British Journal of Nutrition (2016), **115**, 1978–1986
doi:10.1017/S0007114516001197

Decreased hepatic iron in response to alcohol may contribute to alcohol-induced suppression of hepcidin

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(Submitted 13 November 2015 – Final revision received 19 February 2016 – Accepted 3 March 2016 – First published online 15 April 2016)

Abstract

Hepatic Fe overload has often been reported in patients with advanced alcoholic liver disease. However, it is not known clearly whether it is the effect of alcohol that is responsible for such overload. To address this lacuna, a time-course study was carried out in mice in order to determine the effect of alcohol on Fe homoeostasis. Male Swiss albino mice were pair-fed Lieber–DeCarli alcohol diet (20 % of total energy provided as alcohol) for 2, 4, 8 or 12 weeks. Expression levels of duodenal and hepatic Fe-related proteins were determined by quantitative PCR and Western blotting, as were Fe levels and parameters of oxidative stress in the liver. Alcohol induced cytochrome P4502E1 and oxidative stress in the liver. Hepatic Fe levels and ferritin protein expression dropped to significantly lower levels after 12 weeks of alcohol feeding, with no significant effects at earlier time points. This was associated, at 12 weeks, with significantly decreased liver hepcidin expression and serum hepcidin levels. Protein expressions of duodenal ferroportin (at 8 and 12 weeks) and divalent metal transporter 1 (at 8 weeks) were increased. Serum Fe levels rose progressively to significantly higher levels at 12 weeks. Histopathological examination of the liver showed mild steatosis, but no stainable Fe in mice fed alcohol for up to 12 weeks. In summary, alcohol ingestion by mice in this study affected several Fe-related parameters, but produced no hepatic Fe accumulation. On the contrary, alcohol-induced decreases in hepatic Fe levels were seen and may contribute to alcohol-induced suppression of hepcidin.

Key words: Alcoholic liver disease: Iron: Hepcidin

Increased hepatic Fe levels have been reported in patients with alcoholic liver disease (ALD)[1,2] and have been suggested to exacerbate alcohol-induced liver injury[3] and hepatic fibrosis[4]. Transferrin saturation and serum ferritin levels have also been reported to be higher among those who consume >2 alcoholic drinks/d[5]. Several publications are commonly cited to support statements of the association between ALD and hepatic Fe accumulation[1,2,6–9]. However, an in-depth examination of these studies reveals that this association is not invariable. In fact, among these studies, only those by Jakobovits *et al.*[1], Chapman *et al.*[2] and Lundvall *et al.*[6] have determined (and showed data on) hepatic Fe levels. The presence of stainable Fe in liver biopsy samples in these studies varied widely; it was about 57 % in the study by Jakobovits *et al.*[1] and about 29 % in the study by Chapman *et al.*[2], whereas Lundvall *et al.*[6] concluded that there were no increased hepatic Fe stores in their series of patients with ALD. Of the other commonly cited articles, Bell *et al.*[7] reported high serum ferritin and transferrin saturation levels in patients with ALD, whereas Duane *et al.*[8] reported increased (*in vitro*) Fe uptake in duodenal biopsies from patients with ALD. Kohgo *et al.*[9] showed increased transferrin receptor 1 (TfR1) expression in liver tissue from patients with ALD. All of these observations in patients with ALD have indicated Fe overload, but these studies do not show direct evidence of increased hepatic Fe levels. Nonetheless, these publications are commonly cited to support statements that hepatic Fe overload is a feature of ALD.

Hepatic Fe deposition is often seen in the late stages of chronic liver disease, probably due to decreases in hepcidin, which is seen as a consequence of liver damage, thereby eventually leading to Fe overload[10–12]. However, there is no clear evidence from human studies, and very little from studies on rodent models, showing that alcohol *per se* causes liver Fe overload. Our study was designed to address this lacuna in the literature by systematically studying liver Fe levels (and other related events involved in Fe homoeostasis) in a mouse model of chronic alcohol

Abbreviations: ALD, alcoholic liver disease; ALT, alanine transaminase; BMP6, bone morphogenetic protein 6; CYP2E1, cytochrome P2E1; HO-1, haeme oxygenase 1; Tfr1, transferrin receptor 1.

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ingestion, based on the Lieber–DeCarli diet. Such a model has been held to achieve alcohol consumption that is clinically relevant, controls for nutritional status and has provided considerable information in studies on the effects of alcohol on various metabolic events in the liver during early alcoholic liver disease[13,14]. In addition, we studied the effect of alcohol on haeme oxygenase 1 (HO-1) in the liver to ascertain whether induction of the activity of this enzyme (on haeme, thereby releasing Fe) contributed to changes in Fe levels, and also as a marker of oxidative stress.

