

LIVER FIBROSIS: EFFECT OF ANTI-OXIDANT, ALCOHOL WITHDRAWAL

Anti-oxidant ebselen causes the resolution of experimentally induced hepatic fibrosis in rats

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Abstract

Background: Hepatic fibrosis occurs because of injury to the liver parenchyma and biliary system. We have investigated the effect of an organic selenium anti-oxidant, ebselen, in the resolution of experimentally induced hepatic fibrosis, and evaluated its effect on various paradigms involved in hepatic fibrosis.

Methods: Following pretreatment with phenobarbitone, liver fibrosis was induced in male Fischer 344 rats by using carbon tetrachloride treatment for 10 weeks. Carbon tetrachloride-treated rats were randomly assigned into two groups: (i) no ebselen; and (ii) ebselen administered for 3 weeks following a 10-week carbon tetrachloride treatment period. Normal controls were: (i) neither carbon tetrachloride nor ebselen treated; or (ii) ebselen treated for 13 weeks. Liver sections were stained with hematoxylin and eosin, Masson trichrome and stained for reticulin by using silver impregnation. Reverse transcription–polymerase chain reaction was used to analyze the steady-state levels of gene(s) involved in: (i) hepatic fibrosis, namely, transforming growth factor- β 1, procollagen I and III, tissue inhibitor of metalloproteinase-1 and matrix metalloproteinase-13; (ii) oxidative stress, namely, cytochrome P450E1; and (iii) preneoplastic liver foci, namely, the placental form of glutathione-S-transferase.

Results: Histological staining showed that ebselen resolves carbon tetrachloride-induced hepatic fibrosis. Treatment with ebselen reduced steady-state levels of transforming growth factor- β 1, procollagen I and III, tissue inhibitor of metalloproteinase-1, cytochrome P450E1 and placental form glutathione-S-transferase transcripts, and increased transcripts of matrix metalloproteinase-13.

Conclusion: These findings provide evidence that ebselen significantly causes the resolution of carbon tetrachloride-induced hepatic fibrosis in rats.

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Key words: cirrhosis, collagen, cytochrome P450, matrix metalloproteinase-13, placental form of glutathione-S-transferase, selenium, tissue inhibitor of metalloproteinase-1, transforming growth factor- β 1.

INTRODUCTION

Hepatic fibrosis results from the disequilibrium between synthesis and degradation of extracellular matrix (ECM) components.^{1,2} The cytokine, transforming growth factor- β (TGF- β), is expressed strongly in hepatic fibrosis and plays a pivotal role in liver inflammation and in the pathophysiological state.^{3–8} Upon tissue injury, TGF- β , predominantly TGF- β 1, is

released at the site of injury,^{8–10} inducing ECM deposition.^{10–12}

The disproportionate increase in ECM molecules such as the fibrillar collagens (I and III) and the formation of a microheterogeneous architecture results in hepatic fibrosis.^{13–15} The regulation of procollagen I and III synthesis is mediated primarily by TGF- β .^{3,5,7,16}

The tissue inhibitor of metalloproteinase-1 or TIMP-1 is a 28-kDa glycoprotein that promotes the

progression of hepatic fibrosis by inhibiting the degradation of collagens.^{17–19} Collagenase-3²⁰ or matrix metalloproteinase-13 (MMP-13)²¹ belongs to a class of neutral proteinases that require zinc ions for activity, and specifically degrades the native forms of interstitial collagen types I and III.^{18,20,21}

The hepatotoxin, carbon tetrachloride (CCl₄) is frequently used to induce liver fibrosis in animal models.^{4,5,22,23} Treatment with CCl₄ generates free radicals that trigger a cascade of events that result in hepatic fibrosis,²⁴ mimicking the oxidative stress that has a fibrogenic effect on hepatic stellate cells (HSC).^{25,26} The ethanol-inducible form of cytochrome P450 (CYP2E1) is an effective producer of reactive oxygen species and an inducer of oxidative stress, and induces collagen type I mRNA in rat HSC.^{26,27} The CYP2E1 metabolizes CCl₄ in human liver microsomes.²⁸ Carbon tetrachloride treatment also causes the formation of preneoplastic foci²⁹ that express the placental form of glutathione-S-transferase (GST-P), a marker for hepatocarcinogenesis.^{30,31}

Both experimental data and clinical observations suggest that liver fibrosis can be resorbed. Numerous pharmaceutical agents have been tried with varying degrees of success, but have had unacceptable side-effects in long-term therapy.^{16,32–34} One possibility is the use of an anti-oxidant to reduce oxidative stress, which is an important stimulus for the activation of HSC (as reviewed in³⁵).

