

Alternation between dietary protein depletion and normal feeding cause liver damage in mouse

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Abstract The effect of frequent protein malnutrition on liver function has not been intensively examined. Thus, the effects of alternating 5 days of a protein and amino acid-free diet followed by 5 days of a complete

diet repeated three times (3 PFD-CD) on female mouse liver were examined. The expression of carbonic anhydrase III (CAIII), fatty acid synthase (FAS), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and glutathione *S*-transferase P1 (GSTP1) in liver were assessed by proteomics, reverse transcriptase-polymerase chain reaction and Northern blotting. The activities of liver GSTs, glutathione reductase (GR) and catalase (CAT), as well as serum glutamic-oxaloacetic transaminase (SGOT) and glutamic-pyruvic transaminase (SGPT) were also tested. Additionally, oxidative damage was examined by measuring of protein carbonylation and lipid peroxidation. Liver histology was examined by light and electron microscopy. Compared with control mice, 3 PFD-CD increased the content of FAS protein (+90%) and FAS mRNA (+30%), while the levels of CAIII and CAIII mRNAs were decreased (−48% and −64%, respectively). In addition, 3 PFD-CD did not significantly change the content of GSTP1 but produced an increase in its mRNA level (+20%), while it decreased the activities of both CAT (−66%) and GSTs (−26%). After 3 PFD-CD, liver protein carbonylation and lipid peroxidation were increased by +55% and +95%, respectively. In serum, 3 PFD-CD increased the activities of both SGOT (+30%) and SGPT (+61%). In addition, 3 PFD-CD showed a histological pattern characteristic of hepatic damage. All together, these data suggest that frequent dietary

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amino acid deprivation causes hepatic metabolic and ultrastructural changes in a fashion similar to precancerous or cancerous conditions.

Keywords Mouse liver · Cycles of protein depletion · Liver damage · Oxidative stress

Introduction

Protein malnutrition causes severe metabolic changes in mammalian liver [1, 2, 7, 8, 16, 21, 24, 41]. However, the extent to which periodic dietary protein deprivation affects liver function has not been intensively examined.

Previously, we have found that mouse liver protein content decreases by 50% after 5 days of subjecting mice to a diet that lacks amino acids and protein (protein free diet (PFD)), while it is rapidly restored by administration of a complete diet (CD) [10]. In addition, the content of several cytosolic proteins depends on the amino acid intake [31, 33, 36, 37]. It also has been reported that subjecting mice to a 5-day PFD diet produces changes in the liver content of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutathione *S*-transferase subunits (GSTs), carbonic anhydrase III (CAIII), fatty acid synthase (FAS) and Cu/Zn superoxide dismutase (SOD) [15, 33, 36, 37]. Conversely, these changes are reverted by feeding a CD for 2 days [15, 36]. Additionally, we have recently shown that feeding mice a PFD for 5 days causes hepatic oxidative stress [34]. Interestingly, it has been reported that changes in GSTP1 subunit, GAPDH, FAS and CAIII can be used as markers for precancerous, cancerous or senescent conditions in the liver tissue [4, 6, 23].

Based on our previous observations on the effects of a 5-day PFD on mouse liver, we examined how three successive cycles of 5-day PFD followed by 5 days of CD (3 PFD-CD) affect mouse liver function. For this purpose, we measured protein and mRNA content of CAIII, FAS, GSTP1 and GAPDH, as well as the activities of catalase (CAT), GSTs and glutathione reductase (GR), total content of carbonyl groups and lipid peroxidation in liver. In addition, serum markers for liver damage and hepatic ultrastructure were determined. Taken together, our results show that periodic protein malnutrition affects liver function in a harmful manner.

Materials and methods

Animals

Two-month-old female Balb/c mice (body weight, 20–25 g) from IIB, UNMdP-CONICET, Mar del Plata, Argentina were kept in a room at 22°C illuminated from 7:00 to 19:00 hours. They had ad libitum access to complete diet and water and kept in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The local ethical committee for animal research approved the protocols used in this study.

