in SE-treated rats (Fig. 1e). Although the differences in serum transaminases did not reach the level of significance they point to the overall picture showing a hepatoprotective effect of SE in the model of acute CCl_4-induced liver injury.

Effect of the silymarin extract (SE) on pro-inflammatory and pro-fibrogenic gene expression in the model of acute CCl_4-induced liver injury

Heme oxygenase-1 (HMOX-1) is an essential enzyme in the heme catabolism, which is induced by oxidative stress. Quantitative real-time PCR revealed a significant induction of the HMOX-1 expression in response to CCl_4 treatment (Fig. 2a). This increase in HMOX-1 was dose dependently abrogated by SE indicative for reduced oxidative stress (Fig. 2a).

Oxidative stress is a well-known inducer of a pro-inflammatory reactions, and in line with this, we observed a significant induction of hepatic mRNA expression levels of tumor necrosis factor (TNF) and interleukin-1 beta (IL-1ß) in CCl_4-injured livers (Fig. 2b and c). The induction of TNF and IL-1ß was significantly reduced by SE in a dose dependent manner (Fig. 2b and c).

Moreover, the expression of monocyte chemo-attractant protein-1 (MCP-1) was markedly increased in CCl_4-injured livers (Fig. 2d). In rats which had received SE this MCP-1 induction was significantly reduced (Fig. 2d). While TNF and IL-1ß are mainly expressed by resident and infiltrating immune cells in an injured liver hepatic MCP-1 expression levels are dependent on activated HSC [24]. HSC activation is recognized as a central event in the development of hepatic fibrosis and lastly, cirrhosis. Transforming growth factor beta (TGF-β) is also expressed by activated HSC and one of the most potent pro-fibrogenic cytokines in liver disease. We detected a strong induction of TGF-ß in CCl_4-injured livers, and this induction was significantly reduced by SE in a dose dependent manner (Fig. 2e). Analysis of hepatic collagen content revealed only a slight induction of collagen content after 24 h CCl_4-treatment (Fig. 2f), which can be explained by the fact that fibrosis is a time-dependent process and repeated CCl_4-applications are required to induce significant hepatic collagen accumulation over time. Notably, the slight increase of hepatic collagen content in response to CCl_4-treatment was not observed in SE-treated rats (Fig. 2f). Together, these data indicate that SE inhibits the pro-inflammatory and pro-fibrogenic response to CCl_4-induced liver injury. Because it is known that biotransformation of CCl_4 is mainly mediated by CYP2E1 [27, 29], we further analyzed the hepatic expression levels of this cytochrome and found a marked decrease in response to CCl_4 treatment. Interestingly, this decrease was reduced in SE treated rats (Fig. 2g). It can only be speculated whether this less pronounced downregulation of CYP2E1 affected the CCl_4-metabolisms. In any case, it appears that SE exhibited its hepatoprotective effects in response to acute CCl_4-injury at different levels.

Effect of the silymarin extract (SE) on pro-inflammatory and pro-fibrogenic gene expression in activated hepatic stellate cells

The *in vivo* data indicated that SE affected pro-inflammatory and pro-fibrogenic gene expression in HSC. However, they did not allow the differentiation, whether this is an indirect effect *via* reduced tissue damage and inflammation or whether SE exhibits also a direct effect on HSC. To address this question, we stimulated primary activated human HSC with SE *in vitro*. Interestingly, we found that SE significantly reduced the mRNA expression of MCP-1 and TGF-beta in HSC in a dose dependent manner (Fig. 3a,b). Analysis of MCP-1 protein levels in the cell culture supernatants confirmed this result on the protein level (Fig. 3c). Moreover, expression of collagen-I, the predominant extracellular matrix protein in fibrotic human tissues, was significantly impaired by SE stimulation of activated HSCs *in vitro* (Fig. 3d). Together, these findings indicated that SE directly affects pro-inflammatory and pro-fibrogenic gene expression of activated HSC.

