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ORIGINAL ARTICLE

Protein malnutrition during fetal programming induces fatty liver in adult male offspring rats

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Abstract We evaluated the effects of protein malnutrition on liver morphology and physiology in rats subjected to different malnutrition schemes. Pregnant rats were fed with a control diet or a low protein diet (LPD). Male offspring rats received a LPD during gestation, lactation, and until they were 60 days old (MM group), a late LPD that began after weaning (CM), or a LPD administrated only during the gestation-lactation period followed by a control diet (MC). On day 60, blood was collected and the liver was dissected out. We found a decrease in MM rats' total body ($p < 0.001$) and liver ($p < 0.05$) weight. These and CM rats showed obvious liver dysfunction reflected by the increase in serum glutamic pyruvic transaminase (SGOT) (MM $p < 0.001$) and serum glutamic pyruvic transaminase (SGPT) (MM and CM $p < 0.001$) enzymes, and liver content of cholesterol (MM and CM $p < 0.001$) and triglycerides (MM $p < 0.01$; CM $p < 0.001$), in addition to what we saw by histology. Liver dysfunction was also shown by the increase in gamma glutamyl transferase (GGT) (MM, MC, and CM $p < 0.001$) and GST-pi1 (MM and CM $p < 0.001$, MC $p < 0.05$) expression levels. MC rats showed the lowest increment in GST-pi1 expression (MC vs. MM; $p < 0.001$, MC vs. CM; $p < 0.01$). ROS production (MM, CM, and MC: $p < 0.001$), lipid peroxidation (MM, CM, and MC $p < 0.001$), content of carbonyl groups in liver proteins (MM and CM $p < 0.001$, MC $p < 0.01$), and total antioxidant capacity (MM, CM, and MC $p < 0.001$) were increased in the liver of all groups of malnourished animals. However, MM rats showed the highest increment. We found higher TNF-α (MM and CM $p < 0.001$), and IL-6 (MM and CM $p < 0.001$) serum levels and TGF-β liver content (MM $p < 0.01$; CM $p < 0.05$), in MM and CM groups, while MC rats reverted the values to normal levels. Pro-survival signaling pathways mediated by tyrosine or serine/threonine kinases (pAKT) (MM and CM $p < 0.001$; MC $p < 0.01$) and extrasellular signal-regulated kinase (pERKs) (MM $p < 0.01$; CM $p < 0.05$) appeared to be activated in the liver of all groups of malnourished rats, suggesting the presence of cells resistant to apoptosis which would become cancerous. In conclusion, a LPD induced liver damage whose magnitude was related to the developmental stage at which malnutrition occurs and to its length.

Keywords Fetal programming · Low protein diet · Metabolic syndrome · Liver damage · Non-alcoholic fatty liver · Wistar rats

Introduction

Individuals who suffer severe malnutrition during the critical developmental stage have an elevated predisposition to develop metabolic diseases in later life, such as type II diabetes, dyslipidemia, fibrous liver, and/or fatty liver, among others [11, 25, 30], which increases the chances of developing cirrhosis and hepatocarcinoma (HCC) [18, 31, 33]. Fetal intrauterine growth restriction (IUGR) occurs in humans as a consequence of poor maternal nutrition and has been associated with the development of different illnesses, a phenomenon called fetal programming [3]. The association of maladaptive programming with adult diseases has been termed "The

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Barker Hypothesis". IUGR leads to alterations in numerous fetal organs which induce the full metabolic syndrome in the adult [3, 41]. The concept of developmental programming proposes that challenges during an organism's development evoke a persistent physiological response in the offspring [2, 42]. Epidemiological investigations such as those conducted on children conceived during the Dutch famine of 1944–1945 or during the great Chinese famine have highlighted the association between poor maternal nutrition, lowered birth weight, and subsequent adult diseases [37, 39, 40]. Different experimental protocols on animals have been used for the evaluation of developmental programming of metabolism, such as global nutrient restriction [13, 45] or maternal exposure to an isocaloric low protein diet (LPD) [5, 14, 27]. These studies are consistent with the concept that adverse events in utero predispose the offspring toward the later development of metabolic syndrome [22, 34]. Reactive oxygen species (ROS) have a crucial role in human physiological and pathophysiological processes. Under normal conditions, the body has a potent antioxidant system that protects it from the deleterious effects of ROS. When either the production of ROS is increased or the antioxidant defenses inactivated, oxidative stress occurs and ROS can react with cellular macromolecules and enhance the process of lipid peroxidation, cause DNA damage, and/or induce protein and nucleic acid modifications [6, 10]. Increased ROS production is observed at different stages of liver disease and in HCC [17, 19, 43]. In recent years, it became evident that ROS are not only associated with pathologies but also can act as second messengers to activate signaling pathways, such as growth and apoptosis, and hence lead to alterations in gene expression [6, 44]. In addition, interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α are cytokines produced during the inflammatory process and are principal stimulators of acute phase proteins and other markers of chronic inflammation commonly detected in many diseases like diabetes mellitus [10]. Increased expression of pro-inflammatory cytokines is associated with hepatocyte apoptosis but is also essential in hepatocyte proliferation [20] and liver regeneration [46]. By binding to its receptors (TNFR1/TNFR2), TNF-α activates two different signaling cascades either leading to apoptosis or to proliferation [4]. Likewise, transforming growth factor (TGF)-β signaling pathway plays a critical dual role in cancer progression. At an early phase, TGF-β acts as a tumor suppressor, while later, its role switches to tumor progression. TGF-β excess and deficiency are causal for the development of fibrotic and autoimmune liver diseases, respectively [29, 47]. We have previously demonstrated that a LPD during gestation and lactation compromises the integrity of the structural and functional liver in male newborn offspring rats weaned after 24 days of age [35]. The lack of dietary amino acids during the critical developmental stage would generate changes in the oxidative stress status of hepatocytes, inducing an inflammatory process that would result in fatty and fibrous liver and even end in a precancerous condition in adulthood. The aim of this work was to evaluate different protein malnutrition schemes during the critical developmental stage (gestation, lactation, and childhood) and their effects on liver histology, antioxidant status, pro-inflammatory cytokines production, expression of biochemical markers of liver damage, and proliferative capacity versus sensitivity to apoptosis in 60-days-old offspring adult male rats. We also evaluated if nutritional rehabilitation at weaning would revert the liver damage induced by a LPD during gestation and lactation.