Diets

All diets used were based on the PFD outlined by the USP XV Pharmacopoeia and the amino acid composition of bovine β -casein [12, 42]. Normal or complete diet (CD) containing 23% (*w/w*) β -casein was prepared as described by Ronchi et al. [33]. Because carbohydrates replace protein and amino acids, all diets are isocaloric. Mice consumed 2.53 ± 0.29 and 3.38 ± 0.14 g a day of CD and PFD, respectively.

The test mouse group was subjected to three cycles of 5 days of PFD followed by 5 days of CD. The control group was fed a CD throughout the entire experiment. After the treatment, animals were killed by cervical dislocation, and livers were rapidly removed, weighed and placed in buffer A (0.15 M NaCl, 1 mM EDTA, 5 mM β -mercaptoethanol and 20 mM Tris-HCl buffer; pH 7.4).

Cytosol preparation

Livers were homogenized in 5 ml/g fresh weight of cold buffer A. The supernatant obtained by centrifugation at $100,000 \times g$ for 10 min represents the cytosol [37].

Cytosolic enzymes were extracted according to Wiegand et al. [47], with some adjustments. Briefly, each liver was homogenized in 0.1 M potassium phosphate buffer pH 6.5 containing 20% (*v/v*) glycerol, 1 mM EDTA and 14 mM 1,4-dithioerythritol (DTE). Cell debris was removed by centrifugation at $10,000 \times g$ for 10 min. The supernatant was centrifuged at $100,000 \times g$ for 60 min, and the soluble cytosolic fraction was immediately frozen with liquid nitrogen

and stored at -80°C until use. Activity of total glutathione *S*-transferases (GSTs) was determined according to Habig et al. [17]. Glutathione reductase (GR) was tested according to Tanaka et al. [40]. Catalase (CAT) was determined according to Claiborne [9]. Results were expressed in nanokatal per milligram of protein (nkat/mg prot), where 1 kat represents the conversion of 1 mol of substrate per second. Each test was carried out in triplicate.

Protein and nucleic acid determination

Protein concentration was determined according to Bradford [5] using bovine serum albumin as a standard. Nucleic acid concentrations were determined by the method described by Fleck and Munro [14].

Polyacrylamide gel electrophoresis

Cytosol samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% acrylamide under reducing conditions [25] followed by staining with Coomassie blue [28]. Resulting protein patterns were photographed and analyzed with the computer image analyzer system ImageQuant TL v2005. It may be noted that CAIII, FAS and GSTP1 bands were previously identified and quantified using mass spectroscopy combined with sequence analysis of peptide produced by in-gel trypsin digestion [15, 33]. In addition, GAPDH band was identified and quantified by both Western blot test and N-term amino acid sequence [36].

Reverse transcriptase-polymerase chain reaction

Total liver RNA was extracted with Trizol (Invitrogen, Gaithersburg, MD, USA). Extracts were treated with RQ1 RNase-Free DNase (Promega), quantified by measuring the absorbance at 260/280 nm and tested for quality by agarose 1% native gel electrophoresis. First-Strand cDNA synthesis was generated with M-MLV reverse transcriptase (Invitrogen) and oligo dT primers and used for reverse transcriptase-polymerase chain reaction (RT-PCR). Products were obtained after 30–35 cycles of amplification considering the exponential phase for each probe and $55\text{--}65^{\circ}\text{C}$ annealing temperatures. RT-PCR products were separated on 2% agarose gels. Electrophoresis was

carried out at 100 V for 60 min in TBE 1X buffer (0.89 M Tris, 0.89 M borate, 2 mM EDTA). The gels were stained with SYBR[®] Gold nucleic acid stain (Molecular Probes, Eugene, OR, USA). Bands were detected with a Scanner Storm Amersham Biosciences and quantified by ImageQ TL v2005. All the band intensities were related to actin.