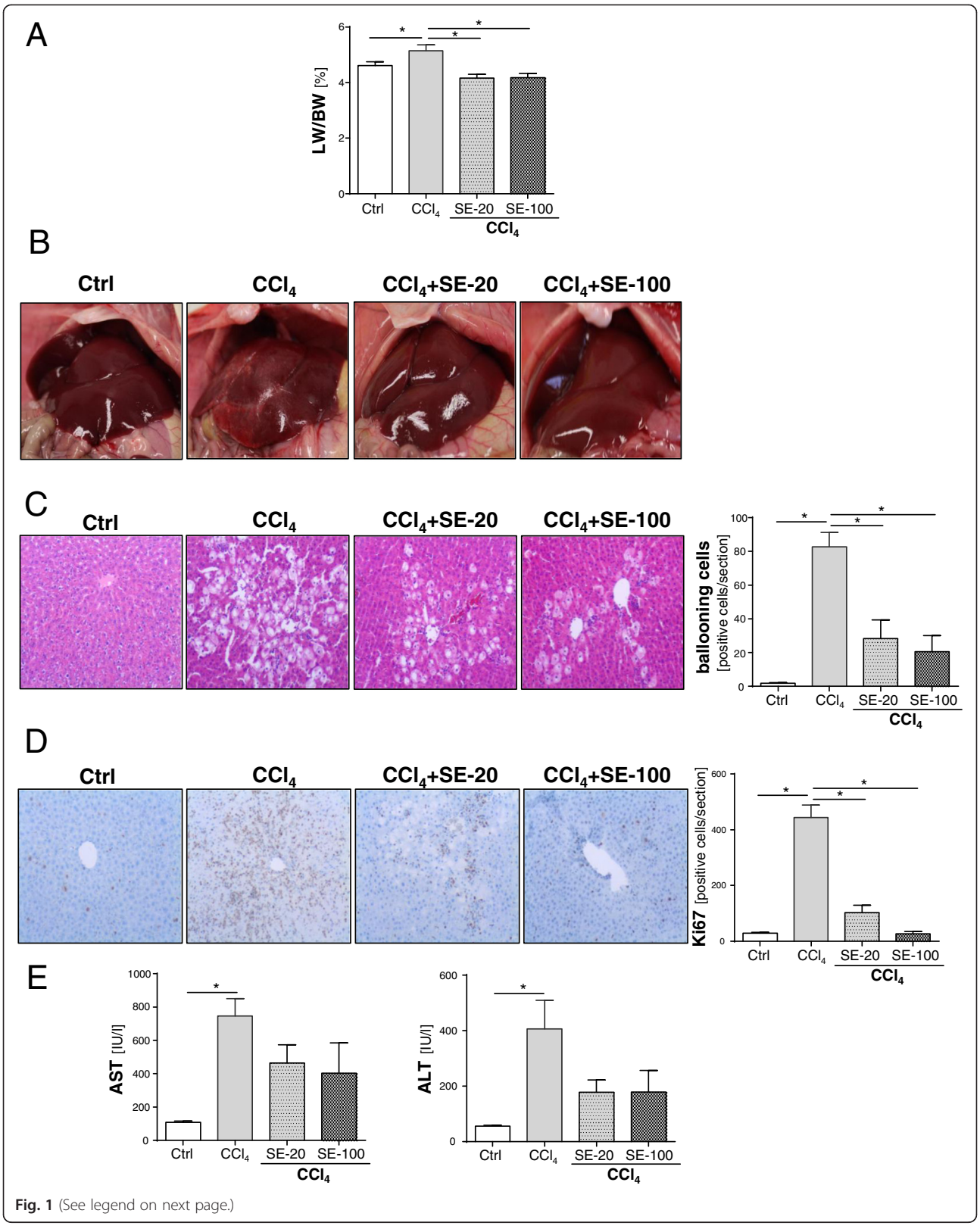

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Fig. 1 Effect of silymarin (SE) on hepatocellular damage in CCl_4- induced liver injury. Rats were treated with MHPC (control) or silymarin (SE) at a single dose of 20 mg/kg BW (SE-20) or 100 mg/kg BW (SE-100) by oral gavage. After 3 h rats were injected with a single dose of CCl_4 or the equal volume of vehicle (control). 24h after CCl_4 injection, rats were sacrificed. **a** Liver-to-body -weight-ratio. **b** Representative macroscopic images of livers from the four treatment groups. **c** H&E staining of liver tissue (Magnification 20x) (left panel); quantification of ballooning hepatocytes in hepatic tissues (right panel). **d** Representative images of Ki67 stained liver tissues (left panel); quantification of Ki67 positive hepatocytes in hepatic tissues (right panel). **e** AST and ALT serum levels. (*: $p < 0.05$)

Discussion

The aim of this study was to assess the effect of oral administration of a Silimarit® dry extract (SE) in the model of acute CCl_4-induced liver injury. Our results reveal a profound hepatoprotective effect accompanied by reduced pro-inflammatory and pro-fibrogenic gene expression. Oxidative stress due to CCl_4-induced free radical production is one of the main mechanism by which hepatocellular damage is induced in this model [12, 26]. Here, we found a significant reduction of CCl_4-induced