Ebselen, 2-phenyl-1,2-benzisoselenazol-3[2H]-one, a synthetic seleno-organic compound, acts as a mimic of glutathione peroxidase³⁶ and contributes to the anti-oxidant defense in tissues.³⁷ Ebselen is a free radical and singlet oxygen quencher³⁸ and inhibits ADP-Fe-induced lipid peroxidation³⁹ and copper- and peroxyl-radical induced low-density lipoprotein (LDL) lipid oxidation.⁴⁰ Ebselen is metabolized by rat liver cells and excreted into the bile. In contrast to inorganic selenium, ebselen has been shown to have low toxicity.⁴¹ Ebselen protects the liver cell line (HepG₂) cells from hydrogen peroxide-induced cytotoxicity, lipid peroxidation and DNA damage.⁴² Fischer rats from aflatoxin B₁-induced hepatocarcinogenesis,⁴³ and mice from galactosamine/endotoxin-induced hepatitis.⁴⁴

In this study, we investigated the effect of ebselen in an experimental model of hepatic fibrosis. We evaluated the mechanism of action of ebselen at the molecular level by investigating its effect on the steady-state transcript expression of factors involved in hepatic fibrosis (TGF-β1, procollagen I and procollagen III, TIMP-1 and MMP-13), oxidative stress (cytochrome P4502E1) and preneoplastic foci formation (GST-P) by using reverse transcription-polymerase chain reaction (RT-PCR).

METHODS

Animals

Consent was obtained from the ethics committee of the hospital (KK Women's and Children's Hospital) before the initiation of animal experiments. All animals

received humane care in compliance with the hospital's and the National Institute of Health's guidelines. Male Fischer 344 rats weighing 175–205 g were obtained from the Laboratory Animal Holding Unit at the National University of Singapore. The rats were randomly assigned, two per cage, and subjected to 12 h day/12 h night cycles. Normal rat chow and water were provided ad libitum.

Ebselen

Ebselen (2-phenyl-1,2-benzisoselenazol-3[2H]-one) was provided by Rhone-Poulenc Rorer, Center de Recherches, Vitry-Alforville, France. One milliliter of a 0.4-mg/mL ebselen suspension in water was administered orally by gavage per 250 g rat, 5 days a week (Monday–Friday), for varying time periods according to the protocol described below.

Reagents

Fentanyl citrate and fluanisone were purchased from Jassen Pharmaceutica, Brussels, Belgium; midazolam from F. Hoffman-La Roche, Basel, Switzerland; RNA extraction buffer TRIZOL reagent, Superscript RNase H⁻ Reverse Transcriptase and agarose from Gibco BRL (Gaithersburg, MD, USA); deoxynucleotide triphosphates (dNTPs) and oligo dT₁₈ primer from New England Biolabs (Beverly, MA, USA); recombinant RNasin ribonuclease inhibitor from Promega (Madison, WI, USA); Taq polymerase and HotStar Taq polymerase from Qiagen GmbH (Hilden, Germany); 100 bp molecular weight ladders from MBI Fermentas (Vilnius, Lithuania) and PCR primers from Genset Singapore Biotech (Singapore).

Treatment of rats

All 40 rats used in the present study were treated with phenobarbitone water (35 mg/dL) for 14 days. Standardized decompensated micronodular cirrhosis was induced in 20 rats by administering CCl₄ orally once a week (on Saturdays) for 10 weeks using a gavage, and following a regimen tailored to individual rats based on weight change response to CCl₄ between each dose.²⁹

Fifteen of the 20 rats survived the 10-week CCl₄ treatment protocol. At the end of 10 weeks, the CCl₄-treated rats were randomly assigned into two groups in a 2:3 ratio for 3 week water (group C, *n*=6) or 3 week ebselen (group EC, *n*=9) treatment. At the time of assigning CCl₄-treated rats into groups C and EC, there was no significant difference in the weights of the 15 rats. The first normal control group, N, consisted of 10 rats that were given neither CCl₄ nor ebselen. To assess the effect of treatment with ebselen alone, a second normal control group, E, consisted of rats fed with ebselen for 13 weeks. None of the rats in the two normal control groups died, indicating that ebselen alone was not toxic to the rat. The treatments are summarized in Table 1.

Table 1 Treatment groups

Group	<i>n</i>	Type of treatment	Experimental protocol
C	6	Disease control	CCl ₄ for 10 weeks, then water for 3 weeks; no ebselen
N	10	Water control	No CCl ₄ , no ebselen
E	10	Ebselen control	Ebselen for 13 weeks
EC	9	Ebselen treatment	CCl ₄ for 10 weeks followed by ebselen for 3 weeks

C, control (treated with carbon tetrachloride (CCl₄) and no ebselen); N, control not treated with CCl₄ or ebselen; E, treated with ebselen only; EC, treated with CCl₄ and then ebselen.

Sacrifice

The rats were killed humanely while under anesthesia. Rats were anesthetized by using an intraperitoneal injection of a mixture of fentanyl citrate (0.063 mg/rat), fluanisone (2 mg/rat) and midazolam (1 mg/rat). A midline laparotomy of the rats was carried out, the liver was excised and samples obtained for histological and transcript analyses.

Histological staining

Liver specimens were preserved in 4% paraformaldehyde in phosphate-buffered saline and dehydrated in a graded alcohol series. Following xylene treatment, the specimens were embedded in paraffin blocks and cut into 5 µm-thick sections that were stained with either hematoxylin and eosin (HE), Masson trichrome or for reticulin by using silver impregnation.