Mouse specific primer sequences for PCR reaction

FAS: FW, 5'-TGC GCC CAG CCT CCT AAG GC-3'; RV, 5'-ATC ACA CGC CGG CAA CAC CTA TCC-3'

CAIII: FW, 5'-TGC CAA AGG GGACAA CCA GT-3'; RV, 5'-GCA CCG GGG GCTCAT TCT C-3'

GAPDH: FW, 5'-ACG GCA AAT TCA ACG GCA CAG TCA-3'; RV, 5'-CAT TGG GGG TAG GAA CAC GGA AGG-3'

GSTP1: FW, 5'-ATG CCA CCA TAC ACC ATT GT-3'; RV, 5'-GTC CAG CAA GTT GTA ATC GG-3'

Actin: FW, 5'-AGT ACT TGC GCT CAG GAG GA-3'; RV, 5'-TCC TCC CTG GAG AAG AGC TA-3'

Northern blotting

Total RNA was extracted from livers using 4 M guanidinium thiocyanate followed by extraction with phenol [35]. The extract was dissolved in water DEPC, quantified and stored at -80°C for further tests. Thirty microgrammes of RNA per condition were denatured for 1 h in GLYOXAL at 55°C [43], separated by electrophoresis on agarose gels 1.5% (*w/v*) and transferred onto a nylon membrane (Amersham, UK).

DNA probes were obtained by PCR amplification with specific primers (see above) using DNA as template. Then, the probes were labelled by random priming (Invitrogen, Life Technologies, DNA labelling System) using [^{32}P] dCTP. Probes were purified by chromatography in G-50 Sephadex columns. Sequential hybridization with the different probes was performed using standard methods. The intensity of the bands was detected by Scanner Storm (Amersham Biosciences) and quantified by ImageQuant TL v2005. All the band intensities were related to actin.

Serum analyses

Blood was collected in 1.5 ml tubes after beheading the animals by guillotine. Blood collection was performed between 8:00 and 10:00 am and kept in 37°C. Within 15 min, the samples were centrifuged for 10 min at 1,000×g (4°C) to separate the serum. Samples were kept at -80°C until determination of serum components.

Serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) were measured by using a commercial kit (Wiener Lab, Rosario, Argentina) and performed according to the appended protocol.

Measurement of protein carbonyl content

The reactive carbonyl content of liver proteins was measured according to Ronchi et al. [34]. Briefly, livers were homogenized at 4°C in 10 ml/g fresh weight of cold 0.15 M NaCl, 1 mM EDTA and 20 mM Tris-HCl, pH 7.4. Two 0.75-ml aliquots per sample containing nearly 1 mg protein were mixed with 0.75 ml of 20% (w/v) trichloroacetic acid (TCA). The pellets were separated by centrifugation at 6,000×g for 5 min, mixed with either 0.75 ml of 2 N HCl (blank) or 0.75 ml of 2 N HCl containing 0.2% (w/v) dinitrophenylhydrazine (DNPH) and agitated for 1 h at 25°C in the dark. Then, they were re-precipitated with 0.75 ml of 20% (w/v) TCA, washed three times with ethanol:ethyl acetate (1:1, v/v), dried and mixed with 0.75 ml of 6 M guanidine HCl at 25°C. After removing the debris by centrifugation, absorbance at 370 nm of DNPH-treated samples after subtraction of blanks was assessed. This value was used to calculate the nanomoles of DNPH incorporated per milligramme of protein based on an average absorption of 21 mM⁻¹ cm⁻¹ for aliphatic hydrazones [22].

Lipid peroxidation test

Thiobarbituric acid reactive substances (TBARS), indicative of lipid peroxidation level, were evaluated as showed by Oakes and Van Der Kraak [30] with adjustments. Briefly, 50 mg of fresh liver were homogenized in 450 µl of a solution containing 0.15 M KCl and 35 µM butylated hydroxytoluene (BHT). Homogenate aliquots of 100 µl were added to

3.9 ml of a reaction mixture containing 37.5 mM TBA, 67 µM BHT, 7.5% (w/v) TCA and 0.4% (w/v) SDS. Then, samples were heated at 95°C for 30 min, cooled to 15°C, and centrifuged at 3,000×g for 10 min. Optical density at 532 nm of supernatants, through a molar extinction coefficient of 1.56×10⁵ M⁻¹ cm⁻¹, allowed assessing the TBARS content in nanomoles by milligramme of tissue [30].