Methods

Animal maintenance and treatment

All procedures performed on animals were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India. Male Swiss albino mice, weighing 28–30 g, were individually housed under standard conditions in the institutional animal facility. They were fed the Lieber–DeCarli liquid alcohol diet (diet no. 710260; Dyets Inc.), providing 20 % of total energy in the form of ethanol. Control mice were pair-fed the Lieber–DeCarli control diet (diet no. 710027; Dyets Inc.), which provided an equivalent amount of energy as the alcohol diet. The Fe content of the control and alcohol diets was 8·7 mg of elemental Fe/l of the diet, corresponding to approximately 35 mg of Fe/kg chow diet, which is considered 'Fe adequate'[15,16]. Mice were fed the diets for 2, 4, 8 or 12 weeks and then killed. Blood samples were used for the estimation of serum alanine transaminase (ALT), hepcidin and Fe levels. Liver and duodenal tissue were frozen immediately in liquid N_2 and stored at −70°C until they were processed further. Quality assurance/quality control/assay performance data for all assays carried out are given in the online Supplementary File 2.

Quantitative real-time PCR

RNA was isolated from liver and duodenal samples using TRI reagent (Sigma) according to the manufacturer's instructions. Integrity of the isolated RNA was confirmed by agarose gel electrophoresis. Isolated RNA (1 μg) was used to synthesise complementary DNA. Quantitative PCR (qPCR) assays (SYBR Green qPCR master mix (no ROX); Eurogentec) were carried out in duplicate, using the BioRad Chromo 4 real-time PCR system (Bio-Rad Laboratories). Expression levels of hepcidin, ferritin (light chain), ferritin (heavy chain), transferrin receptor 1 and 2, bone morphogenetic protein 6 (BMP6) and HO-1 were normalised to that of ribosomal protein L19 (RPL19), which was used as the reference gene. Sequences of all primers used are listed in the online Supplementary File 1, Table S1. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) checklist and qPCR validation data are provided in the online Supplementary File 2, Tables S1 and S2.

Western blotting

Liver and duodenal samples were homogenised in radioimmunoprecipitation assay (RIPA) buffer (Tris-HCl 10 mM, NaCl 140 mM, EDTA 1·5 mM, sodium deoxycholate 0·1 %, Triton-X 1 %, SDS 0·1 %, pH 8·0), containing a cocktail of protease inhibitors (Sigma). Samples were centrifuged at 14 000 ***g*** for 20 min. Protein content of the supernatant was estimated using the Pierce bicinchoninic acid (BCA) protein assay kit, and 50 μg protein was separated by SDS-PAGE using 10 % gels. For cytochrome P2E1 (CYP2E1) and HO-1 blots, hepatic microsomes were used, isolated as described previously[17]. For TfR1, protein isolated from TRI-reagent extracts was used. Separated proteins were transferred onto polyvinylidene fluoride membranes (Millipore) at 80 V for 2 h and probed with specific antibodies (listed in the online Supplementary File 1, Table S2). Bands were detected using an enhanced chemiluminescence system (SuperSignal West Dura Extended Duration Substrate kit; Thermo Scientific). Band intensities were normalised to that of β-actin, which was used as a loading control.

Estimation of cytochrome P2E1 and haeme oxygenase activity

The activity of CYP2E1 was estimated in liver microsomal preparations by measuring the hydroxylation of *p*-nitrophenol to *p*-nitrocatechol[18]. In brief, 500 μg of microsomal protein was incubated with 0·2 mM *p*-nitrophenol and 0·6 mM NADPH for 30 min at 37°C. The reaction was stopped by precipitation of protein by adding 0·6 N perchloric acid. Following centrifugation, 0·1 ml of 10 N NaOH was added to 0·9 ml of the supernatant and the absorbance was measured at 546 nm using a UV/visible spectrophotometer.

HO activity was measured by a spectrophotometric method[17]. In brief, the reaction mixture consisted of 500 μg microsomal protein, 1 mg rat liver cytosol (as a source of biliverdin reductase), 25 μM haemin and 1 mM NADPH in 0·1 M $MgCl_2$ phosphate buffer (pH 7·4). The samples were incubated in a 37°C water bath in the dark for 1 h. The reaction was stopped by placing the tubes on ice. Bilirubin formed was extracted with 1 ml chloroform. The amount of bilirubin formed was calculated from the difference in absorbance at 454 and 530 nm using an extinction coefficient of $5{\cdot}63 \times 10^4\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$. HO activity was expressed as pmol of bilirubin formed per mg of protein per h.

Measurement of glutathione, thiobarbituric acid reactive substances and alanine transaminase

Levels of thiobarbituric acid reactive substances (TBARS), a surrogate marker of lipid peroxidation, were estimated in liver homogenates using a spectrophotometric assay as described previously using 1,1,3,3 tetramethoxypropane as the standard[19]. In brief, thiobarbituric acid reacts with malondialdehyde and related products of lipid peroxidation in an acidic medium to yield a pink colour, which is measured at 532 nm.

Levels of glutathione in liver tissue were estimated using a commercially available kit (Cayman Chemicals no. 703002; Cayman Chemical Company). The assay is based on the reaction between the sulfhydryl group of GSH and 5,5′-dithio-bis-2-nitrobenzoic acid (Ellman's reagent) to yield yellow-coloured 5-thio-2-nitrobenzoic acid (TNB) and a mixed disulphide, GSTNB (between GSH and TNB). Glutathione reductase reduces GSTNB and GSSG to GSH in order to produce more

TNB. Measurement of the absorbance of TNB at 405 nm provides an estimate of total glutathione in the sample. The assay was modified to measure GSSG by first derivatizing the sample with 2-vinylpyridine.