Analysis of transcripts

Liver sections were harvested into TRIZOL solution, frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted by using the TRIZOL solution. The expression of various transcripts was analyzed by using non-radioactive, densitometric analysis of RT-PCR products,⁴⁵⁻⁵⁰ and were comparable to northern blot analysis.⁵¹ The conditions for the analyses of TGF-β1, procollagen I, procollagen III, TIMP-1, MMP-13 and GST-P were as described previously.^{43,51} The sequences of the PCR primers for the amplification of CYP2E1 were: CYP2E1 upper primer: 5'-AGC GCT TCG GGC CAG TGT TC-3'; CYP2E1 lower primer: 5'-CAG GGT GCG CAG CCA ATC AG-3' (this paper, based on Accession No. AF061442). The amplification of CYP2E1 specific fragments was carried out for 27 cycles at 95°C for 30 s, 57.6°C for 30 s and 72°C for 30 s. The levels of expression of all the transcripts were normalized to that of the constitutively expressed housekeeping gene glyceraldehyde-3-phosphate dehy-

Table 2 Percentage weight gain and final liver weights

Group	<i>n</i>	Percentage weight gain (SE)*	Final liver weight (SE)
C	6	63.0 (2.7)	8.6 (0.4)
N	10	73.5 (6.0)	9.6 (0.4)
E	10	73.3 (3.2)	9.6 (0.2)
EC	9	55.5 (6.0)	8.7 (0.6)

*Percentage weight gain = ((final weight—initial weight)/initial weight) × 100%. C, control (treated with carbon tetrachloride (CCl₄) and no ebselen); N, control not treated with CCl₄ or ebselen; E, treated with ebselen only; EC, treated with CCl₄ and then ebselen.

drogenase (G3PDH) mRNA in the same tissue sample. All PCR reactions were carried out in the linear, quantitative phase of the amplification process. An excess of dNTPs (200 µmol/L each dNTP), PCR primers (100 pmol) and thermostable DNA polymerase (2 units per 20 µL volume) was used to ensure that these were not limiting factors in the PCR analysis.

The identities of all the PCR products were verified by sequence analysis. The PCR products were size-fractionated on agarose gels, visualized by using ethidium bromide staining and photographed. The photographs were scanned with a GS-700 imaging densitometer (Bio-Rad, Hercules, CA, USA), and the density of the bands was quantified by using Molecular Analyst software (Bio-Rad, Hercules, CA, USA). In order to compare mRNA levels between different animals, the transcripts were normalized against the corresponding PCR condition-matched control G3PDH transcript.

Statistical analysis

Statistical analysis of the mean values were carried out by using SPSS software (SPSS Inc., Chicago, IL, USA). When the data were normally distributed, the parametric Scheffé test with a significance level of 0.05 was carried out. When the data were not normally distributed, the Mann-Whitney *U* Wilcoxon rank sum *W* tests were performed. *P* values less than or equal to 0.05 were considered significant.

RESULTS

Weight gains and liver weights

The general condition of the rats was monitored every day, and the bodyweights of individual rats were recorded twice weekly. At the time of the killings, both the body- and liver weights of all the rats were recorded (Table 2). There was no significant difference in the percentage weight gains and liver weights of both groups

of rats treated with CCl_4 (groups C and EC). However, both these groups had significantly lower weight gains and liver weights compared to non- CCl_4 -treated rats (groups N and E).

Ebselen improved liver histology

A primary consideration in assessing the effectiveness of a potential therapeutic agent in the resolution of hepatic fibrosis is its effect on liver histology. Liver specimens from the normal untreated group N were non-fibrotic (Fig. 1a). Treatment with CCl_4 caused deposition of ECM components (Fig. 1b). The treatment group EC (Fig. 1c) showed a marked improvement in liver histology, as seen by the reduction in the amount of collagen fibers detected. Treatment with ebselen alone resulted in normal liver histology (results not shown), indicating that long-term treatment with ebselen had no deleterious effect on the liver.

Ebselen reduced transforming growth factor- β 1

The effect of treatment with ebselen on the transcription of various factors associated with hepatic fibrosis was assessed by using RT-PCR, normalized to that of the constitutively expressed housekeeping gene, G3PDH, in the same sample.

Amplification of fragments specific to TGF- β 1 and G3PDH yielded fragments of 162 and 209 bp, respectively (Fig. 2a). The mean TGF- β 1 : G3PDH ratio of the disease control group C was 46% higher than that of the untreated normal control group N. Treatment with ebselen resulted in a marked reduction of the TGF- β 1 transcript, with rats in group EC having a 41% lower TGF- β 1 : G3PDH ratio than those in group C. Treatment with ebselen alone (group E) did not change the level of expression of the TGF- β 1 transcript.

Ebselen reduced procollagen I and III

Amplification of procollagen I and III yielded fragments of 319 (pC I, Fig. 3a) and 514 bp (pC III, Fig. 3b), respectively. Treatment with CCl_4 in group C resulted in a 68% increase in the level of expression of mean procollagen I : G3PDH compared to group N (Fig. 3c). There was a reduction of the mean procollagen I : G3PDH mRNA ratio by 35% in group EC compared with group C. Treatment with ebselen alone (group E) did not cause significant change in the procollagen I transcript expression compared with group N livers.

The disease control group C had a 55% higher procollagen III : G3PDH ratio than group N (Fig. 3d). Treatment with ebselen significantly decreased the procollagen III : G3PDH ratio by 40% (group EC), compared with group C. Treatment with ebselen alone (group E) did not cause significant change in procollagen III mRNA expression, as compared with group N.