Materials and methods

Care and maintenance of animals

All experiments were approved by our Institutional Animal Care Committee (RD 140/15). Wistar rats were kept in a room with controlled temperature (25 ± 1 °C) and with artificial dark/light cycle (light on from 8 am to 8 pm). Aged 3 months, virgin female rats were caged with one male. Once pregnancy was determined by the presence of sperm in the vaginal smears after mating with an adult male for 24 h, they were individually housed in plastic cages with free access to water and food. Once separated, rats were fed either 23% casein (control diet, AIN-93G) or an 8% casein isocaloric diet (LPD=low protein diet). Food was provided in the form of a large flat biscuit which was retained behind a grill through which the rats nibbled. Food was provided ad libitum. To ensure homogeneity of study subjects, litters of over 12 pups or less than 7 were not included in the study. On day 24, pups per mother were counted and sex was determined. Male rats were housed in groups of 6 animals each. Two animals per litter from each group of mothers (control or LPD) were randomly distributed in the four different experimental groups as follows: CC in which dams received the control diet during pregnancy and lactation, and then the litters were fed with control diet between days 24 and 60; MM in which dams received the LPD during pregnancy and lactation, and then the litters continued being fed with LPD between days 24 and 60; CM in which dams received the control diet during pregnancy and lactation, and then the litters received the LPD between days 24 and 60; and MC in which dams received the LPD during pregnancy and lactation, and then the litters were provided with the control diet between days 24 and 60. The daily feed intake rate of all experimental groups was calculated. The difference between recorded food consumption rates was not statistically significant.

The animals were euthanized on day 60 using a CO_2 chamber, with 1-h fasting. For consistency, only male offspring were used for the study because fetal programming is known to occur in a sexually dimorphic manner [12]. Blood was collected and processed to obtain serum, which was

individually kept at −80 °C until assay. The liver was dissected out. Body and liver weights were recorded.

Metabolite determinations

Serum total protein, glucose, triglycerides, and cholesterol concentration

It was determined by enzymatic assays from Wiener Laboratories, Argentina. 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. Protein concentration in the liver was measured with bicinchoninic acid protein assay [35].

IL-6 and TNF-α levels were quantified by enzyme linked immunosorbent assay (ELISA) kits (BD Biosciences PharMingen) following the manufacturer's directions. The minimum detectable concentration was 4.5 pg/ml for TNF-α and 10 pg/ml for IL-6.

Tissue lipids

Tissue samples of approximately 0.2 g, together with 2 ml of chloroform/methanol (2:1) solution were homogenized. The tubes were then centrifuged at 3000 rpm. An aliquot of chloroform/methanol extract was transferred to another tube and dried under a stream of nitrogen gas. These samples were redissolved in 100 μl isopropyl alcohol, after which cholesterol and triglyceride levels in the isopropyl alcohol were measured by conventional enzymatic methods (Wiener Lab, Rosario, Argentina).

Tissue glycogen

Approximately 0.2 g of tissue was dropped into a centrifuge tube containing 1 ml of 30% potassium hydroxide solution. The tissue was then digested by heating the tube in a boiling water bath for 20 min. When the tissue was dissolved, 0.5 ml of saturated sodium sulfate was added and the glycogen was precipitated by the addition of 3 ml of 95% ethanol. The tube and contents were heated again until the mixture began to boil, then cooled, and centrifuged at 3000 rpm. The mother liquor was decanted, and the test tube was allowed to drain. The precipitated glycogen was redissolved in 1 ml of distilled water and reprecipitated with 1.5 ml of 95% ethanol, the alcoholic supernatant liquid decanted, and the tube drained as before. The purified glycogen was redissolved in 1 ml of distilled water, and glycogen in this solution was measured by anthrone reagent.

Measurement of liver redox state

Detection of cytosolic ROS production

The rate of cytosol H_2O_2 production was determined by using the BIOXYTECH® H_2O_2-560™ (Oxis International, Inc.). The assay is based on the oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) by hydrogen peroxide (H_2O_2) under acidic conditions. Fe^{3+} binds to the indicator dye xylenol orange (3,3′-bis [N,N-di (carboxymethyl)-aminomethyl]-*o*-cresolsulfone-phthalein, sodium salt) to form a stable colored complex whose absorbance can be measured at 560 nm.

Trolox equivalent antioxidant capacity assay

The Trolox equivalent antioxidant capacity (TEAC) assay is based on the scavenging of the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical converting it into a colorless product. A reaction mixture containing 20 μl of H_2O_2 (100 μmol/l), 100 μl of metmyoglobin (6.1 μmol/l), ABTS (610 μmol/l), and 4 μl of Trolox (concentration range, 0–1.65 μmol/l) was incubated and subjected to spectrophotometry (measured at 414 nm, Bio-Tek Instruments) [32].

Lipid peroxidation test

Briefly, the following reagents were added sequentially: 0.25 mM $FeSO_4$, 25 mM H_2SO_4, 0.1 mM xylenol orange, and water to a total of 0.9 ml. A sample of tissue extract (2–100 μl) was then added, and the final volume was adjusted to 1 ml with water. Blanks were prepared by replacing tissue extract with water. Samples were incubated at room temperature until the reaction was complete (30 min to 2 h), and absorbance at 580 nm was then read. With tissue samples, a further addition was then made of 10 μl of 0.8 mM H_20_2, and absorbance at 580 nm was again read.

Protein carbonyl content

The reactive carbonyl content of liver proteins was measured according to Ronchi et al. [38]. Briefly, the 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 6000×*g* for 5 min, mixed with either 0.75 ml of 2 nmol HCl or 0.75 ml of 2 nmol HCI containing 0.2% (*w*/*v*) dinitrophenylhydrazine (DNPH), and agitated for 1 h at 25 °C in the dark. Then, they were reprecipitated with 0.75 ml of 20% (*w*/*v*) TCA, washed three times with ethanol:ethyl acetate (l:l, *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 milligram of protein based on an average absorption of 21 Mm-1 cm-1 for aliphatic hydrazones.

