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Maternal high-fat diet consumption programs male offspring to mitigate complications in liver regeneration

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Abstract

In the last decades, obesity and nonalcoholic fatty liver disease (NAFLD) have become increasingly prevalent in wide world. Fatty liver can be detrimental to liver regeneration (LR) and offspring of obese dams (HFD-O) are susceptible to NAFLD development. Here we evaluated LR capacity in HFD-O after partial hepatectomy (PHx). HFD-O re-exposed or not to HFD in later life were evaluated for metabolic parameters, inflammation, proliferation, tissue repair markers and survival rate after PHx. Increasing adiposity and fatty liver were observed in HFD-O. Despite lower IL-6 levels, Ki67 labeling, cells in S phase and Ciclin D1/PCNA protein content, a lower impact on survival rate was found after PHx, even when re-exposed to HFD. However, no difference was observed between offspring of control dams (SC-O) and HFD-O after surgery. Although LR impairment is dependent of steatosis development, offspring of obese dams are programmed to be protected from the damage promoted by HFD.

Introduction

Nonalcoholic fatty liver disease (NAFLD) may be defined as the hepatic manifestation of a metabolic syndrome.[1] It is characterized by accumulation of hepatic fat in the absence of insults like drugs or alcohol consumption and inherited disturbances.[2,3] The NAFLD affects between 25% and 45% of the population, independently of age, gender, and ethnicity.[2,4] It has been associated to diabetes mellitus, dyslipidemia, obesity, and the consumption of high-calorie diets, which are important factors that lead to hepatic lipid accumulation.[5]

Many different experimental models have been used to investigate NAFLD,[6–13] and the literature shows that maternal diets play an important role in offspring NAFLD development.[14,15]

In primates, the occurrence of nonalcoholic steatosis (NASH) and metabolic diseases was reported due to the exposure to lipids excess from maternal diet,[16] Previous data from our group using a mice model showed that maternal high-fat diet (HFD) consumption lead to fatty liver and insulin resistance in adult mice offspring.[17,18] However, HFD re-exposure in adulthood aggravated both metabolic disturbances.[19,20] Surprisingly, recently we found that survival rate after LPS-induced sepsis in mice offspring of HFD dams (HFD-O) was higher than offspring of control dams (SC-O), suggesting that despite the impairment in insulin signaling and liver lipid homeostasis, maternal programming induced by HFD confer advantage to offspring (data not shown).

It is estimated that about 50% of individuals with NAFLD progress to NASH and may need some type of liver resection surgery.[21] The liver has a unique and complex regenerative capacity, and when 70% or more of the organ is removed (partial hepatectomy-PHx), the replication of existing hepatocytes without activation of cell progenitors is observed.[22] During this process, an inflammatory response driven by interleukin-6 (IL-6) occurs, culminating in the activation of several genes involved in cell replication.[22,23]

The liver regeneration (LR) process has been studied in order to uncover all the factors involved with this highly regulated condition whose success is essential for the survival of the individual in cases of surgery. Presence of hepatic steatosis and obesity, have been related to impairment in regeneration after liver resection, probably due to preexisting inflammation.[24] However, studies that used experimental models of diet induced obesity, such as high-sucrose

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diet, high-fructose diet, HFD, high-cholesterol diet, choline-methionine-deficient diet, show development of different degrees of steatosis and inflammation,[6–13] probably due to the type of diet and consumption time used in each model. Although offspring of dams whose obesity was induced by a high-calorie diet developed hepatic steatosis, there are no studies in the literature that investigated the LR process in the context of metabolic programming, i.e., without the direct dietary interfering.

In this study, we hypothesized that although steatosis and greater adiposity are complicating factors for LR, maternal consumption of HFD during pregnancy and lactation, despite promoting liver steatosis, could provide protection to the offspring mitigating an impairment in LR. Our results suggest that HFD-O mice reached success in regenerating the liver after PHx even when rechallenged to HFD in later life for short-term.