Histological studies

For morphological observation by light microscopy, liver tissues were fixed in 10% neutral formaldehyde solution, dehydrated in an ascending ethanol series and embedded in paraffin. Sections were stained with haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). The structures of liver lobules, hepatocytes, portal tracts and sinusoidal and perisinusoidal acini were analyzed.

For electron microscopy analysis, the samples were fixed in glutaraldehyde 2% in buffer phosphate (pH 7.4) for 2 h and washed twice with buffer solution for 30 min. Then, they were post-fixed in 1% osmium tetroxide, dehydrated in a series of alcohol and embedded in Epon 812 for ultra-thin sectioning. Ultrathin sections, 60–80-nm thick, were cut with a diamond knife on an ultramicrotome (ultramicrotome supernova, Reichert-Jung) and then viewed under a transmission electron microscope (JEM 1200EX II, Jeol).

Statistical analysis

All data are presented as means ± SEM. Data comparison was performed using unpaired two-tailed Student's *t* test (InStat, Graph Pad software). *P* values lower than 0.05 were considered significant. Data analysed include at least three separate experiments.

Results

General properties

After 3 PFD-CD, body weight of treated mice declined to a value near 20% lower than the control (Fig. 1). Figure 1 also shows that major body weight loss happened during the PFD periods. The treatment

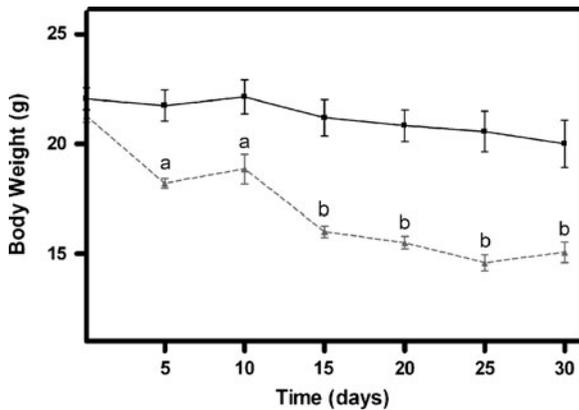


Fig. 1 Changes in body weight. Treatment groups were (gray triangle) PFD-CD and (black square) CD. Weights are the means \pm SEM of three independent experiments ($n=6$ for each experiment). Values significantly different from the CD group: ^a $P<0.001$, ^b $P<0.0001$

did not change either the weight or DNA content of the liver but showed a tendency to increase RNA (+19%) and protein (+23%) content. Control mice (CD diet) displayed the following properties: liver weight, 1.03 ± 0.03 g; DNA, 1.47 ± 0.26 mg/liver; RNA, 4.47 ± 0.43 mg/liver; total protein, 144.12 ± 9.30 mg.

Liver content of CAIII, FAS, GAPDH and GSTP1

The effect of 3 PFD-CD on CAIII, FAS, GAPDH and GSTP1 content was assessed by densitometry of SDS-PAGE bands (Fig. 2). FAS increased 90% and CAIII decreased 48%, while both GAPDH and GSTP1 did not show a significant change. Western blotting confirmed the results obtained by SDS-PAGE for both FAS and GSTP1 (not shown).