Western blot analysis

Total protein extracts were obtained using a lysis buffer containing 30 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycolate, 0.1% SDS, and 10% glycerol. Pellets were incubated for 1 h in lysis buffer at 4 °C, sonicated for 5 s, boiled for 5 min, and centrifuged at 13,000 rpm for 10 min at 4 °C. Western blot procedure was carried out as described previously [35]. The antibodies used were the following: rabbit anti β actin (clone A2066; Sigma Aldrich); anti phospho Akt (Ser473) (CS-9271), anti phospho p44/42 MAPK (Thr202/Tyr204) (pERK1/2) (CS-9101), anti TGF-β (CS-3711), and anti Ikβα (CS-4812). Antibodies were used at 1:1000, except β actin (1:5000). Antibody binding was visualized using Amersham ECL (GE Healthcare, Madrid, Spain).

Analysis of gene expression

Ultra Clean Tissue & Cells RNA Isolation Kit (MO BIO Lab) was used for total RNA isolation. First strand complementary DNA (cDNA) synthesis was generated with MMLV reverse transcriptase (Invitrogen) and oligo dT primers and then used for reverse transcriptase polymerase chain reaction (RT-PCR). Semiquantitative PCRs were performed using the following specific primers for rat samples:

Gamma glutamyl transferase (GGT): F 5″ ATCCGAAC CTTGCCGTCCTTA 3′, R 5″ ACCACTCACCCAAC CGCCTAC 3′; Glutathione S transferase pi 1 (GST-pi1): F 5″ GATGAGGGTAAATATTTGCATCG 3′, R 5″ TGAGTCCACACCTCTGTCTACCGC 3′; ribosomal RNA 18S: F 5′ GCGAAAGCATTTGCCAAGAA 3′; R 5′ ATCACAGACCTGTTATTGC 3′.

Products were obtained after 30–35 cycles of amplification and 59–65 °C of annealing temperature and were electrophoresed in 1.5% agarose gels.

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 hematoxylin and eosin (H&E) and reticulin. The structures of the liver lobules, hepatocytes, portal tracts, sinusoidal and perisinusoidal acini, and reticular fiber networks were analyzed.

Statistical analysis

All data are presented as means ± SEM. Data comparison was performed using non parametric unpaired two-tailed Student's *t* test (InStat, Graph Pad software). *P* values lower than 0.05 were considered significant. In each experiment, two male offspring from three different mothers were separated; finally total $N = 6$, for each experimental group.

Results

Effect of a LPD during the developmental stage on body and hepatic weights in offspring rats

Body and hepatic weights were characterized in 60-day-old offspring adult male rats belonging to the different malnourished groups studied (Fig. 1a). We found a significantly lower body weight in MM group (91 ± 8; $p < 0.001$) compared to that of CC animals (163 ± 12), while it remained statistically the same in that of CM and MC (Fig. 1a). The weight of the livers in MM group was significantly lower (5 ± 0.6; $p < 0.05$) than that in control rats (7 ± 0.7) (Fig. 1b).

Measurement of serum and liver biochemical parameters in malnourished offspring rats

Liver determinations

We found higher levels of triglycerides (MM 3.05 ± 0.46; $p < 0.01$, CM 3.69 ± 0.22; $p < 0.001$ vs. CC 0.97 ± 0.19) and cholesterol (MM 4.17 ± 0.08; $p < 0.001$, CM 3.56 ± 0.06; $p < 0.001$ vs. CC 1.42 ± 0.07), and lower levels of glycogen (MM 0.34 ± 0.02; $p < 0.001$, CM 6.29 ± 3.18; $p < 0.01$ vs. CC 19.5 ± 0.50), and proteins (MM 102.86 ± 9; $p < 0.001$; CM 143.94 ± 9; $p < 0.01$ vs. CC 220.04 ± 22) in the liver's hepatocytes of MM and CM malnourished rats, when compared to those of control group (Table 1). Similarly, the MC rats' liver showed higher levels of cholesterol (3.84 ± 0.23; $p < 0.001$) and lower levels of glycogen (9.57 ± 1.43; $p < 0.001$), (Table 1). However, this group reverted the content of triglycerides to normal levels (0.9 ± 0.18) and surprisingly increased the protein content, in relation to that of the control group (292.53 ± 8; $p < 0.05$) (Table 1).

Serum determinations

We found lower levels of serum triglycerides (MM 73.6 ± 5; $p < 0.001$, CM 82 ± 6; $p < 0.001$, vs. CC 150.3 ± 9), cholesterol (MM 58.3 ± 4; $p < 0.001$, CM 61 ± 4; $p < 0.001$, vs. CC 112.6 ± 7), and proteins (MM 4.7 ± 0.3; $p < 0.001$, CM 4.5 ± 0.3; $p < 0.001$, vs. CC 6.2 ± 0.3) in MM and CM

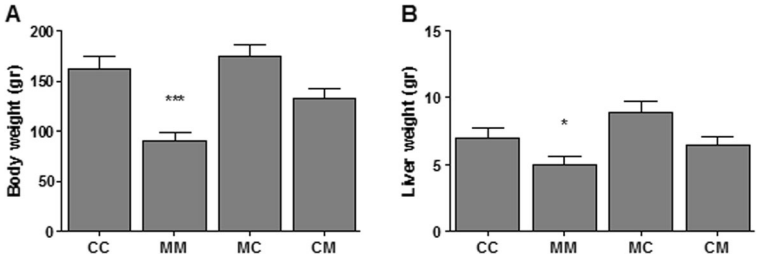

Fig 1 Effect of a LPD on body and liver weights in offspring adult male rats. Animals at 24 days of age, born from pregnant and lactating mothers fed with control diet or LPD, were fed after weaning with control diet or LPD until they were 60 days old. Body (**a**) and hepatic (**b**) weights were measured. *CC* control rats born from dams which received the control diet during pregnancy and lactation, and then after weaning were fed with control diet between days 24 and 60; *MM* rats born from dams which received the LPD during pregnancy and lactation, and then after weaning continued being fed with LPD between days 24 and 60; *CM* rats born from dams which received the control diet during pregnancy and lactation, and then after weaning received the LPD between days 24 and 60; and *MC* rats born from dams which received the LPD during pregnancy and lactation, and then after weaning were provided with the control diet between d 24 and 60. Results were expressed in grams. The mean ± SEM is plotted. *, significant difference ($p < 0.05$); *** ($p < 0.001$) compared to those of CC group; $n = 6$ for each group

malnourished groups, compared to those of control rats (Table 2). We also examined whether a LPD modified the activity of two enzymes commonly known as molecular markers of liver damage (Table 2). MM and CM rats showed an increase in SGPT activity (MM 217.3 ± 11; $p < 0.001$, CM 195 ± 11; $p < 0.001$, vs. CC 92 ± 7), while SGOT activity only increased in MM group (391 ± 25; $p < 0.001$ vs. CC 220 ± 18), when compared to those of CC (Table 2). Rats in MC group reverted all the serum parameters studied to normal levels (Table 2). The content of glucose did not differ between groups (data not shown).