Serum ALT activity was measured in the mice as a marker of liver damage using a commercially available kit (Cayman Chemicals no. 700260; Cayman Chemical Company). ALT in the sample converts alanine to pyruvate. The rate of formation of pyruvate, which is proportional to ALT activity in the sample, is measured using a coupled reaction system using lactate dehydrogenase and NADH. The rate of oxidation of NADH to NAD+ is measured as a decrease in absorbance at 340 nm and used for the calculation of ALT activity.

Estimation of serum hepcidin

Serum hepcidin levels were measured by a competitive enzyme immunoassay using the Bachem ELISA kit (Peninsula Laboratories). Serum samples were diluted 1:250 in the diluent provided by the manufacturer before analysis.

Estimation of liver and serum iron content

Fe content was measured by a spectrophotometric assay, as described previously[20]. Liver tissue was dried in a hot air oven at 48°C for 48 h, weighed and digested in acid reagent (3 M hydrochloric acid and 10 % TCA) (10 mg of dried liver tissue/100 µl acid reagent) at 65°C for 24 h. A sample of 10 µl of acid extract (diluted 1:10 in 0·1 N-HCl) was mixed with 0·2 ml of chromogen reagent (4 M sodium acetate, 0·01 % bathophenanthroline disulphonic acid, 0·1 % thioglycollic acid) and incubated for 15 min. The absorbance of the reaction mixture was measured at 540 nm, using a microplate reader, and was compared with a set of Fe standards treated identically. For serum measurements, 20 µl of serum was incubated with 20 µl of acid reagent and digested at 65°C for 24 h. Digested samples were centrifuged at 12 000 ***g*** for 10 min; 10 µl of the supernatant was mixed with 0·1 ml chromagen reagent and incubated for 15 min. Absorbance was measured at 540 nm as described earlier.

Light microscopy and Prussian blue staining

Liver tissue was fixed in 10 % buffered formalin for histopathological examination. The samples were embedded in paraffin and sectioned. Tissue sections were stained with haematoxylin–eosin for light microscopy. Perl's Prussian blue stain was used to detect Fe in sections[21], along with a positive control.

Statistical analysis

Unless stated otherwise, data are presented as average fold changes in alcohol-fed mice, compared with pair-fed control mice. The Kolmogorov–Smirnov and Shapiro–Wilk tests for normality of data distribution showed that the data were not normally distributed. Therefore, non-parametric tests were used for statistical analysis. The Kruskal–Wallis test was used to detect statistically significant changes occurring in the different groups of mice. The Mann–Whitney test was used for all pairwise comparisons. Statistical analyses were carried out using Statistical Package for Social Scientists (SPSS), version 16.0.

Results

Average daily alcohol consumption and body weight changes in mice

The average daily alcohol consumption by the mice in the study ranged between 10 and 12 g/kg body weight per day; these amounts were not significantly different in the time periods up to 8 weeks of consumption. However, there was a small, but significant, decrease in consumption of alcohol in mice fed for 12 weeks, compared with those fed for 8 weeks (Fig. 1(a)). There were no significant differences in weights of pair-fed mice (data not shown).

Alcohol-induced effects on cytochrome P2E1 activity and protein levels in the liver

Microsomal fractions isolated from the livers of mice fed alcohol showed an increase in CYP2E1 activity compared with control

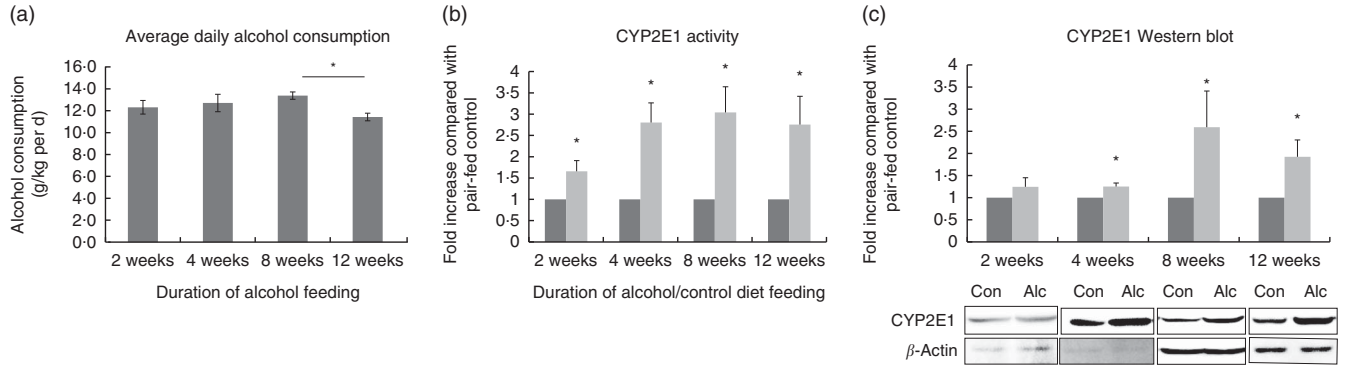

Fig. 1. Average daily alcohol consumption and effect of alcohol on cytochrome P2E1 (CYP2E1). (a) Average alcohol consumption (grams of alcohol consumed per day per kg body weight) by mice on the Lieber–DeCarli diet for various periods (n 6–10). (b) CYP2E1 activity in liver microsomes isolated from control and alcohol-fed mice (n 3–6). (c) Representative images for CYP2E1 Western blots for pair-fed animals at each time point (2, 4, 8 and 12 weeks), with densitometric quantification of bands obtained (n 3–6). * Mean value was significantly different compared with that in corresponding pair-fed control animals at the corresponding time point ($P < 0·05$). Values are means, with standard errors represented by vertical bars. ■, Control (Con); □, alcohol (Alc).