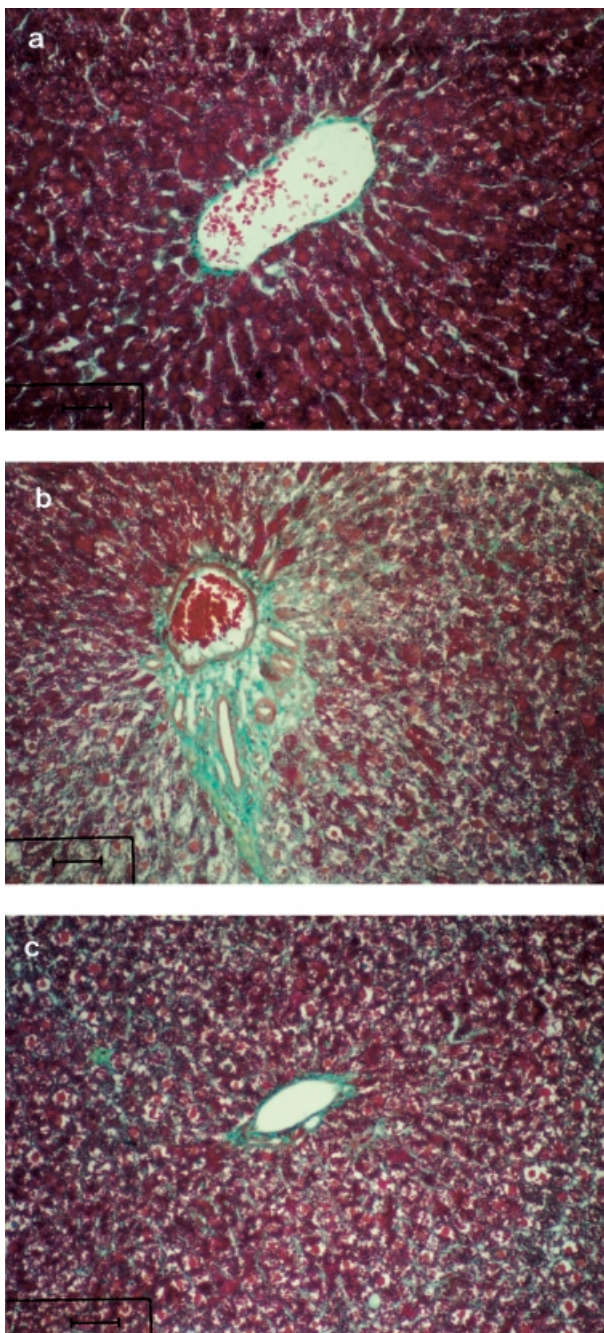


Figure 1 Treatment with ebselen improved liver histology. Hepatic fibrosis is revealed by Masson trichrome staining. Collagen fibers in the extracellular matrix (ECM) are stained blue-green while the cytoplasm is stained red. Representative liver sections from experimental groups N, C and EC. Group N, normal rat liver (untreated control); group C, liver section showing marked hepatic fibrosis; group EC, note that there is less fibrosis than in group C (Scale bar = 40 μm). C, control (treated with carbon tetrachloride (CCl_4) and no ebselen; N, control not treated with CCl_4 or ebselen; EC, treated with CCl_4 and then ebselen. (a) Group N, (b) group C, (c) group EC.

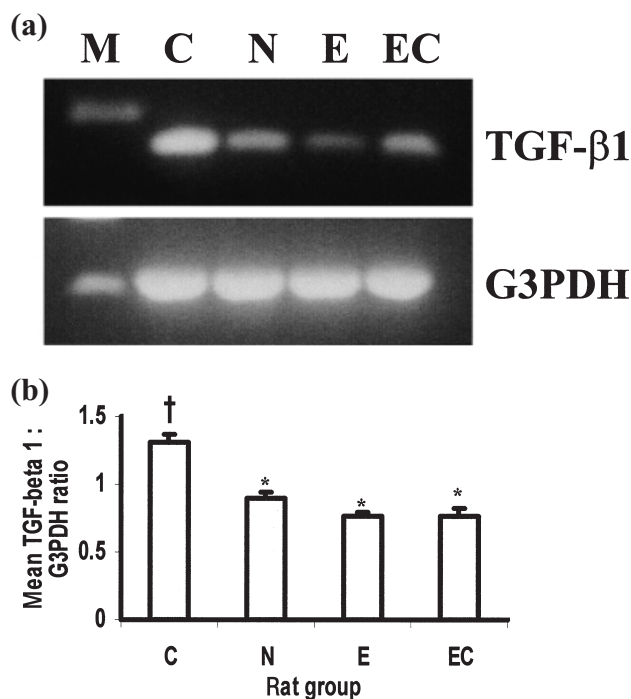


Figure 2 Treatment with ebselen decreased the transforming growth factor (TGF)-β1 mRNA. (a) Amplification of TGF-β1 (162 bp) or glyceraldehyde-3-phosphate dehydrogenase (G3PDH, 209 bp) in representatives from each treatment group. The fragments of the 100 bp molecular weight ladder (M) are indicated. (b) The density of TGF-β1 to G3PDH mRNA was expressed as the TGF-β1 : G3PDH ratio. The error bar indicates SE. The mean TGF-β1 : G3PDH ratios for groups C, N, E and EC (±SE) were 1.31 ± 0.05 , 0.90 ± 0.05 , 0.77 ± 0.03 and 0.77 ± 0.05 , respectively. The number of rats were: $n=6$ (Group C), 10 (Groups N and E) and nine (Group EC). *Statistically significant differences between a group and the disease control group C ($P<0.05$), †represent significant difference between a group and the untreated normal control group N ($P<0.05$). The Scheffé test was used for statistical analysis. C, control (treated with carbon tetrachloride (CCl₄) and no ebselen; N, control not treated with CCl₄ or ebselen; E, treated with ebselen only; EC, treated with CCl₄ and then ebselen.