Serum levels of pro-inflammatory cytokines in offspring rats fed with a LPD

The values of serum pro-inflammatory cytokines were more elevated in MM and CM groups, compared to those of CC group. TNF-α: MM (206 ± 11; $p < 0.001$), CM (128 ± 6; $p < 0.001$), vs. CC (18 ± 1.8), (Fig. 2); IL-6: MM (63 ± 4; $p < 0.001$), CM (51 ± 3; $p < 0.001$), vs. CC (28 ± 1.5) (Fig. 2). However, the highest values of cytokines were found in MM malnourished rats: TNF-α: (MM vs. CM $p < 0.001$) and IL-6: (MM vs. CM $p < 0.05$). Rats in MC group reverted the values of the cytokines evaluated to normal levels after a nutritional rehabilitation at weaning: TNF-α (25 ± 4); IL-6 (31 ± 5) (Fig. 2).

Malnutrition leads to enhanced oxidative stress

ROS production and oxidative stress are important cellular hallmarks for apoptosis and/or transformation processes [6, 10]. For this reason, we decided to measure different oxidative stress markers in the liver of rats fed with a LPD. We found that all markers studied: ROS production (MM 88.20 ± 3.71; $p < 0.001$, CM 99.70 ± 3.80; $p < 0.001$, MC 29.70 ± 1.43; $p < 0.001$, vs. CC 9.8 ± 1.52) (Fig. 3a), lipid peroxidation (MM 530 ± 18.78; $p < 0.001$, CM 435.2 ± 11.18; $p < 0.001$, MC 418.6 ± 8.49; $p < 0.001$, vs. CC 284.2 ± 10.29) (Fig. 3b), content of carbonyl groups in liver proteins (MM 26 ± 0.89; $p < 0.001$, CM 22 ± 0.44; $p < 0.001$, MC 15 ± 0.89; $p < 0.01$, vs. CC 10 ± 0.89) (Fig. 3c), and level of total antioxidant capacity (MM 391 ± 9.83; $p < 0.001$, CM 288 ± 8.94; $p < 0.001$, MC 275 ± 8.05; $p < 0.001$, vs. CC 220 ± 6.70) (Fig. 3d) were increased in the liver of all malnourished groups of animals, compared to those the control (Fig. 3a–d). However, the liver of MC rats showed the lowest increment in ROS production (MC vs. MM; $p < 0.001$, MC vs. CM; $p < 0.001$), and the liver of MM malnourished rats showed the highest increment in lipid peroxidation levels

Table 1 Hepatic metabolites content. Tissue lipids, glycogen, and total proteins: cholesterol and triglycerides, after homogenized and chloroform/methanol (2:1)-extracted, were determined by conventional enzymatic methods (Wiener Lab, Rosario, Argentina). Glycogen, following the protocol described in the "Methodology section" and afterwards determined by the technique of anthrone reagent. Protein concentration was measured with bicinchoninic acid protein assay. Results were expressed in mg/g of liver tissue, by the mean ± SEM. *, significant difference ($p < 0.05$); **, ($p < 0.01$); ***, ($p < 0.001$) compared to those of control; $n = 6$ for each group

Groups	Glycogen	Triglycerides	Cholesterol	Proteins
CC	19.5 ± 0.50	0.97 ± 0.19	1.42 ± 0.07	220.04 ± 22
MM	0.34 ± 0.02***	3.05 ± 0.46**	4.17 ± 0.08***	102.86 ± 9***
MC	9.57 ± 1.43***	0.9 ± 0.18	3.84 ± 0.23***	292.53 ± 8*
CM	6.29 ± 3.18**	3.69 ± 0.22***	3.56 ± 0.06***	143.94 ± 9**

Table 2 Serum metabolites. Serum proteins (g/dl), triglycerides (mg/dl), and cholesterol (mg/dl) concentrations were determined by enzymatic assays from Wiener Laboratories, Argentina. SGOT and SGPT were expressed in UI/l and measured by using a commercial kit (Wiener Lab, Rosario, Argentina). Results were expressed by the mean ± SEM. ***, significant difference ($p < 0.001$), compared to those of control; $n = 6$ for each group

Groups	Triglycerides mg/dl	Cholesterol mg/dl	Proteins g/dl	SGOT UI/l	SGPT UI/l
CC	150.3 ± 9	112.6 ± 7	6.2 ± 0.1	220 ± 18	92 ± 7
MM	73.6 ± 5***	58.3 ± 4***	4.7 ± 0.3***	391 ± 25***	217.3 ± 11***
MC	140.3 ± 11	89 ± 8	5.9 ± 0.2	187 ± 21	95 ± 7
CM	82 ± 6***	61 ± 4***	4.5 ± 0.3***	275 ± 22	195 ± 11***

(MM vs. MC; $p < 0.001$, MM vs. CM; $p < 0.01$), oxidized proteins (MM vs. MC; $p < 0.001$, MM vs. CM; $p < 0.0001$), and total antioxidant capacity (MM vs. MC; $p < 0.001$, MM vs. CM; $p < 0.001$).