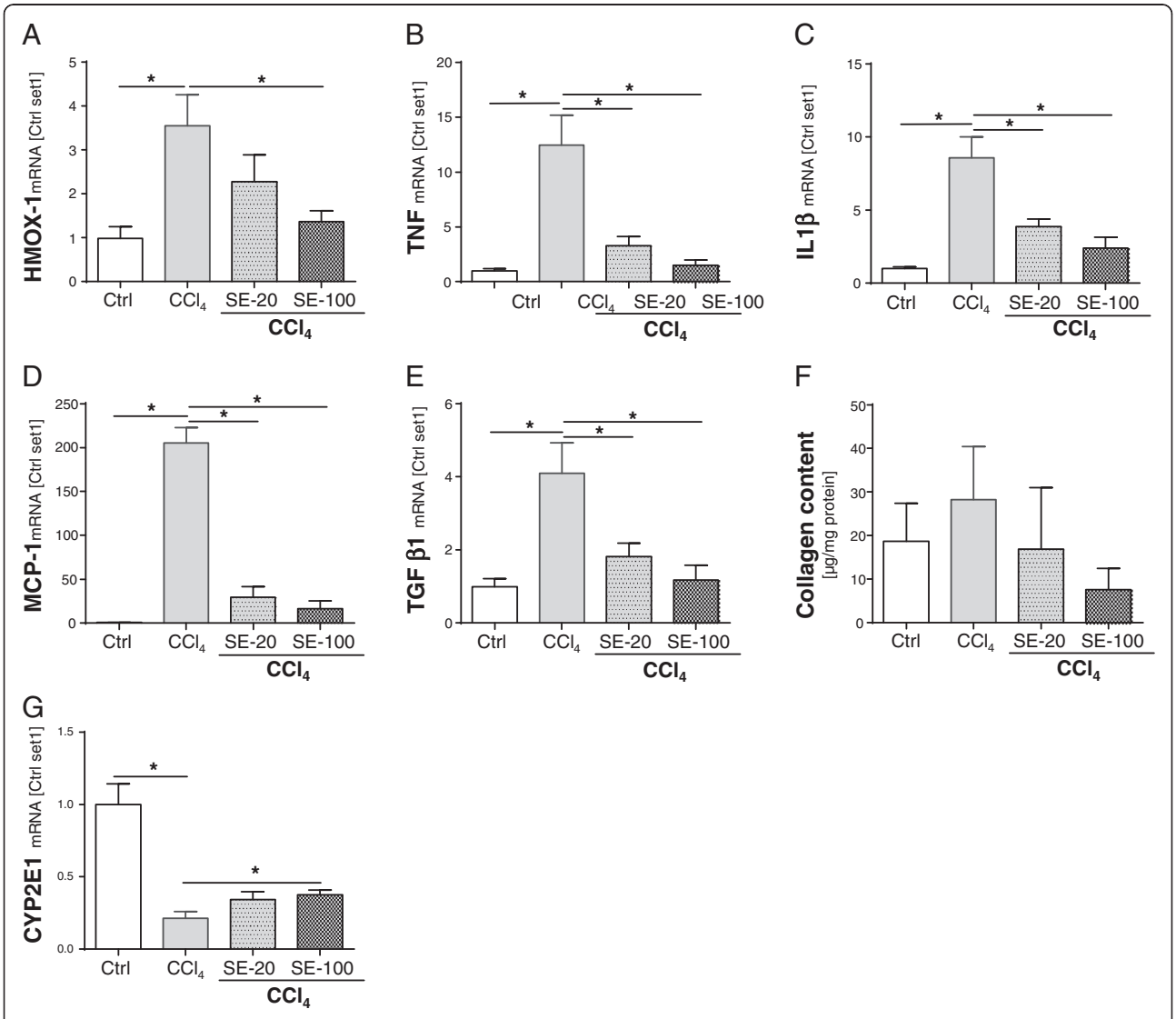

Fig. 2 Effect of silymarin (SE) on the pro-inflammatory and pro-fibrogenic response in CCl_4- induced liver injury. Rats were treated with MHPC (control) or silymarin (SE) at a single dose of 20 mg/kg BW (SE-20) or 100 mg/kg BW (SE-100) by oral gavage. After 3 h rats were injected with a single dose of CCl_4 or the equivalent volume of vehicle (control). 24h after CCl_4 injection, rats were sacrificed. Analysis of hepatic mRNA levels of (**a**) HMOX-1 (**b**) TNF (**c**) IL1-ß (**d**) MCP-1 and (**e**) TGFß1 by quantitative RT-PCR. **f** Analysis of the hepatic collagen content. **g** Analysis of hepatic mRNA levels of CYP2E1 by quantitative RT-PCR. (*: $p < 0.05$)