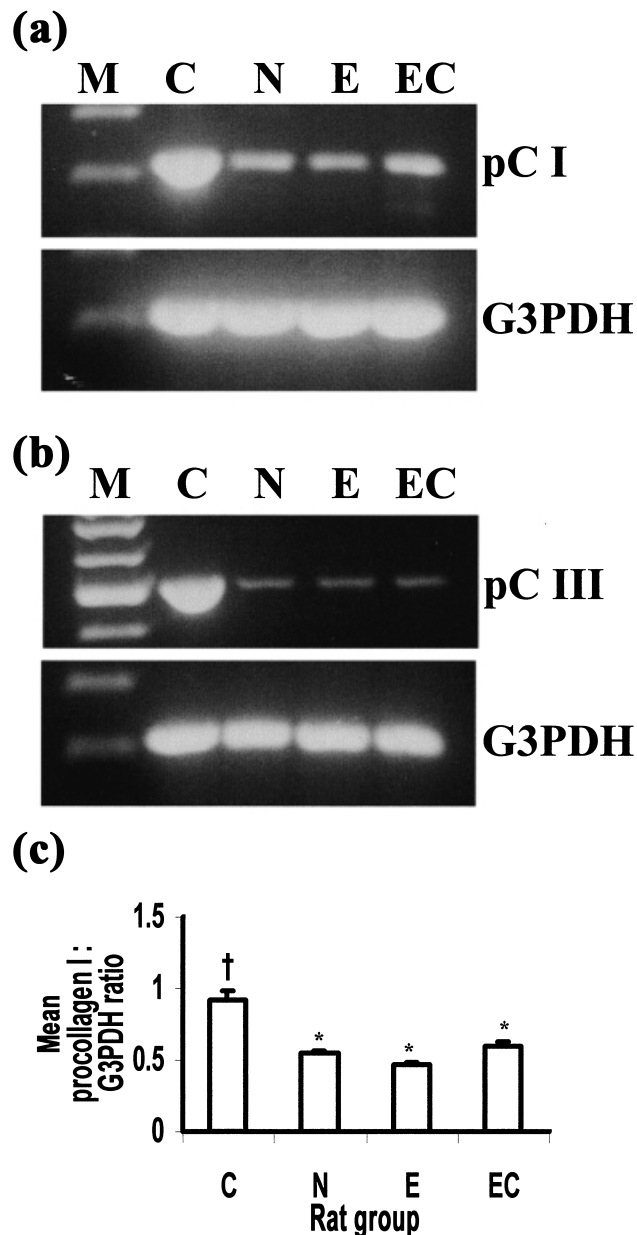
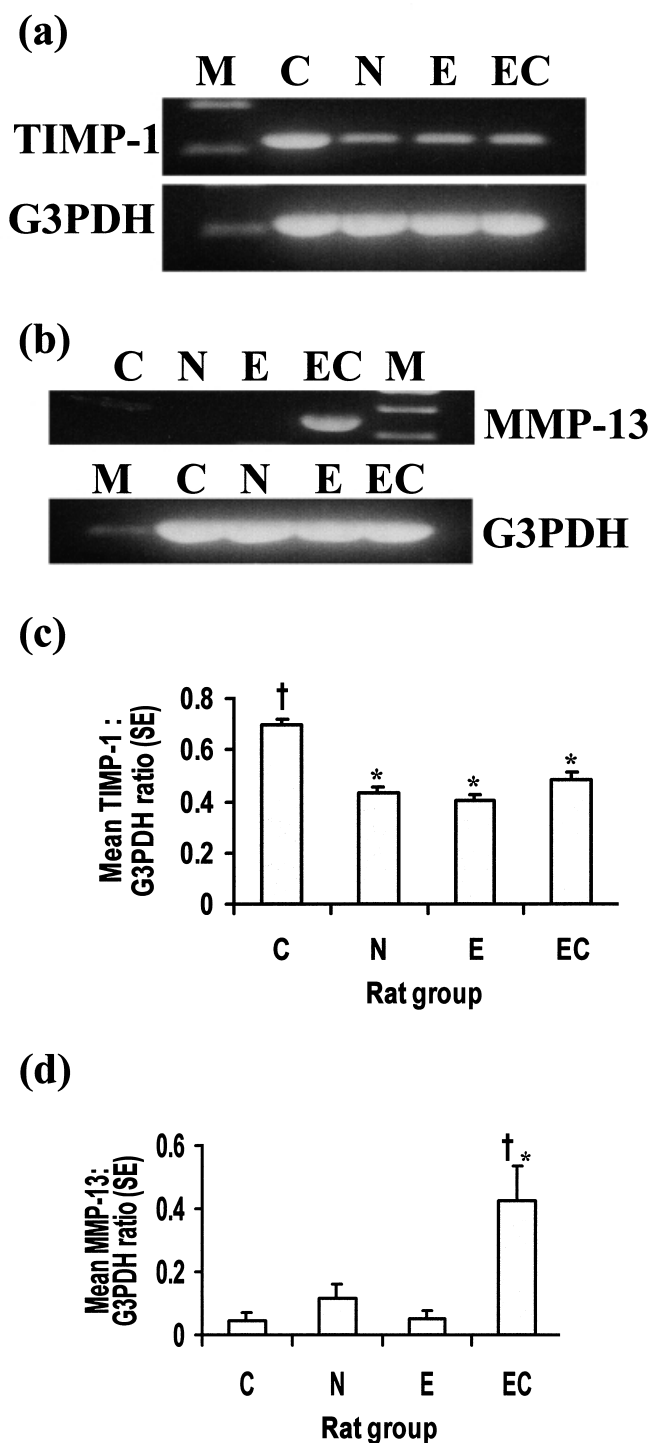