Expression levels of GGT and GST-pi1 enzymes in the liver of malnourished offspring rats

Because GGT and GST-pi1 are markers of liver damage [1, 23], we decided to evaluate the expression of these enzymes using a semiquantitative RT-PCR in the liver of rats fed on a LPD. Figure 4 shows the relative expression of GGT and GST-pi1. Expression levels of both enzymes were significantly higher in all the malnourished groups compared to those of the control: GGT (MM 9.36 ± 0.8; $p < 0.001$, MC 1.98 ± 0.1; $p < 0.001$, CM 2.59 ± 0.2, $p < 0.001$, vs. CC 0.11 ± 0.01) and GST-pi1 (MM 8.82 ± 0.7, $p < 0.001$, MC 2.21 ± 0.3, $p < 0.05$, CM 4.88 ± 0.36, $p < 0.001$, vs. CC 1.09 ± 0.1) (Fig. 4). However, the liver of MC rats showed the lowest increment in GST-pi1 expression (MC vs. MM; $p < 0.001$, MC vs. CM; $p < 0.01$), and the liver of MM malnourished rats showed the highest increment in the expression of both enzymes: GGT (MM vs. MC; $p < 0.001$, MM vs. CM; $p < 0.01$) and GST-pi1 (MM vs. MC; $p < 0.001$, MM vs. CM; $p < 0.01$).

Effect of a LPD on the proliferative capacity vs. sensitivity to apoptosis in the offspring rats' hepatocytes

The cytokine TGF-β, a physiological inducer of hepatocyte apoptosis [29, 47], was altered in the livers of CM and MM malnourished rats. These groups showed higher levels of TGF-β compared to those of the control: MM (2.4 ± 0.2; $p < 0.01$), CM (1.52 ± 0.1; $p < 0.05$), vs. CC (0.9 ± 0.1) (Fig. 5a, b). Likewise, malnutrition induced a decrease in the levels of phosphorylated IκBα, an inhibitor of NF κB, but only in the MM rats' liver (0.36 ± 0.02) vs. CC (1.16 ± 0.2); $p < 0.05$, (Fig. 5a, c). Interestingly, pro-survival signaling pathways appeared to be activated in the liver of malnourished rats. We found higher levels of pAKT in all groups of animals fed on a LPD, in comparison to those of the control: MM (1.94 ± 0.11; $p < 0.001$), MC (1.01 ± 0.05; $p < 0.01$), CM (1.52 ± 0.05; $p < 0.001$), vs. CC (0.56 ± 0.05) (Fig. 5a, d). However, the liver of MC rats showed the lowest increment of pAKT (MC vs. MM; $p < 0.01$, MC vs. CM; $p < 0.01$). The enzyme pERKs was increased in MM (1.94 ± 0.11; $p < 0.01$) and CM (1.52 ± 0.05; $p < 0.05$) malnourished rats, compared to those of CC (1.17 ± 0.05) (Fig. 5a, e). However, the MM rats' liver showed the highest increment of pERKs (MM vs. CM; $p < 0.05$), as well as pAKT enzyme (MM vs. MC; $p < 0.01$, MM vs. CM; $p < 0.05$).

Liver histology of malnourished offspring adult rats

The liver of CC rats showed the typical lobular structure with normal central veins and portal tracts (Fig. 6a). The hepatocytes showed normal cytological structures with well-preserved staining and morphological characteristics of the cytoplasm and nucleus. Liver sections from MM rats showed a completely altered

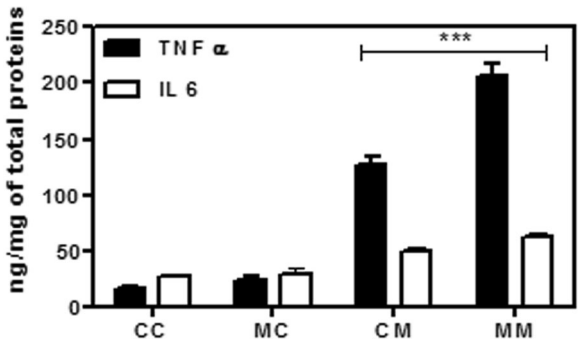

Fig. 2 Increment of pro inflammatory cytokines production in response to a LPD in offspring rats. Serum IL-6 (*white bars*) and TNF-α (*black bars*) levels were quantified by enzyme linked immunosorbent assay (ELISA) kits (BD Biosciences PharMingen). Results were expressed by the mean ± SEM. ***, significant difference ($p < 0.001$), compared to those of control; $n = 5$ for each group