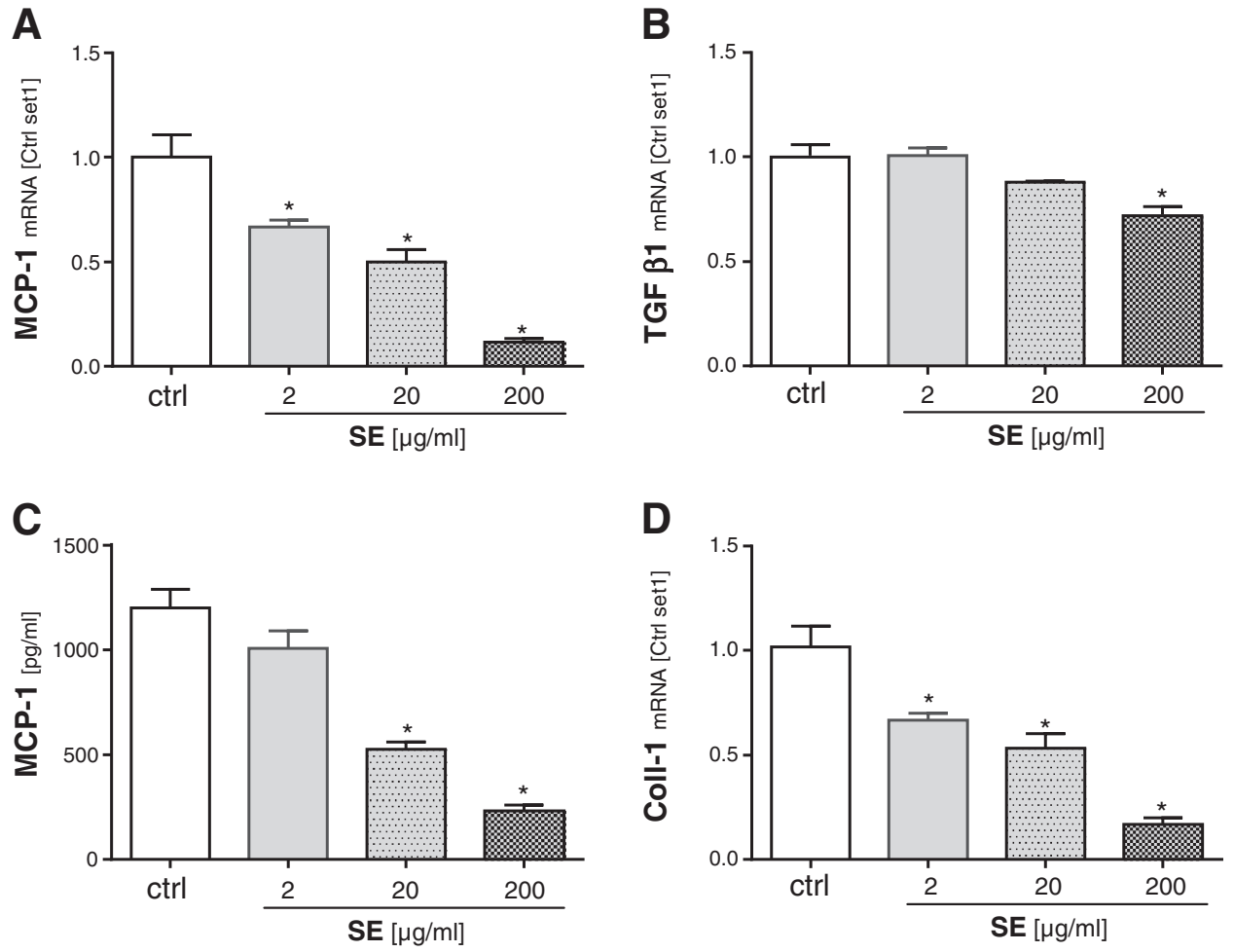

Fig 3 Effect of the silymarin extract (SE) on pro-inflammatory and pro-fibrogenic gene expression in activated hepatic stellate cells (HSC). Primary activated human HSC were stimulated *in vitro* for 24 h with SE in the indicated doses. **a** Analysis of mRNA levels of MCP-1, **b** TGFß1 and **d** Coll-I by quantitative RT PCR. **c** Analysis of MCP-1 protein levels in the cell culture supernatant by ELISA. (*: $p < 0.05$)