mice. Significant increases were seen from 2 weeks of alcohol feeding up to 12 weeks (Fig. 1(b)). CYP2E1 proteins levels were also increased in response to alcohol, with significant increases seen from 4 weeks onwards (Fig. 1(c)). These observations show that alcohol was metabolised in the liver in these mice.

Alcohol-induced effects on iron content and iron-related proteins in the liver

The Fe content of the liver tended to increase after 4 weeks of alcohol consumption. This increase was, however, not statistically significant when compared with levels in pair-fed controls. Subsequently, liver Fe levels in alcohol-fed mice dropped and became significantly lower than levels in control mice after 12 weeks of alcohol consumption (Fig. 2(a)). Similar changes were seen in ferritin (L) and TfR1 protein levels in the liver (Fig. 2(b) and (c)). TfR1 mRNA levels were significantly increased at 4 and 8 weeks, but returned to control levels at 12 weeks (Fig. 2(d)). No consistent changes were seen in the protein levels of liver divalent metal transporter 1 (DMT1) (online Supplementary Fig. S1(a)). Levels of ferroportin in the liver tended to be higher at 4 and 12 weeks, but not significantly (online Supplementary Fig. S1(b)). Hepatic transferrin receptor 2 (TfR2) protein levels were significantly decreased after 4 and 8 weeks of alcohol ingestion and returned to control levels at 12 weeks (online Supplementary Fig. S1(d)). TfR2 mRNA levels showed similar trends (online Supplementary Fig. S1(c)). BMP6 mRNA levels, which are known to be regulated by hepatic Fe content, showed no significant changes in response to alcohol (data not shown).

Changes in hepatic hepcidin expression and serum levels of hepcidin and iron

Liver hepcidin expression (Fig. 3(a)) dropped progressively over the study period to become significantly lower at 12 weeks; serum hepcidin levels were also found to be significantly lower after 12 weeks of alcohol feeding (Fig. 3(b)). Correlation analysis showed a significant positive correlation between hepatic hepcidin expression and serum hepcidin levels (Spearman's correlation coefficient: 0·540; *P*-value: 0·009). A progressive increase in serum Fe levels was seen over time, with levels becoming significantly higher at 12 weeks (Fig. 3(c)).

Alcohol-induced effects on iron-related proteins in the duodenum

Protein expression of duodenal DMT1 was found to be significantly increased at 8 weeks (Fig. 4(a)) and that of ferroportin at 8 and 12 weeks (Fig. 4(b)). No consistent effect was seen on the expression of duodenal ferritin (H) (Fig. 4(c)).