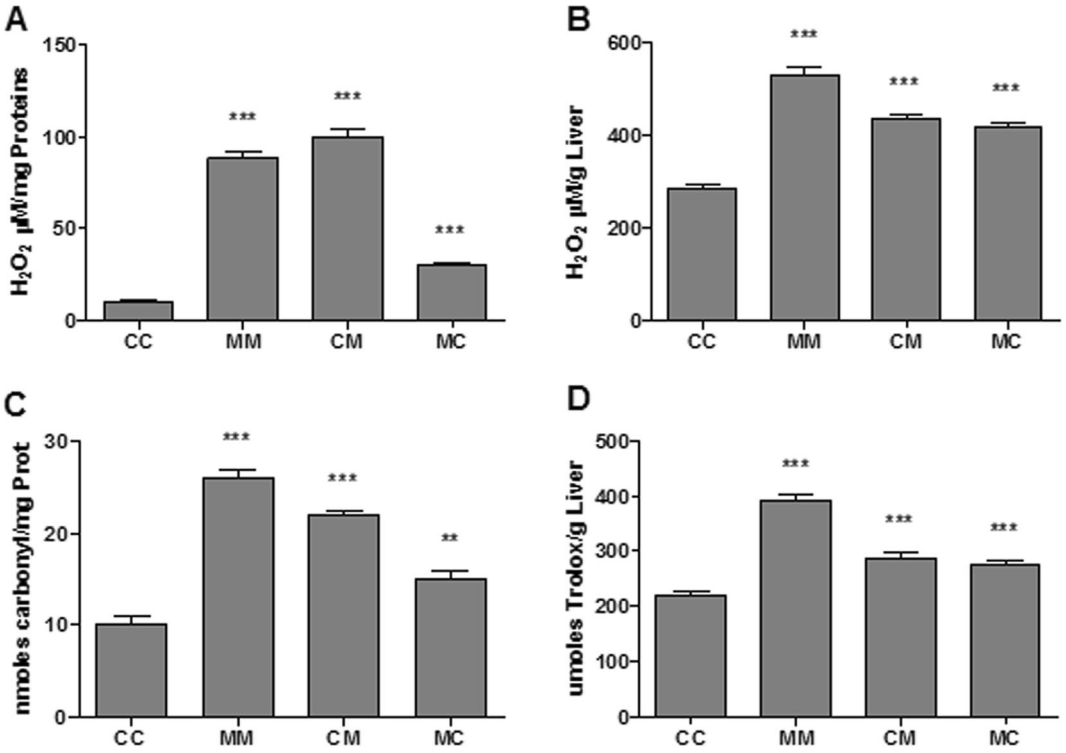

Fig. 3 Protein restriction diet induces changes in the oxidative status of hepatocytes. The levels of ROS (**a**), lipid peroxidation (**b**), carbonyl groups in liver proteins (**c**), and total antioxidant capacity (**d**) were determined for each nutritional condition: CC, MM, CM, and MC. The rate of cytosol H_2O_2 production was determined by using the BIOXYTECH® H_2O_2-560™ (Oxis International, Inc.). For lipid peroxidation tests, xylenol orange was used. The content of carbonyl groups in liver proteins was evaluated by their reaction with DNPH. For the antioxidant capacity assays, a solution of Trolox was employed. Results were expressed by the mean ± SEM. **, significant difference ($p < 0.01$); ***, ($p < 0.001$), compared to those of control; $n = 5$ for each group

hepatic architecture. The nuclei were displaced by large lipid vacuoles (Fig. 6a). In MC group, the hepatic structure was partially preserved. The presence of cellular swelling was detected (Fig. 6a). Liver sections from CM rats displayed a large quantity of lipid vacuoles; however, we observed a considerable number of normal hepatocytes (Fig. 6a). Figure 6b shows reticulin staining. Since reticulin provides the stromal support for the parenchyma, the reticulin stain provides important information about the architecture of the liver. When hepatocytes are damaged and undergo necrosis, the reticulin fibers surrounding them collapse in the empty space left behind. Areas of reticulin crowding thus indicate focal hepatocyte loss. Large areas of cell necrosis appear as reticulin collapse. In liver sections of CC rats, we observed an orderly and normal position directly related to the provision of the hepatic parenchyma preserved (Fig. 6b). In MM group, we found a dashed fiber network characterized by the presence of agglomerates thereof (Fig. 6b). In MC rats, we observed that the arrangement of reticular fibers was similar to that one in controls, with an orderly disposition (Fig. 6b). Finally, liver sections of CM rats showed a fiber network partially preserved but dashed due to the presence

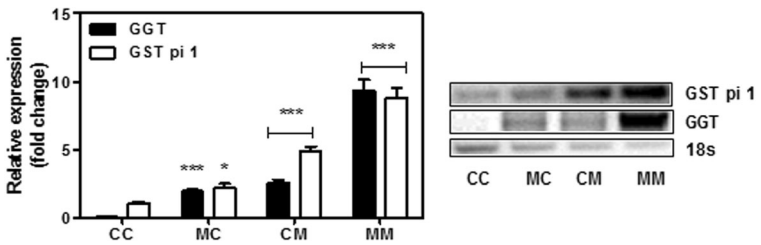

Fig 4 Analysis of GGT and GST-pi1 expression levels in the liver of malnourished offspring rats. RNA was collected for semiquantitative PCR analysis. The ribosomal RNA 18S was used as a control of DNA integrity. The *right panel* shows a representative experiment and the *left panel* indicates the densitometric analysis of at least three independent experiments. Results were expressed by the mean ± SEM. *, significant difference ($p < 0.05$), ***, ($p < 0.001$), compared to those of CC group