Methods

Experimental design

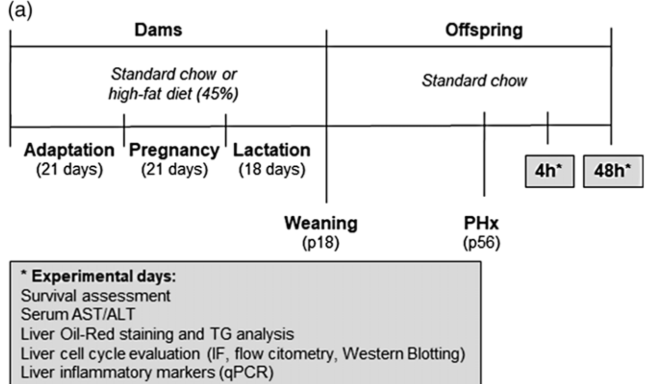
Swiss (Unib:SW) female mice (5 weeks old, $n = 10$ per group) were provided by the State University of Campinas Animal Breeding Center (CEMIB, Brazil) and randomly separated into two groups: group fed standard chow (SC, composed of 73% kcal carbohydrate, 17,5% kcal protein, and 9,5% kcal lipids) and group fed HFD (for growth, composed of 35% kcal carbohydrate, 19% kcal protein, and 45% kcal lipids) *ad libitum* during adaptation period, gestation, and lactation (Fig. 1a). All mice were maintained in individual polypropylene micro-isolators at 22 ± 1°C and lights on from 06:00 to 18:00 h. Swiss males from the same age fed SC were used for mating (2 females:1 male). After birth, both litters of control dams (SC-O) and HFD dams (HFD-O) were adjusted to eight male pups per dam and females were discarded. Offspring were weaned and fed SC *ad libitum* until 8-week-old (p56), when they were subjected to PHx procedure. In order to evaluate the effect of acute re-exposure to HFD in adulthood on LR, the offspring were fed HFD (for maintenance) for 3 days (p53 to p56) prior to PHx and evaluated for 7 days after surgery (p63) (Fig. 1b). One male pup of each litter was used for analysis. HFD were prepared according to AIN-93 Guidelines, as described previously[19] and Nuvilab® CR-1 commercial feed was used as standard chow. All mice were culled by decapitation after receiving a mixture containing ketamine (139.2 mg/kg bw), diazepam (4 mg/kg bw), and xylazine (18.4 mg/kg bw).

Induction of hepatic regeneration by 2/3 partial hepatectomy (PHx)

To assess liver regenerative capacity, a PHx procedure was performed at p56 and 2/3 of the liver was removed under anesthesia with a mixture of isoflurane and oxygen (2%:2L/min), as previously described.[25] After surgery mice received analgesia immediately and every 24 h until the sacrifice (carprofen – 10 mg/kg of body weight). Analyses were performed using the same animal as control, with time 0 h referring to the left lobe removed during surgery. For 4 and 48 h after PHx, the right lobe was evaluated.

Immunoblotting

Liver samples (100 mg) were homogenized in RIPA buffer and protein concentrations were determined using Biuret Reagent. 50 ug of proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking the membrane was incubated overnight with antibodies to p-STAT3 (Cell Signaling Technology, #9145, 1:1000), STAT3 (Santa Cruz Biotechnology, sc-482, 1:500), p-ERK1/2 (Cell Signaling Technology, #9106, 1:1000), Cyclin D1 (Cell Signaling Technology, #2978, 1:1000), PCNA (Abcam, ab29, 1:1000), β-actin as endogenous control (Abcam, ab8227, 1:1000) and then incubated with a secondary antibody. Band intensity was detected by chemiluminescence in GeneGnome equipment (Syngene) and evaluated by densitometry using Scion Image software (Scion Corporation).