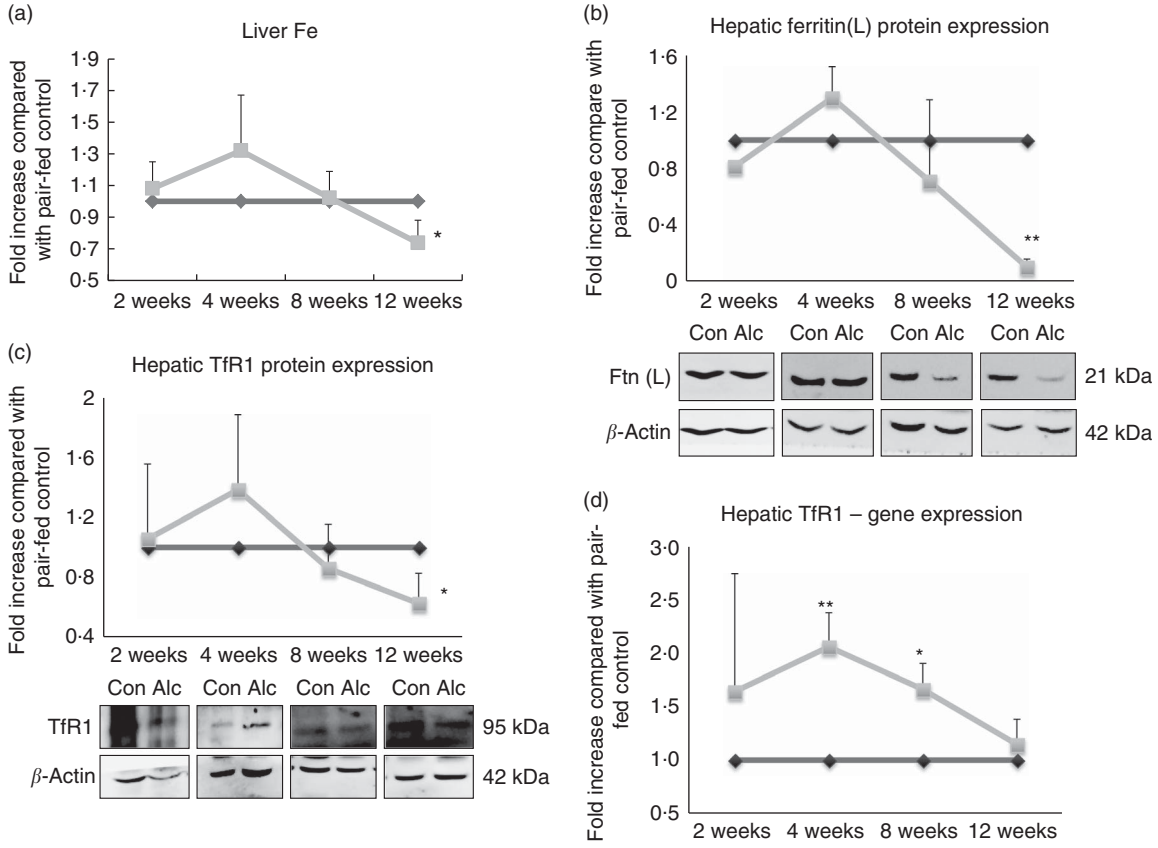

Fig. 2. Effect of alcohol on iron-related parameters in the liver. (a) Liver iron content (*n* 6–9). (b and c) Hepatic ferritin (light chain) (b) and transferrin receptor 1 (TfR1) (c) protein levels by Western blotting. Representative blots with densitometric quantification of the blots are shown (*n* 3 in each case). (d) Gene expression of TfR1 by quantitative PCR (qPCR) (*n* 3–6). Expression levels were normalised to that of β-actin. Values are means, with standard errors represented by vertical bars. Mean value was significantly different from that for corresponding pair-fed controls: * $P<0·05$, ** $P<0·01$. —◆—, Control (Con); —■—, alcohol (Alc).

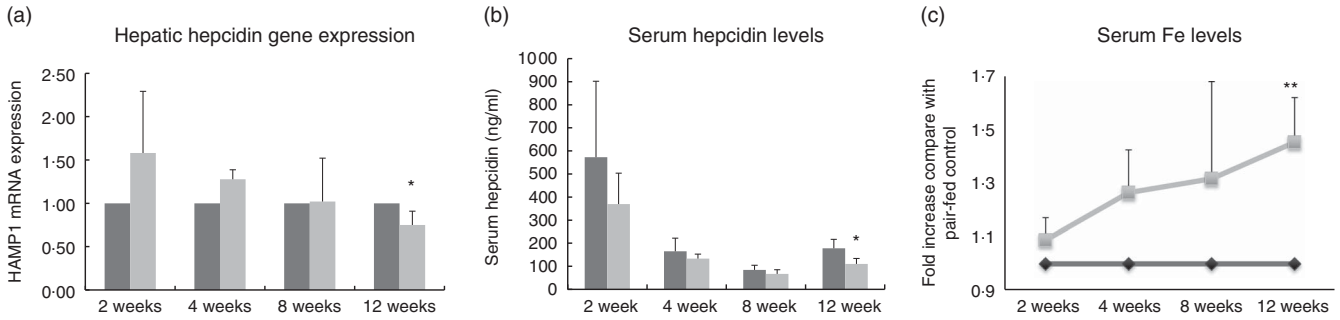

Fig. 3 . Effect of alcohol on hepcidin and serum iron levels. (a) Hepatic hepcidin antimicrobial peptide (HAMP) mRNA expression by quantitative PCR (n 3–6). (b) Serum hepcidin levels (n 3–4). (c) Serum iron levels (n 6–10). Values are means, with standard errors represented by vertical bars. Mean value was significantly different from that for corresponding pair-fed controls: * $P<0{\cdot}05$, ** $P<0{\cdot}01$. ■, Control; ■, alcohol; —◆—, Control; —■—, alcohol.