Liver content of CAIII, FAS, GAPDH and GSTP1 mRNAs

To find out whether the effects of 3 PFD-CD observed on CAIII, FAS, GAPDH and GSTP1 content depend on changes in mRNA content, Northern blot and RT-PCR experiments were carried out. In agreement with protein content, FAS mRNA increased 30% and CAIII mRNA decreased by 64%. On the other hand, GSTP1 mRNA increased 20%, while GAPDH mRNA did not change significantly,

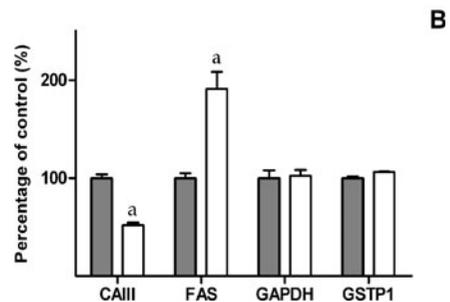
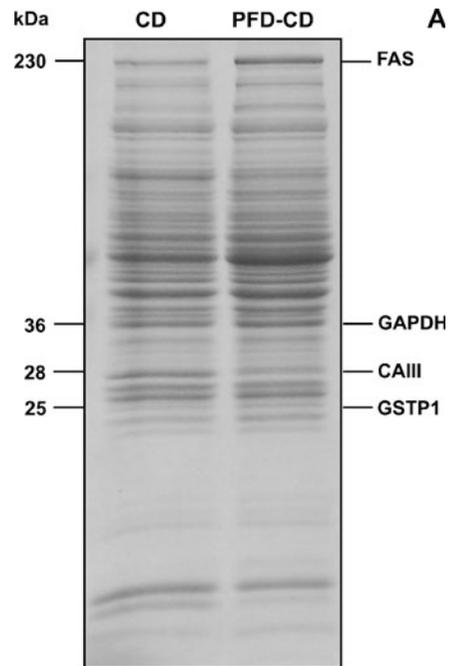


Fig. 2 Effect of 3 PFD-CD on cytosolic protein composition. **a** SDS-PAGE representative analysis of protein samples (25 μ g) from CD and 3 PFD-CD. Positions of size markers are signalled on the left. Bands of FAS, GAPDH, CAIII and mGSTP1 are signalled on the right. **b** Average percentages of FAS, GAPDH, CAIII and mGSTP1 band densities in 3 PFD-CD (white bars) with respect to CD (grey bars). Values represent the means \pm SEM of three independent experiments (CD and 3 PFD-CD with $n=6$ each experiments). Values significantly different from the CD condition: ^a $P<0.001$

though individual data displayed high variability (Figs. 3 and 4).

Liver activities of CAT, GSTs and GR

We also examined whether 3 PFD-CD affect the enzymatic activities of CAT, GSTs and GR in liver. Both CAT and GSTs decreased 66% and 26%, respectively, while GR did not change (Table 1).

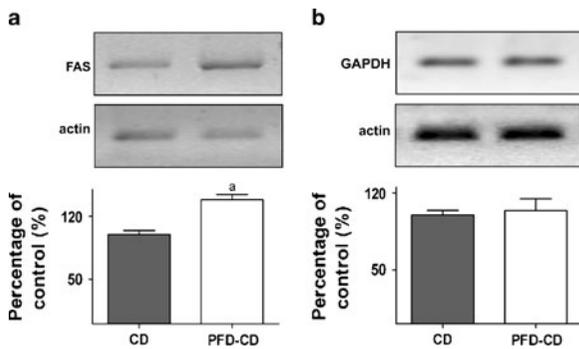


Fig. 3 Effect of 3 PFD-CD on FAS and GAPDH mRNA content. **a** RT-PCR analysis of FAS mRNA. **b** RT-PCR analysis of GAPDH mRNA. After dietary treatments, total liver mRNA was isolated, and the levels of FAS and GAPDH mRNAs were assessed by RT-PCR analysis. Actin content was used for RNA normalization. Relative changes in FAS and GAPDH mRNA content were assessed by scanning densitometry of RT-PCR bands. Data are expressed as the intensity of bands determined by densitometry and related to the CD group. Values represent the means \pm SEM of three independent experiments (CD and 3 PFD-CD with $n=6$ each experiments). Values significantly different from CD condition: ^a $P<0.05$

Carbonyl-group content of protein

We examined the extent of liver oxidative stress by measuring the total protein carbonyl content. This was evaluated by the reaction with DNPH. Carbonyl