HMOX-1 expression in the liver tissue of SE-treated rats indicating reduced ROS formation as one mechanism by which the hepatoprotective effect of the extract was mediated. Moreover, we found that SE treatment inhibited the CCl_4-induced hepatic TNF and IL-1ß expression in a dose dependent manner which indicates reduced activation of Kupffer cells, the resident macrophages in the liver, and less hepatic immune cell infiltration, respectively. While TNF and IL-1ß are mainly expressed by resident and infiltrating immune cells in an injured liver, activated HSC contribute to hepatic MCP-1 expression levels [24]. HSC activation is recognized as a central event in the development of hepatic fibrosis and occurs early in response to liver injury. Consequently, in the model of acute CCl_4-induced liver injury, HSC activation has also been described as a crucial event by us and others [25, 30]. Thus, reduced MCP-1 expression indicates attenuation of liver injury after SE treatment. Upon hepatic injury, HSC transform in an activated myofibroblast-like cell type that is responsible for the excessive hepatic matrix deposition in chronically damaged livers [8]. Transforming growth factor beta (TGF-β) that is one of the most potent pro-fibrogenic cytokines in liver disease is expressed by activated HSC. TGF-β leads to an autocrine induction of fibrogenic gene expression in HSC. SE treatment reduced the CCl_4-induced expression of TGF-β and collagen-I which play crucial roles in liver fibrosis also in the CCl_4 model [6, 7, 9, 16]. In our study, a remarkable decrease of pro-fibrogenic and pro-inflammatory mRNAs was observed, leading to the conclusion that the used silymarin extract has protective effects on the formation of fibrosis after acute liver injury induced by CCl_4. These findings add to the results of other studies showing reduced fibrosis in injured liver tissue after silymarin treatment in chronic

models of liver injury [2, 4, 14, 21, 23]. The protective role of silymarin extract on early stages of fibrosis and liver damage – as shown in this study – may also be relevant in chronic liver disease. In a recent study Shaker and co-workers demonstrated antifibrotic effects of a milk thistle preparation and chemotherapeutic agents in chronic thioacetamide (TAA)-induced liver injury. Although, the chemical agents showed a profound anti-fibrotic potential, they exhibited marked toxicity on hepatocytes. The advantage of milk thistle treatment was that no toxic side effects on the liver were observed [21]. Also in our study we did not observe any negative side effects in SE-treated rats (data not shown).

In humans as well as rodents the biotransformation of CCl_4 is mainly mediated by CYP2E1 and very marginally by other CYPs (CYP2B and CYP3A) (reviewed in [27]). Accordingly, CYP2E1 knockout mice are resistant to CCl_4 hepatotoxicity [29]. A recent study conducted with guinea pigs (*Cavia porcellus*) showed increased hepatic CYP2E1 expression in response to chronic alcohol administration, while silymarin supplementation significantly reduced this induction in comparison with alcohol abstention group [2]. Here, we found that CCl_4-treatment caused a considerable decrease of hepatic CYP2E1 expression, which was in line with a previous study of Wong et al. [29]. Interestingly, the inhibitory CCl_4-effect on CYP2E1 expression was alleviated by SE-treatment. These findings indicate that silymarin has a modulating effect on hepatic CYP2E1 expression, which can be altered by different hepatotoxins. Together, the hepatoprotective effects and anti-fibrotic effect of SE combined with the absence of toxicity makes it a good candidate for further clinical investigations.

Importantly, our *in vitro* studies in primary human HSC revealed that SE directly affects the expression of pro-fibrogenic factors in HSC. These data indicate that direct effects of the SE on HSC also contribute to the hepatoprotective effect of SE *in vivo*. With regards to the effect of SE on HSC and pro-fibrogenic gene expression, respectively, it has to be noted that we applied the SE orally in our study. After oral administration peak levels of Silibinin, a major component of SE, are reached in liver, lung, and stomach [31].

Conclusions

In conclusion, we found that the silymarin extract studied exhibited profound hepatoprotective effects in a model of acute liver injury *via* different mechanisms. Particularly, the inhibitory effects on fibrogenic factors in this model as well as the direct anti-fibrogenic effects of the extract on human HSC *in vitro* further indicate the potential of this natural compound in the prevention and treatment of hepatic fibrosis in patients with (chronic) liver disease.

Abbreviations

SE: Silymarin extract; CCl_4: Tetra-carbon chloride; HSCs: Hepatic Stellate Cells.

Competing interests

The study was in part funded by Bionorica SE. PP, AL and JH are employees of Bionorica SE. AM, AK, BC and CH have no competing interests to declare.

Authors' contributions

AM, AK and BC performed the experiments and acquired the data; AM and CH analyzed and interpreted the data; PP, AL, JH and MM provided material support; CH designed and supervised the study; CH and AM drafted the manuscript and all authors critically revised and approved the manuscript.

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