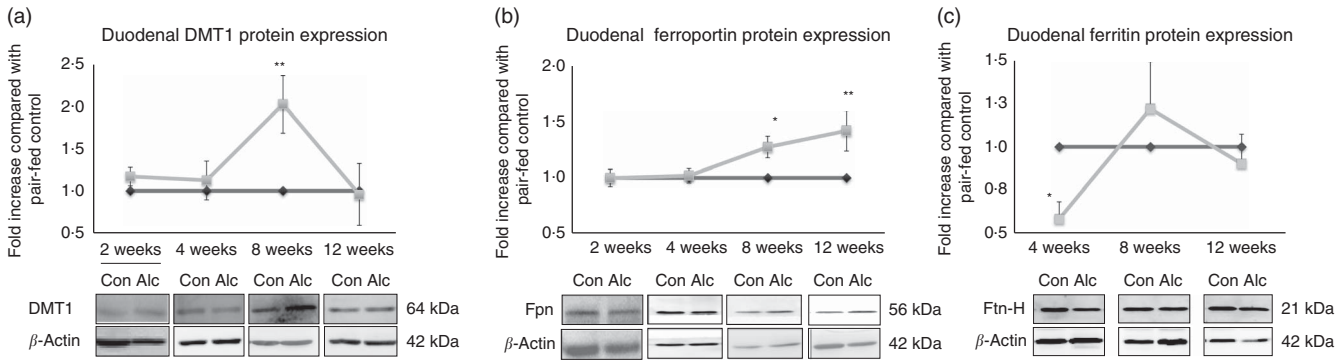

Fig. 4. Effect of alcohol on iron-related proteins in the duodenum. Duodenal divalent metal transporter 1 (DMT1) (a), ferroportin (b) and ferritin (heavy chain) (c) protein levels by Western blotting. Representative images for the blots with densitometric quantification of the bands are shown. Values are means (n 3), with standard errors represented by vertical bars. Mean value was significantly different from that for corresponding pair-fed controls: * $P<0{\cdot}05$, ** $P<0{\cdot}01$. —◆—, Control (Con); —■—, alcohol (Alc).

Changes in haeme oxygenase 1 and parameters of oxidative stress as well as markers of hepatic damage in response to alcohol feeding

HO-1 mRNA (Fig. 5(a)) and enzyme activity (Fig. 5(b)) in the liver were induced in response to alcohol. Total glutathione levels were found to be significantly lower at 8 weeks (Fig. 5(c)). The ratio of GSH:GSSG decreased progressively, with increasing periods of alcohol consumption, to become significantly lower at 12 weeks (Fig. 5(d)). Tissue levels of TBARS (online Supplementary Fig. S2(a)) (a marker of lipid peroxidation) and serum ALT levels (online Supplementary Fig. S2(b)) (a marker of hepatic damage) were not significantly affected by alcohol at any of the time points studied.

Histological studies

Light microscopic examination of the liver sections showed focal centrilobular microvesicular fatty change at 12 weeks in alcohol-fed mice (online Supplementary Fig. S3(a–d)). There was no stainable Fe at any of the time points studied (online Supplementary Fig. S3(e) and (f)).

Discussion

Animal models of alcohol ingestion, used to study the pathogenesis of ALD, have shown that the effects of alcohol are both time and dose dependent[22,23]. Lieber & DeCarli[14] standardised a rodent model of chronic alcohol ingestion based on *ad libitum* consumption of a liquid diet containing known amounts of alcohol. The advantage of this diet is that it allows for control animals to be pair-fed an isoenergetic control diet lacking alcohol, thus ensuring that alcohol-fed and control animals consume equivalent amounts of energy. In studies that have used the Lieber–DeCarli diet in rodents, the energy supplied in the form of alcohol ranged from 20 to 35 % of total energy [24–27]. Periods of administration of the diet varied from 4[28,29] to 16 weeks[26]. In the present study carried out over 12 weeks, we used a diet that provided 20 % of energy in the form of alcohol, based on the study by Heritage *et al.*[27], in which they investigated the effects of alcohol on Fe homoeostasis.

It has been shown that if only alcohol is administered no known rodent model replicates adequately the progression of pathological changes seen in ALD in humans[13,22,23,30]. In fact, rodent models of ALD have often used other agents such as Fe[31], high-fat diet[32] or lipopolysaccharide[33] in addition to alcohol to provide a second 'hit', as alcohol itself was not found to reproduce the pathological changes seen in human ALD. This is a limitation in the use of a rodent model of ALD that uses only alcohol. However, the use of such a model (Lieber–DeCarli diet) was appropriate for our study, as our aim was to determine whether alcohol *per se* affects Fe homoeostasis and causes Fe accumulation in the liver. Our systematic time-course study has shown that hepatic Fe accumulation did not occur in

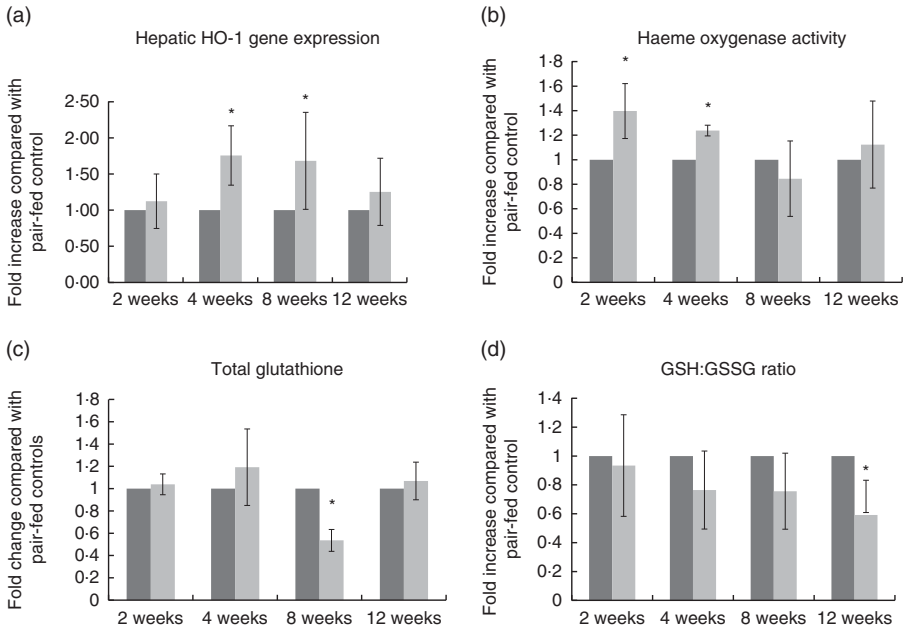

Fig. 5. Effect of alcohol on oxidative stress parameters in the liver. (a) Hepatic haeme oxygenase 1 (HO-1) mRNA expression by quantitative PCR (n 3–6). (b) HO activity (n 3–6). (c–d) Total glutathione (c) and ratio of GSH:GSSG (d) (n 3–6). Values are means, with standard errors represented by vertical bars. * Mean value was significantly different compared with that in corresponding pair-fed controls ($P < 0{\cdot}05$). ■, Control; □, alcohol.