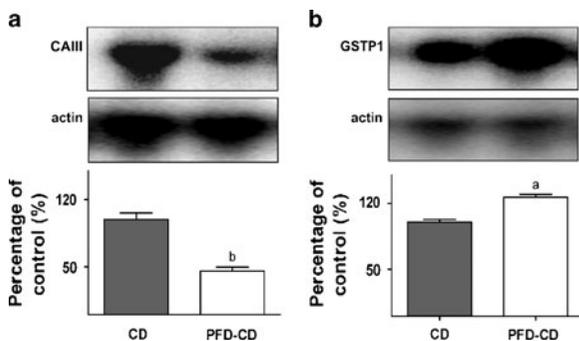


Fig. 4 Effect of 3 PFD-CD on CAIII and mGSTP1 mRNA content. **a** Northern blot analysis of GSTP1 mRNA. **b** Northern blot analysis of CAIII mRNA. After dietary treatments, total liver RNA was isolated. Thirty micrograms were subjected to Northern blot analysis using specific cDNA probes for CAIII and GSTP1 mRNAs. Actin cDNA probe was used for RNA normalization. The radioactivity in the Northern blots with different probes was measured by scanning densitometry. Data are expressed as intensity of bands determined by densitometry and related to the CD group. Values represent the means \pm SEM of three independent experiments (CD and 3 PFD-CD with $n=6$ each experiments). Values significantly different from CD condition: ^a $P<0.05$, ^b $P<0.001$

Table 1 Activities of liver cytosolic enzymes after dietary treatments

	Enzymes activity (nkat/mg)		
	CAT	GSTs	GR
CD	341.29 \pm 70.69	17.81 \pm 1.18	0.63 \pm 0.06
3 PFD-CD	117.04 \pm 67.65*	13.23 \pm 0.79**	0.56 \pm 0.03

Values are means \pm SEM of two independent experiment ($n=4$ each experiments). Values significantly different from CD group

* $P<0.05$; ** $P<0.005$

content significantly increased with 3 PFD-CD when compared with CD, as evidenced by their values (17 ± 1.5 and 11.2 ± 0.9 , respectively, $P<0.0001$).

Lipid peroxidation

The effect of 3 PFD-CD on liver lipid peroxidation was investigated. The 3 PFD-CD increased 95% the TBARS levels of liver compared with CD (0.232 ± 0.02 and 0.119 ± 0.01 , respectively, $P<0.002$).