Figure 3 Treatment with ebselen decreased procollagen I and III transcripts. Analysis of (a) procollagen I (pC I; 319 bp) and (b) procollagen III (pC III; 514 bp) transcripts by using RT-PCR. The expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) is shown for comparison. The results of the densitometric analyses of (c) pC I and (d) pC III are shown. The mean ratios of procollagen I : G3PDH (±SE) for groups C, N, E and EC were 0.91 ± 0.07 , 0.54 ± 0.02 , 0.46 ± 0.03 and 0.59 ± 0.03 , respectively. The mean ratios of procollagen III : G3PDH (±SE) for the same groups were 0.93 ± 0.02 , 0.60 ± 0.04 , 0.50 ± 0.04 and 0.56 ± 0.04 , respectively. The Scheffé test was used for statistical analysis. * $P<0.05$, † $P<0.05$. CYP2E1, cytochrome P450; GST-P, placental form of glutathione-S-transferase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; pC I, procollagen I; pC III, procollagen III; C, control (treated with carbon tetrachloride (CCl₄) and no ebselen; N, control not treated with CCl₄ or ebselen; E, treated with ebselen only; EC, treated with CCl₄ and then ebselen; M, 100 bp molecular weight ladder.

Ebselen reduced tissue inhibitor of metalloproteinase-1 and increased matrix metalloproteinase-13

In the liver, collagen levels are modulated by enzymes that degrade it, namely matrix metalloproteinases (MMP), and enzymes that inhibit their action, namely tissue inhibitors of metalloproteinases (TIMPs). Fragments specific to the transcripts encoding TIMP-1



(218 bp, Fig. 4a) and MMP-13 (365 bp, Fig. 4b) were amplified by using RT-PCR.

Treatment with CCl₄ in group C resulted in a 60% increase in the level of expression of the mean TIMP-1 : G3PDH ratio compared with group N (Fig. 4c). Treatment with ebselen caused a reduction of the mean TIMP-1 : G3PDH ratio by 43.5% in group EC compared with group C. Treatment with ebselen alone (group E) did not change the level of expression of the TIMP-1 transcript.

The levels of expression of the MMP-13 transcript in groups C, E and N were very low, with several rats in each of these groups having no detectable liver MMP-13 transcripts (Fig. 4b,d). Treatment with ebselen after the cessation of the CCl₄ treatment regimen, however, caused a 740% increase in the mean MMP-13 : G3PDH ratio in group EC compared with group C (Fig. 4d). This level was 268% higher than that in the normal liver (group N).

Ebselen decreased cytochrome P450E1 and glutathione-S-transferase P

The induction of CYP2E1 in liver tissue is associated with an increase of CYP2E1 mRNA.⁵² We determined the effect of ebselen treatment on oxidative stress by assessing the level of expression of CYP2E1 by using RT-PCR. Amplification of CYP2E1 yielded a fragment of 346 bp (CYP2E1, Fig. 5a). Treatment with CCl₄ in group C resulted in a 30% increase in the CYP2E1 : G3PDH ratio (Fig. 5b) compared with group N. Ebselen treatment resulted in a reduction of CYP2E1 : G3PDH ratio by 25% (group EC) compared with group C. Treatment with ebselen alone (group E) did not cause significant change in the CYP2E1 transcript expression compared with the group N.