response to alcohol (comprising 20 % of the energy consumed) for 12 weeks, but that steatosis did, observations that are in line with the existing literature.

Most studies involving mouse models of ALD have studied different effects (of interest) over 4–6 weeks of alcohol administration, with very few studies using protocols that have exceeded 8–12 weeks of administration. Our results, obtained for up to 12 weeks, contribute to information on the interactions between alcohol and Fe homoeostasis over this prolonged period of feeding. We have shown that the expression levels of several Fe-related genes changed in a time-dependent manner; this indicates that the duration of alcohol feeding and the amount of alcohol consumed appear to be important factors that influence changes in the parameters of Fe homoeostasis in response to the Lieber–DeCarli diet. Such a systematic time-course study has not been reported before, as far as we have been able to ascertain.

Alcohol ingestion has been shown to reduce hepcidin expression in the liver. The mechanism of this down-regulation is not clearly known, although a variety of mechanisms have been suggested[34–37]. However, most of these studies have not determined liver Fe levels, which are known to regulate hepcidin expression, with low levels suppressing it and high levels increasing its expression[38,39]. Therefore, our results provide useful data that liver Fe levels in mice decreased over time in response to alcohol and that this was associated with decreased hepcidin levels. It is possible that such a reduction in hepatic Fe levels may contribute to alcohol-induced decreases in hepcidin expression, which has been often reported in response to alcohol. Such a possible time-course sequence of events has not been reported before.

The interest in hepatic Fe levels in ALD stems from the fact that increased liver Fe has been suggested to play a role in the pathogenesis of irreversible liver injury and hepatocellular carcinoma[3,40]. However, a detailed review of the literature (as outlined in the first few paragraphs) shows that hepatic Fe accumulation is not an invariable feature of human ALD. Results from animal studies are also inconsistent, with some showing an increase in liver Fe content[41,42], whereas others have documented decreased Fe stores[31,43], following ethanol administration. These differences are possibly due to differences in the methodology and animal models used. What is apparent is that alcohol ingestion does not produce very consistent effects on hepatic Fe levels in rodents or humans. In our model, liver Fe levels decreased significantly at the end of 12 weeks of alcohol feeding; the lack of induction of BMP6 observed in our study is in concordance with this finding. Such a decrease in liver Fe in response to alcohol has been reported in other studies carried out in rats[31,43].

The reason for the reduction in liver Fe levels in our mouse model is not clear. Our observations on patients with ALD also do not show evidence of Fe overload (JV James, J Varghese, K Rasalkar, R Raghavan, CE Eapen and M Jacob, unpublished results). It is possible that the hepatomegaly and steatosis that developed in alcohol-fed mice resulted in decreased Fe content per gram of dry liver weight. Such a possibility has been postulated earlier by Tsukamoto *et al.*[31], who have reported decreased hepatic Fe levels (and induction of steatosis) in rats in response to alcohol for 16 weeks. Alternatively, alcohol may induce re-distribution of Fe from the liver to other tissues. Such a phenomenon has been shown to occur in other conditions associated with hepatic steatosis, such as obesity, where it has been shown that high-fat feeding induced hepatic steatosis and a reduction in liver Fe levels and an increase in adipose tissue Fe[44]. We have shown that hepatic ferroportin protein levels tended to increase after 4 and 12 weeks of alcohol feeding (online Supplementary Fig. S1(b)), suggesting increased efflux

of Fe from the liver, which may be responsible for the decreased cellular Fe content. This is supported by increases seen in serum Fe levels over the course of the study (Fig. 3(c)). However, we have not been able to measure Fe levels in the adipose tissue of our experimental mice. Further studies are required to determine whether such events occur.

An additional factor that may play an important role in determining liver Fe stores in this setting is the Fe content of the Lieber–DeCarli diet. The Fe content of both control and alcohol diets was 8·7 mg of elemental Fe per litre of the diet. For a mouse that consumes approximately 15 ml of diet/d, it provides 0·13 mg of Fe/d. This is well below the quantum of Fe provided by the National Institutes of Health – 31 open-formula rodent diet (NIH-31 diet) (commonly used as normal chow for rodents) that contains 300 mg/kg of Fe. A mouse consuming 5 g of this diet would consume 1·5 mg of elemental Fe/d. Therefore, the Lieber–DeCarli diet used in this study provided less than one-tenth the amount of Fe provided by a standard rodent chow such as the NIH-31 diet. However, even this low amount of Fe has been shown to be 'Fe adequate' in mice[15,16].