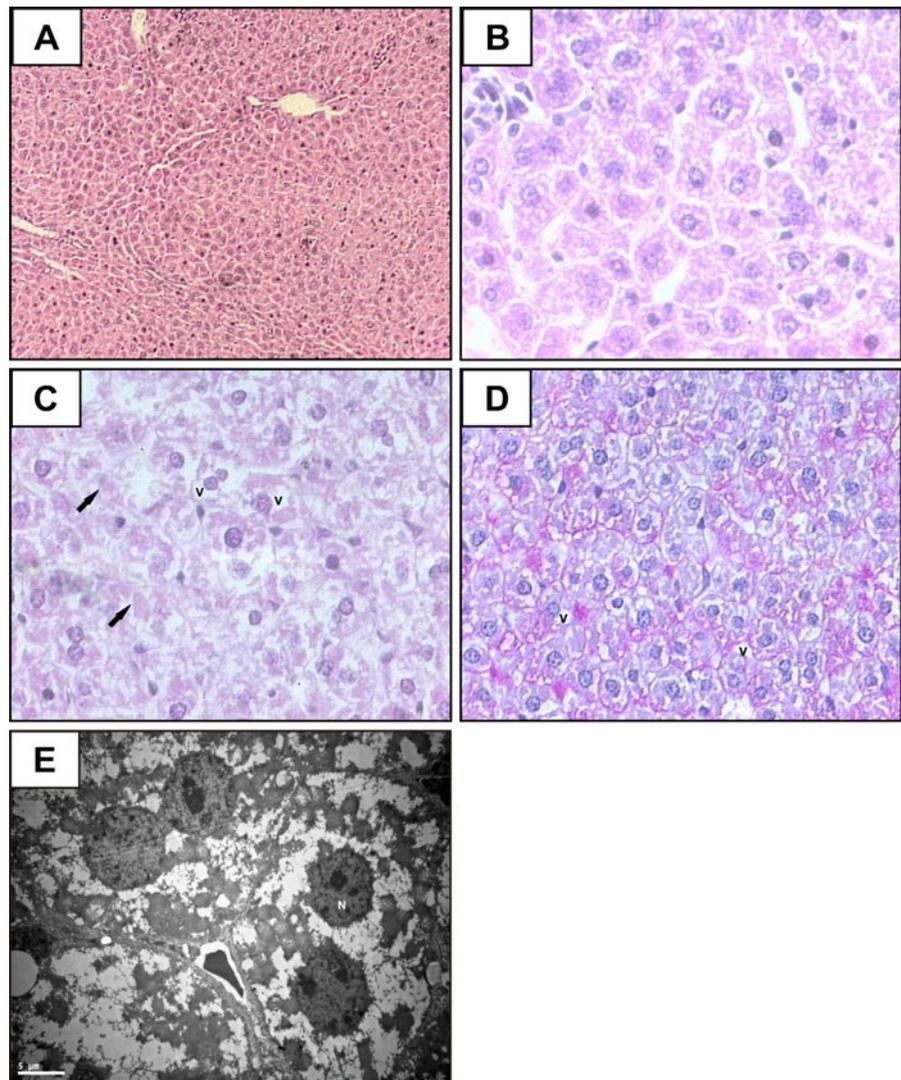
Serum markers of liver damage

We also examined whether 3 PFD-CD changes the serum content of molecular markers for liver damage. Activities in U/L \pm SEM of serum glutamic-oxaloacetic (SGOT) and serum glutamic-pyruvic (SGPT) transaminases were SGOT, 472.83 ± 25.87 and 611.20 ± 10.64^b for CD and 3 PFD-CD, respectively; SGPT, 182.25 ± 13.99 and 293.50 ± 45.09^a , respectively. Values correspond to means \pm SEM of two independent experiments ($n=4$); values were considered significantly different from the CD group at ^a $P<0.05$ and ^b $P<0.001$.

Liver histology

The liver of CD mice showed the typical lobular structure with normal central veins and portal tract (Fig. 5a). The hepatocytes showed normal cytological structures with well-preserved staining and morphological characteristics of cytoplasm, nucleus and nucleolus (Fig. 5b). Some PAS positive inclusions were found in the cytoplasm of hepatocytes. After 3 PFD-CD, liver sections showed that although the

Fig. 5 Histology of the liver after 3 PFD-CD treatment. **a** CD mouse liver shows typical lobular structure with normal central vein and portal tract; H&E, $\times 100$. **b** CD mouse liver; H&E, $\times 400$. **c** The hepatocytes show a vacuolated cytoplasm (v). In some areas, nuclei are absent (arrow); H&E, $\times 400$. **d** The cytoplasmic vacuoles are negative to PAS reaction (v). PAS-haematoxylin; $\times 400$. **e** Cytoplasmic vacuolization and lack of normal organelle organization (n, nucleus); TEM, $\times 5,000$



lobular organization was preserved, the hepatocytes displayed a vacuolated cytoplasm. Also, nuclei were absent in some areas (Fig. 5c). In addition, the cytoplasmic vacuoles were negative to PAS and H&E stain (Fig. 5d). Presence of hepatic damage after 3 PFD-CD was confirmed by electronic microscopy. Hepatocytes from these mice showed a decreased nuclear size with indented nuclear membrane and an increase of the perinuclear aggregation of heterochromatin. Mitochondria organization was abnormal and showed swelling and cristolysis. Vast accumulation of vacuoles representing lipid droplets were also observed in the liver of mice exposed to 3 PFD-CD (Fig. 5e).

Discussion

This work was aimed to address whether three cycles of feeding a diet lacking amino acids for 5 days followed by a complete diet for 5 days (3 PFD-CD) produce irreversible changes in the protein composition and function of mouse liver.