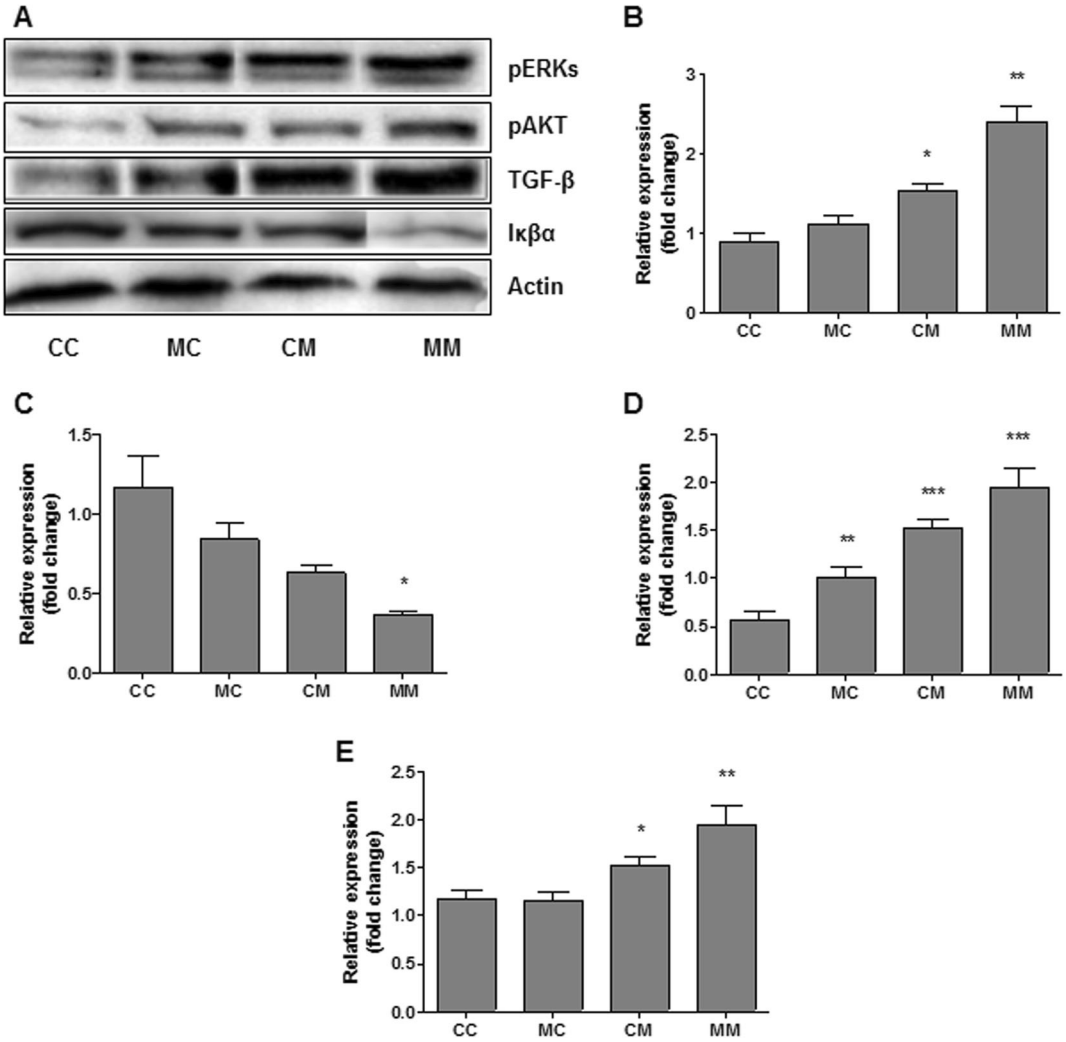

Fig 5 Western blot for the analysis of TGF-β, Iκβα, pERKs, and pAKT levels in the malnourished rats' hepatocytes. β actin was used as loading control. A representative experiment is shown (a). **b** TGF-β, **c** Iκβα, **d** pAKT, and **e** pERKs indicate the densitometric analysis of at least three independent experiments in each case. Results were expressed by the mean ± SEM. *, significant difference ($p < 0.05$); **, ($p < 0.01$); ***, ($p < 0.001$) compared to those of CC

of broken fibers (Fig. 6b). Fibrous tissue composed of collagen type I appears brown on a reticulin stain and thus can be distinguished from reticulin fibers.

Discussion

IUGR occurs as a consequence of poor maternal nutrition and has been associated with the development of different illnesses, a phenomenon called fetal programming [3]. Nutrition can influence this phenomenon in a gender-related manner. An unfavorable programming could thus lead to different susceptibilities to diseases between males and females, though the underlying cause of the sexual dimorphism reported until now is unclear [12, 28]. This work was aimed at addressing the effects of protein malnutrition on liver morphology and physiology particularly in male rats subjected to different malnutrition schemes during the critical developmental stages (gestation, lactation, and childhood). Rats received a LPD during the gestation, lactation and until they were 60 days old after weaning (on day 24), or received a late LPD that began after weaning, or a LPD administrated only during the gestation-lactation period followed by a control diet. In this study, we found a decrease in the total body and liver weight of rats malnourished during gestation, lactation, and up to the 60th day of life (MM group), which is in agreement with our previous results [35] and with other authors' reports [8, 9, 14, 16]. This is consistent with the reduction of serum triglycerides, cholesterol, and proteins; and glycogen and proteins liver content found in these rats. Although we saw a similar tendency in serum and liver biochemical parameters found in rats which received a late LPD (CM group), their reduction on body and hepatic weight did not reach a statistical significance, which highlights the importance of

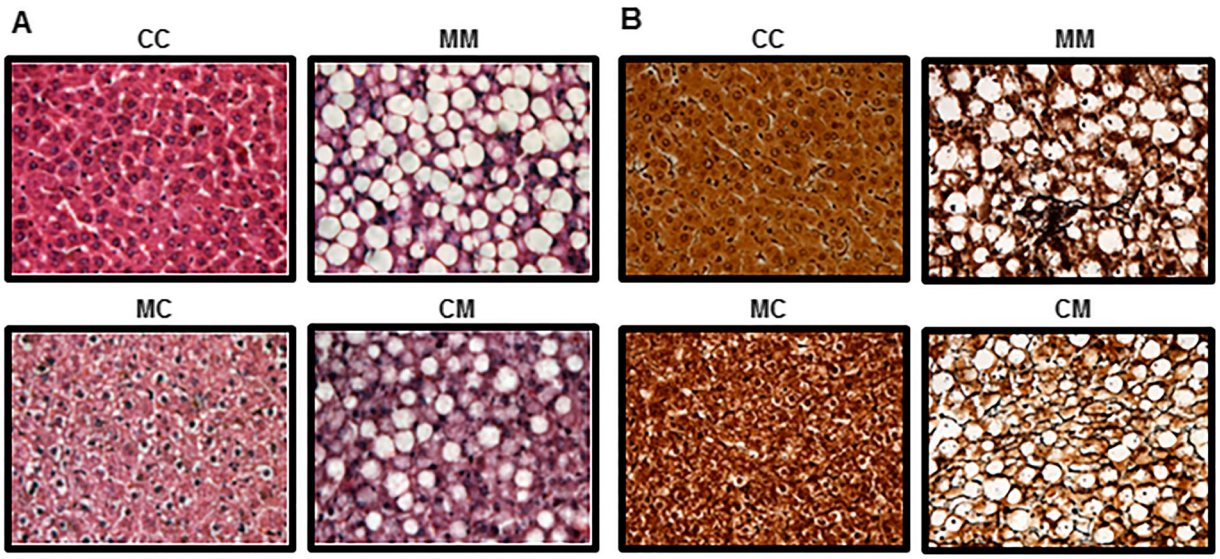

Fig 6 Histology of the liver in malnourished 60-day-old offspring rats. **a** Liver H&E stain, ×400. The liver of *CC* rats showed the typical lobular structure with normal central veins and portal tract. The liver of *MM* rats showed a completely altered hepatic architecture. The nuclei were displaced by large lipid vacuoles. The liver of *MC* group showed a partially preserved hepatic structure with cellular swelling. The liver of *CM* rats displayed a large quantity of lipid vacuoles, but likewise a considerable number of normal hepatocytes. **b** Liver reticulin stain, ×400. The liver of *CC* rats showed an orderly and normal disposition of the fibers. The liver of *MM* rats showed a dashed fiber network. The liver of *MC* rats showed an arrangement of reticular fibers similar to those of CC group. The liver of *CM* rats showed a fiber network partially preserved but dashed due to the presence of broken fibers. A representative experiment is shown