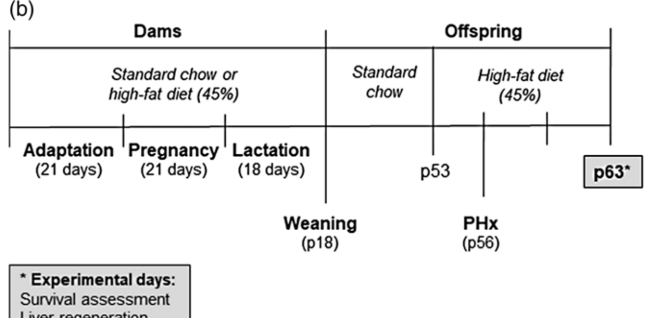


Fig. 1. Experimental design. Dams were randomly fed standard chow (SC, 9.5% fat) or a high-fat diet (HFD, 45% fat) during an adaptation period, pregnancy, and lactation. They were mated with standard chow male mice and at the delivery day were adjusted to eight pups per litter (at least three different litters per group). After weaning, offspring was only fed SC until p56 (protocol **a**) or p53 (protocol **b**, when the diet was switched to HFD). At p56 offspring from SC-O or HFD-O were subjected to 2/3 hepatectomy (PHx) and euthanized on 0, 4, and 48 h postoperation (protocol **a**) or on 7 days postoperation (protocol **b**).

Real-time quantitative PCR (qPCR)

Total RNA from liver samples (50mg) was extracted using RNAzol reagent (Sigma-Aldrich) according to the manufacturer's recommendations and quantified using NanoDrop ND-2000. Reverse transcription was performed with 3 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Relative expression was determined using a Taqman system and primers for the following targets: Gfer, Tnfα, Il1β, and Il6. Rplp0 was used as endogenous control. Each PCR contained 20 ng of complementary DNA and was performed on the ABI 7500 Fast System. Data were expressed as relative values determined by the threshold cycle comparison method (2-ΔΔCt), according to the manufacturer's recommendation.

Immunofluorescence

For immunofluorescence analyses, 5 µm of liver frozen sections were fixed in 4% paraformaldehyde solution for 30 min, washed

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in PBS and then incubated in PBS solution containing 0.5% Triton X-100 and 0.05% SDS for 30 min. After washing, slides were blocked with 1% albumin diluted in PBT (0.1M PBS + 0.25% Triton X-100) for 2 h at room temperature. Slides were then incubated with Ki67 (Abcam, ab15580, 1:100) and CK18 (Abcam, ab668, 1:100) primary antibodies diluted in blocking solution overnight at 4°C. The sections were visualized using IMMU-Mount immunofluorescence medium (Thermo Fisher Scientific) with DAPI solution (Sigma, D9542, 1:2000) in a fluorescence optical microscope (LEICA DMI 4000B) under 200× magnification and quantified with Image J software.

Oil red

Lipid droplets were analyzed in 5 µm of liver frozen sections by oil red staining (Oil Red O, Sigma-Aldrich) as described by Mehlem *et al.*[26] Images were obtained in an optical microscope (LEICA DMI 4000B) under 200× and 1000× magnification.

Serum determination of alanine and aspartate amino transferase (ALT/AST)

Blood samples were centrifuged at 2500 rpm for 30 min and serum ALT/GLP and AST/GOT were detected using enzymatic kits (Labtest) according to the manufacturer's instructions.

Liver triglyceride measurement

Lipids from liver tissues were extracted by the Folch method. Briefly, hepatic samples (200 mg) were homogenized in 4-ml Folch solution (2:1 v/v chloroform/methanol) and centrifuged at 2500 rpm for 10 min. Around 3 ml of the lower phase (lipid-containing fraction) was collected and dried (evaporated) and lipids were resuspended in 1 ml of isopropanol. Triglyceride (TG) contents were measured with the GPO-PAP assay kit (Roche Diagnostics), according to the manufacturer's instructions.

Cell cycle assay

In order to determine the distribution of cell cycle stages, hepatocytes were extracted and isolated according to Severgnini *et al.*[27] 5×10^5 cells/mL were resuspended in 100 ul of PBS + propidium iodide (ImmunoChemistry Technologies) at a concentration of 20 ug/ml and flow cytometric analysis was performed in a BD Accuri C6 cytometer on channels FL2 (585/40 nm) and FL3 (610/20 nm). A total of 10,000 events were counted in gate P2.