Similar to rats exposed to three cycles of fasting–re-feeding [24], 3 PFD-CD decrease the total body weight while preserving liver weight. This result is consistent with the fast recovery of liver weight in mice fed with a normal diet after 5 days under PFD [10]. Similarly, despite body weight loss, liver weight of fasted rat nearly reaches that of control within few

days of re-feeding with normal diet [18]. Liver growth during CD feeding is favoured because PFD is isocaloric and taken in rather high amount than CD [26]. Therefore, liver may regained its normal weight during each period of PFD. In addition, 3 PFD-CD does not change liver DNA content but increases slightly RNA and protein levels. These results agreed with those displayed by mice that were fed a CD after the intake of a PFD during 6 days [10].

Because FAS is regulated at the transcriptional level, 3 PFD-CD produces an increase in FAS content caused by an increase in its mRNA. Besides its control by hormonal and nutritional factors [19], it has been recently published that signalling pathways such as AMPK-S6K1 might be involved in FAS induction. Furthermore, is also known that lipogenic enzymes are markedly induced in hepatocellular carcinomas [13, 45, 48]. Thus, the increase in FAS caused by 3 PFD-CD could be associated with the development of hepatocellular carcinoma. In contrast, although GAPDH content increases in hepatic carcinogenesis [3], it remains unmodified after 3 PFD-CD. Based on our previous studies [36], the last 5 days under normal diet in the present experimental model would be enough to restore the content and the mRNA levels of GAPDH.

We had previously noticed that liver content of CAIII decreases after feeding a PFD for 5 days [37]. In this study, although the last feeding period of 3 PFD-CD is CD, both CAIII content and CAIII mRNA decrease with 3 PFD-CD. Besides preserving the acid–base balance, CAIII acts as an oxygen radical scavenger, protecting cells from oxidative damage [27, 32]. Thus, the down-regulation of this enzyme might promote liver damage and, possibly, lead to hepatocellular carcinoma [23].

GSTs have a prominent role in protecting cells against oxidative stress. Among them, GSTP1 has been proven to increase during detoxification of electrophilic drugs and carcinogens [44]. The content of GSTP1 does not change after 3 PFD-CD, but its mRNA does. Transcription of the GSTP1 gene is activated in both chemically induced and spontaneous precancerous lesions [20, 38]. Thus, the observed GSTP1 mRNA increase could be a marker for an early stage of hepatocarcinogenesis.

In this study, we have also shown that 3 PFD-CD markedly decreases GSTs and CAT activities in liver, therefore increasing oxidative stress, which is

revealed by an increment in protein carbonylation and lipid peroxidation. In addition, 3 PFD-CD increases the activities of SGPT (+61%) and SGOT (+30%), which have been reported as markers for liver damage [50].

Our histological observations support the changes produced by 3 PFD-CD in both liver and blood serum. In hepatocytes, 3 PFD-CD induces indented nuclear membrane and perinucleolar aggregation of heterochromatin material. This suggests that a nuclear disorganization might be occurring [46], which eventually might lead to apoptosis [39]. Interestingly, the changes in mitochondria organization have been proposed to reflect a disturbance in the redox balance of the liver cell [11]. Further, damage of mitochondria is a common response of the hepatocyte to stress [29], which can undergo significant swelling to show “hydropic degeneration” [49]. It is likely that 3 PFD-CD induced vacuolation of the cytoplasm is caused by accumulation of lipid droplets, as it had been shown elsewhere [11]. Since extensive vacuole accumulation can be a cause of cell death, this further points to 3 PFD-CD as an inducer of liver damage.

In conclusion, shifting mice from protein deprivation to a complete diet three times changes both liver metabolic status and ultrastructure like it has been reported for precancerous and cancerous conditions [33, 44, 48]. These changes might have their origin in an oxidative stress caused by 3 PFD-CD. However, the mechanisms that trigger such adjustments are not entirely known and should be a focus of future work to understand the influence of nutrition on liver metabolism.

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