We have previously shown a correlation between the expression of GST-P mRNA and protein levels in pre-neoplastic liver foci during hepatocarcinogenesis.⁴³ In addition, it is known that long-term liver fibrosis increases the risk for hepatocellular carcinoma. We

Figure 4 Treatment with ebselen decreased tissue inhibitor of metalloproteinase-1 (TIMP-1) transcript and increased matrix metalloproteinase (MMP)-13 transcript. Fragments corresponding to (a) TIMP1 (218 bp), (b) MMP-13 (365 bp) and G3PDH from representatives of each group of rats and histograms of the ratios of (c) TIMP-1 or (d) MMP-13 to G3PDH (\pm SE). The mean ratios of TIMP-1 : G3PDH for groups C, N, E and EC were 0.69 ± 0.02 , 0.43 ± 0.02 , 0.40 ± 0.02 and 0.48 ± 0.03 , respectively. The values for MMP-13 : G3PDH for the same groups were 0.05 ± 0.02 , 0.11 ± 0.04 , 0.05 ± 0.03 and 0.42 ± 0.12 , respectively. Statistical analysis was performed by using the Scheffé test (TIMP-1) or the Mann-Whitney *U*-test (MMP-13). * $P < 0.05$, † $P < 0.05$. G3PDH, glyceraldehyde-3-phosphate dehydrogenase; C, control (treated with carbon tetrachloride (CCl₄) and no ebselen; N, control not treated with CCl₄ or ebselen; E, treated with ebselen only; EC, treated with CCl₄ and then ebselen; M, 100 bp molecular weight ladder.

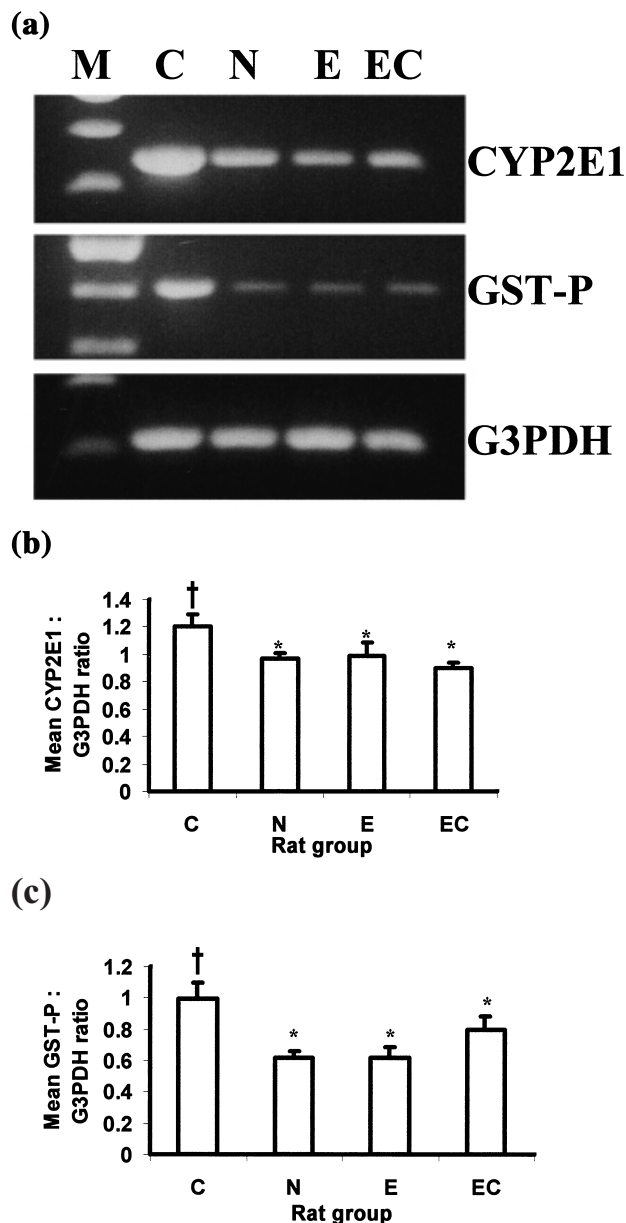


Figure 5 Treatment with ebselen decreased cytochrome P450 (CYP2E1) and glutathione-S-transferase (GST-P) transcripts. Fragments corresponding to (a) CYP2E1 (346 bp), GST-P (409 bp) and G3PDH from representatives of each group of rats and histograms of the ratios of (b) CYP2E1 or (c) GST-P to G3PDH (\pm SE). The mean ratios of CYP2E1:G3PDH for groups C, N, E and EC were 1.20 ± 0.09 , 0.96 ± 0.04 , 0.99 ± 0.09 and 0.9 ± 0.04 , respectively. The values for GST-P:G3PDH for the same groups were 0.99 ± 0.1 , 0.61 ± 0.04 , 0.62 ± 0.06 and 0.79 ± 0.09 , respectively. Statistical analysis was performed by using the Mann-Whitney *U*-test. G3PDH, glyceraldehyde-3-phosphate dehydrogenase; pCI, procollagen I; pCIII, procollagen III; CYP2E1, cytochrome P450; GST-P, placental form of glutathione-S-transferase; C, control (treated with carbon tetrachloride (CCl_4) and no ebselen; N, control not treated with CCl_4 or ebselen; E, treated with ebselen only; EC, treated with CCl_4 and then ebselen; M, 100 bp molecular weight ladder.

therefore determined the effect of ebselen on CCl_4 -induced micronodule formation by assessing GST-P mRNA levels by using RT-PCR. Amplification of GST-P gave rise to a 409-bp fragment (GST-P, Fig. 5a). The disease control group C had a 62% higher GST-P:G3PDH ratio than group N (Fig. 5c). Treatment with ebselen significantly decreased the level of expression of GST-P:G3PDH by 20% (group EC), compared with group C. Treatment with ebselen alone (group E) did not cause significant change in GST-P mRNA expression compared with group N.