Increased expression of liver TfR1 has been reported in alcoholics[9]. TfR1 mediates Fe uptake by cells by binding to and internalising Fe-bound transferrin (holo-transferrin). In our study, gene expression of TfR1 peaked at 4 weeks of alcohol feeding and then decreased to control levels by 12 weeks (Fig. 2(d)). TfR1 protein levels followed a similar trend with significantly lower levels seen at 12 weeks (Fig. 2(c)). The changes in TfR1 expression mirrored the changes in liver Fe levels (Fig. 2(a)) and liver ferritin levels (Fig. 2(b)). TfR1 and ferritin levels are reciprocally regulated in response to intracellular free Fe by the Fe-regulated protein and Fe response element (IRP-IRE) system[45]. In this study, however, the changes in hepatic TfR1 were mirrored by similar changes in ferritin, suggesting that the effects of alcohol on TfR1 expression are independent of its effects on intracellular Fe levels.

BMP6, which is known to be regulated by hepatic Fe content, was not significantly affected in response to alcohol (data not shown). Tang *et al.*[42] showed evidence of increased hepatic Fe levels in mice in response to alcohol and have suggested that this is secondary to ethanol-induced suppression of BMP6, Mothers against decapentaplegic homolog 4 (SMAD4) signalling and hepcidin expression in their model[35]. However, they have not shown time-course data to show that this chronological sequence of events did indeed occur. The reason for the different findings in their study, compared with ours, is possibly related to the different strains of mice used (C57Bl/6 mice were used by Tang *et al.*[35,42] and Swiss albino mice were used in the present study) and the higher amounts of alcohol ingested and longer duration of feeding (30 % energy from alcohol for 15 weeks used by Tang *et al.*[35,42] compared with 20 % alcohol for up to 12 weeks in the present study). These fundamental methodological differences make it difficult to make direct comparisons of their studies with ours. However, our observations of decreased hepatic Fe levels and lack of induction of BMP6 over the period of our study are in concordance with one another.

Alcohol ingestion resulted in the up-regulation of CYP2E1 levels and activity in the liver. The level of CYP2E1 induction was comparable with other studies that used diets providing 35 % energy as alcohol[28,46]. Evidence of oxidative stress in the liver was also seen in the present study, as shown by the decrease in GSH:GSSG ratios and induction of HO-1 (Fig. 5(a), (b) and (d)). All of these are known responses to alcohol.

Chronic activation of HO-1 has been suggested to contribute to tissue Fe overload[47] by catalysing the breakdown of haeme with subsequent release of free Fe as one of the products. However, in the present study, we found that the increases seen in HO-1 gene expression and activity were variable (Fig. 5(a) and (b)), and therefore are not likely to affect hepatic Fe levels.

As shown in Fig. 1, mice fed alcohol for 12 weeks consumed slightly lower amounts of alcohol than the group fed the same diet for 8 weeks. This difference in amounts of alcohol consumed was found to be statistically significant. On further analysis, we found that it was the data of a sub-group of mice among those fed alcohol for 12 weeks that were contributing to the overall decrease in average consumption in this group. The reason for the decreased alcohol consumption in this sub-group is not clear. All the mice in this sub-group were from the same batch of mice allotted for the study from the in-house mouse breeding facility. When data from this sub-group of mice that consumed lower amounts of alcohol were removed from the overall data, the results obtained did not differ significantly from when the total data were considered. Furthermore, all other characteristics of this sub-group were similar to the rest of the mice fed for 12 weeks. We therefore believe that this difference in alcohol consumption has not affected the overall observations and conclusions of this study.

In conclusion, the results of this study show that alcohol feeding resulted in time-dependent effects on several Fe-related parameters in the liver and duodenum. It is important that this is kept in mind when extrapolating findings from studies that look at the effects of alcohol at a single time point. Our data show that many Fe-related proteins were affected by alcohol, causing dysregulation of the processes involved in Fe homoeostasis, but that Fe did not accumulate in the liver in response to alcohol ingestion for up to 12 weeks. On the basis of our observations, it is our contention that hepatic accumulation of Fe does not always occur in response to alcohol. Additional factors (a second 'hit') may be involved in those patients with ALD who do develop such an overload. We also suggest that alcohol-induced decreases in hepatic Fe levels may contribute to suppression of hepcidin levels in response to alcohol. This aspect warrants further study.

Acknowledgements

This study was funded by grants from the Department of Biotechnology, Government of India, awarded to J. V. (BT/PR10978/GBD/27/133/2008) and M. J. (BT/PR12738/Med/30/214/2009) and by a Fluid Research Grants awarded to M. J. by the Christian Medical College, Vellore (Institutional Review Board min numbers 6589 dated 11 June 2008 and 7714 dated 12 December 2011).

J. V. and J. V. J. carried out most of the experimental work, analysed and interpreted the data and wrote the manuscript. S. S., S. C. and A. S. carried some of the experimental work and were involved in the statistical analysis and interpretation of the data from their parts of the work. B. R. carried out the histopathological analysis and interpreted the data. M. J.

conceptualised and designed the study, supervised the work carried out, analysed and interpreted the data and reviewed the manuscript.

The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary material/s referred to in this article, please visit http://dx.doi.org/doi:10.1017/S0007114516001197

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