maternal nutrition in the growth of their offspring. Surprisingly, offspring rats fed with a control diet after being weaned from mothers which had received a LPD during pregnancy and lactation (MC group) showed body and hepatic weights similar to that of the control group and reverted all the serum biochemical parameters studied to normal values, including SGOT and SGPT levels, which have been reported as markers for liver damage [48]. According to Desai M. and others, the factor that determines whether the animal recovers appears to be the time at which malnutrition occurs. The earlier the undernutrition, the less likely the recovery after the insult is discontinued [8]. In contrast to MC group, MM and CM groups showed obvious liver dysfunction reflected on the increase in serum SGOT and SGPT enzymes and liver cholesterol and triglycerides, which would suggest that hepatocytes suffered alterations affecting their integrity in addition to what we saw by histology. Liver dysfunction was also observed by the increase in GGT and GST-pi1 expression levels. GGT is a marker of hepatic injury [23], used extensively in humans [15, 21, 42] and rats [7, 23, 42]. Some authors have reported that high levels of GGT are associated with risk of atherosclerotic cardiovascular disease, fatty liver, insulin resistance, type 2 diabetes, obesity, and other metabolic risk factors [15]. In particular, striking rises of serum GGT are common at the alcoholic fatty liver stage, and its determination is useful for assessing early stages of liver disease due to alcohol abuse [42]. GST-pi1 is a marker for hepatotoxicity in rodent system, which also plays an important role in carcinogenic detoxification [1]. We found higher expression levels of both enzymes in all the malnourished groups compared to those of the control, even in MC rats. However, the liver of this last group showed the lowest increment in GST-pi1 expression. Our results are in part in accordance with those obtained by Vianna de Oliveira I.M. and Fujimori E., who found a significant rise of the pups' liver GGT activity promoted by protein restriction at day 10 of lactation, indicating a special influence of protein malnutrition during gestation and lactation on this parameter of hepatic injury [7]. In addition, Caballero and co-workers found that 2-month-old female Balb/c mice subjected to three cycles of 5 days of protein-free diet followed by 5 days of control diet produced an increase in GST-pi1 messenger RNA (mRNA) level and even more, increased the serum activities of SGOT and SGPT [5], which is consistent in part with our observations. In relation to MC group, the results obtained suggest that perinatal administration of a LPD induced an irreversible long-term imprinting effect on different liver biochemical parameters in offspring adult male rats, although some parameters reverted to normal levels after a nutritional rehabilitation at weaning.

It has been shown that protein malnutrition causes oxidative stress in liver [24, 36], which is an important cellular hallmark for apoptosis and/or transformation processes [10, 19]. Moreover, increased ROS production has been observed at different stages of liver disease and in HCC [17, 19, 43]. In this work, we found all markers of oxidative stress studied (ROS production, lipid peroxidation, content of carbonyl groups in liver proteins, and level of total antioxidant capacity) increased in the liver of all groups of malnourished

animals. However, the liver of MM rats showed the highest hepatic injury. We also observed higher TNF-α and IL-6 levels in the serum of MM and CM malnourished groups. However, the rats which received a nutritional rehabilitation post weaning (MC group) reverted the values to normal levels similar to those found in control rats. In the liver, the major source of TNF-α and IL-6 is the Kupffer cells, which constitute 15% of the total liver cell population [26]. IL-6 and TNF-α are cytokines produced during inflammatory processes [10]. Increased expression of pro-inflammatory cytokines is associated with hepatocyte apoptosis but is also essential in hepatocyte proliferation [20] and liver regeneration [46]. By binding to its receptors (TNFR1/TNFR2), TNF-α activates two different signaling cascades either leading to apoptosis or to proliferation [4]. In the latter case, TNF-α-stimulation leads to IKK activation resulting in IκBα phosphorylation and subsequent IκB degradation. NF kB translocates into the nucleus inducing downstream gene targets. Activation of NF kB ultimately results in enhanced proliferation and cell survival [19]. We did not find increased levels of IκBα in the malnourished groups. The liver of MC and CM rats showed levels of this inhibitor of NF κB similar to those observed in the control group, while livers from MM group showed lower levels compared to that of the control. These results suggest that TNF-α would not be activating the signaling cascade leading to proliferation, and instead it would be inducing apoptosis, which is in agreement with the TGF-β levels found in malnourished rats. It is well-known that TGF-β signaling pathway also plays a critical dual role in cancer progression. At an early phase, it acts as a tumor suppressor, inducing apoptosis, while later its role switches to tumor progression [29, 47]. In this study, we observed altered levels of TGF-β in the liver of MM and CM malnourished rats, which were higher compared to those of control group, while MC group reverted the levels to control values. Interestingly, however, pro-survival signaling pathways mediated by pAKT and pERKs appeared to be activated in the liver of all groups of malnourished rats, even pAKT levels in MC group. Therefore, although a LPD induced an inflammatory microenvironment that predisposed hepatocytes to apoptosis, pro-survival signals may indicate the presence of cells resistant to programmed cell death which would become cancerous.

In conclusion, a LPD induces structural and functional liver damage whose magnitude is related to the developmental stage at which malnutrition occurs and to its length.

In this study, we found as expected that MM rats showed the highest liver damage. However, rats fed on a LPD after weaning (CM group) also showed hepatic injury although to a lesser extent. In relation to MC group, it is important to highlight that even though most of the parameters studied reverted to control levels after a nutritional rehabilitation at weaning, the results obtained in this work altogether suggest that perinatal administration of a LPD induced an irreversible long-term imprinting effect in the liver of offspring adult male rats, at least at the time studied. Our results support the hypothesis of IUGR-fetal programming and contribute to demonstrate that protein malnutrition during the developmental stage predisposes to suffer metabolic diseases in adulthood, which could even end in a precancerous or cancerous condition.

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Author contributions Conceived and designed the experiments: SC, EP, SME, and ANC. Analyzed the data: SC, EP and ANC. Wrote the first draft of the manuscript: SC. Contributed to the writing of the manuscript: SC and ANC. Agree with manuscript results and conclusions: SC, EP, SME, and ANC. Jointly developed the structure and arguments for the paper: SC and ANC. Made critical revisions and approved final version: SC, EP, SME, and ANC. All authors reviewed and approved the final manuscript.

Compliance with ethical standards

All experiments were approved by our Institutional Animal Care Committee (RD 140/15).

Conflict of interest The authors declare that they have no conflict of interest.

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