DISCUSSION

The CCl_4 -treated rat is frequently used in studies of hepatic fibrosis.^{22,23,29} Carbon tetrachloride is activated by oxidases in the liver to yield the trichloromethyl ($\text{CCl}_3\cdot$) free radical, which either extracts a hydrogen atom from unsaturated membrane lipids to initiate lipid peroxidation or reacts with the sulfhydryl proteins. The hepatotoxicity of CCl_4 is potentiated by chemicals that induce cytochrome P450 isozymes, such as phenobarbitone.⁵³ As the anti-oxidant activity of ebselen was known to be mediated by its ability to inhibit lipid peroxidation,^{37-40,42} we decided to investigate the role of this seleno-organic compound in CCl_4 -induced hepatic fibrosis in the Fischer 344 rat.

In the present study, we have examined: (i) body- and liver weights; (ii) liver sections and assayed the levels of expression of various paradigms involved in (iii) hepatic fibrosis (TGF- β 1, procollagen I, procollagen III, TIMP-1 and MMP-13); (iv) oxidative stress (CYP2E1); and (v) the induction of preneoplastic foci (GST-P). All data consistently demonstrate that ebselen effectively causes the resolution of CCl_4 -induced hepatic fibrosis and liver damage.

Treatment with ebselen resulted in a marked improvement in the histology of the CCl_4 -treated rat liver (Fig. 1). One of the strategies in the development of antifibrotic drugs is the search for effective inhibitors of TGF- β 1 activity (as reviewed in³⁵). Treatment with ebselen significantly reduced the steady-state level of TGF- β 1 mRNA (Fig. 2), indicating that ebselen could resolve CCl_4 -induced hepatic fibrosis.

The measurement of serum procollagen I and III levels is routinely used in the assessment of hepatic fibrosis,^{22,54,55} although there are limitations. The presence of procollagen I in serum is transient and not easily detectable.^{22,56} Conversely, serum procollagen III is persistent and its detection would not distinguish between pre-existing, degraded, and new collagen III levels. Consequently, serum collagen III levels may not be indicative of liver collagen III levels.^{22,49} In the present study, we have investigated the effect of ebselen on liver collagen I and III levels by using RT-PCR of the corresponding transcripts, a technique which gives an indication of the effect of treatment with ebselen on steady-state levels of procollagen I and III mRNA. The low level of procollagen I mRNA in normal liver is increased during hepatic fibrosis.^{3,5,15,55-60} Procollagen III, the most abundant collagen in normal liver,⁵⁸ is also induced in hepatic fibrosis, although to a lesser extent

than procollagen I.^{15,55,57,60} Treatment with ebselen reduced the levels of expression of procollagen I and III transcripts to levels comparable to normal, non-CCl₄-treated rat livers (Fig. 3).

The net deposition of interstitial collagen in chronic hepatic fibrosis occurs not only as a result of an increase in fibrillar collagen synthesis, but also as a result of a decrease in the degradation of these fibers by collagenases. As with previously published work, we have observed elevated levels of TIMP-1^{11,17,19,51} and decreased expression of MMP^{11,17,51,61,62} upon treatment with CCl₄. The disruption of the balance between TIMP-1 and MMP promotes accumulation of fibrillar collagens and causes development of hepatic fibrosis.¹⁹ Treatment with ebselen was effective both in decreasing the level of TIMP-1 and in markedly increasing the level of MMP-13 in group EC (Fig. 4), thereby contributing to the resolution of hepatic fibrosis in this group of rats.

Cytochrome P450 (CYP2E1) is involved in the pathogenesis of alcoholic liver disease by enhancing the production of reactive oxygen intermediates, release of free radicals and lipid peroxidation (as reviewed in⁶³). The resultant oxidative stress caused by the induction of CYP2E1 production consequent to CCl₄ treatment was effectively reduced upon treatment by the anti-oxidant ebselen (Fig. 5b). The inhibition of CYP2E1-mediated oxidative stress by ebselen probably contributed to the decrease in procollagen expression, as previously observed in rat HSC.²⁶

The 10-week treatment of rats using CCl₄ induces liver fibrosis and micronodular cirrhosis. One of the early markers of hepatocarcinogenesis, GST-P,^{30,31} is expressed in preneoplastic liver foci in aflatoxin B₁-treated Fischer rats.⁴³ Treatment with CCl₄ increased the steady-state levels of GST-P mRNA in the liver (Fig. 5c). This CCl₄-induced GST-P expression was effectively resolved by treatment with the anti-oxidant ebselen.

Wendel and Tiegs have previously reported that oral pretreatment with 600 mg/kg ebselen was not effective in the protection against acute CCl₄ intoxication in mice when CCl₄ was given in a single, large dose of 3.2 g/kg bodyweight.⁴⁴ The authors, however, conceded that their findings did not exclude the beneficial effects of ebselen in chronic disease states, such as the one we have used in the present study.

Lipid hydroperoxides are normally minimized by selenium-containing enzymes, mainly glutathione peroxidase.⁶⁴ Ebselen has been shown to have glutathione peroxidase-like activity *in vitro*,³⁶ and it also inhibits lipid peroxidation.^{39,40,42} The results, as demonstrated here, were therefore not surprising.

In conclusion, our data provide compelling evidence that ebselen significantly causes the resolution of CCl₄-induced hepatic fibrosis.

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