Statistical analysis

The results are expressed as mean±SEM. Student's *t*-test was used to compare two groups. One-way, Two-Way or Factorial Analysis of variance (ANOVA) was applied for multiple comparisons, and a post hoc test (Bonferroni) was used to determine a significance level of $p \leq 0.05$. Considering a statistical power of 80%, with a type I error (alpha) of 5% and taking into account the three R's concept of Russell and Burch[28] a minimum sample size of 5 animals was applied. Statistical analysis of each result is described in each figure legend. Data were analyzed using GraphPad Prism, version 7 (GraphPad Software, Inc. USA).

Results

Effects of maternal obesity on liver regenerative capacity of male offspring

Since the increase in BMI negatively impacts liver regenerative capacity in humans (2) and offspring of obese dams (HFD-O) are programmed to develop an obesogenic phenotype, we evaluated if alterations in hepatic regenerative capability could be detected in HFD-O.

HFD dams presented increased weight gain from week 2 to 4 of the adaptation period and higher body weight before mating, despite no differences being observed in caloric intake (Supl. Fig. 1).

The HFD-O group presented higher body weight from the second week of life compared to offspring of control dams (SC-O) (Fig. 2a), as shown in our previous studies.[18–20] At postnatal day 56 (p56), the HFD-O group also presented greater adiposity (2.4-fold, $p < 0.05$), demonstrating characteristics of an obese phenotype triggered by metabolic programming (Fig. 2b).

After PHx, the SC-O group had 100% survival rate, while the HFD-O group was only slightly lower, about 90% survival rate (Fig. 2c). In order to understand the regenerative response, the liver was analyzed by investigating the factors involved in initial and late postoperative response. AST and ALT activity after PHx was evaluated (Fig. 2d, 2e). As expected, these enzymes reached high indices with 4 h (more than 21-fold for AST and ALT, $p < 0.05$) and then returned to basal levels in 48 h, in both groups. Both SC-O and HFD-O mice developed transient steatosis due to higher TG accumulation after 48 h (Fig. 2f, 2g).

Maternal diet seemed to differentially affect hepatocytes ability to progressing in the cell cycle. The HFD-O group showed lower Ki67 positive labeling (twofold, $p < 0.05$) in hepatocytes (Fig. 3a, 3b), as well as few cells in the S phase 48 h after PHx compared to SC-O (6.8% HFD-O *vs* 16.3% SC-O) (Fig. 3c). On the other hand, the percentage of cells in subG0/G1 phase was greater in the HFD-O group (13.0% HFD-O *vs* 3.5% SC-O) at the same time point (Fig. 3d, 3e, 3f, 3g). We also evaluated mRNA levels of *Gfer*, a gene that encodes hepatopoietin, described as to promote hepatocytes growth in the regenerating liver. The results showed similar tendency between SC-O and HFD-O group regarding *Gfer* expression, which diminished 4 h after PHx and returned to baseline levels 48 h after PHx, demonstrating that the gene was not modulated in offspring by the maternal diet (Fig. 3h).

Regarding the inflammatory markers, after 4 h PHx, only IL6 levels increased in the liver of SC-O group (Fig. 4a). The analysis of the IL6 signaling pathway showed that phosphorylation of STAT3 occurred 4 h after PHx in both groups, which is consistent with the initial inflammatory response of the regenerative process (Fig. 4b), even though there was no pronounced increase in IL6 in the HFD-O group. After 48 h, p-STAT3 levels showed a slightly increase in SC-O compared to HFD-O (Fig. 4c). In order to evaluate the proteins involved in cell proliferation, MAP Kinase ERK1/2 phosphorylation was determined as well as cyclin and PCNA content. p-ERK1/2 remained lower 4 h and 48 h after PHx in both SC-O and HFD-O (Fig. 4b, 4c). Consistent alterations in cyclin and PCNA content were observed only as a result of late postoperative response (48 h after PHx), despite the surprising PCNA reduction in both SC-O and in HFD-O 4 h after PHx. Accordingly, PCNA levels were higher only in SC-O 48 h after PHx, and cyclin D1 tended to decrease in HFD-O 48 h after PHx (Fig. 4c).

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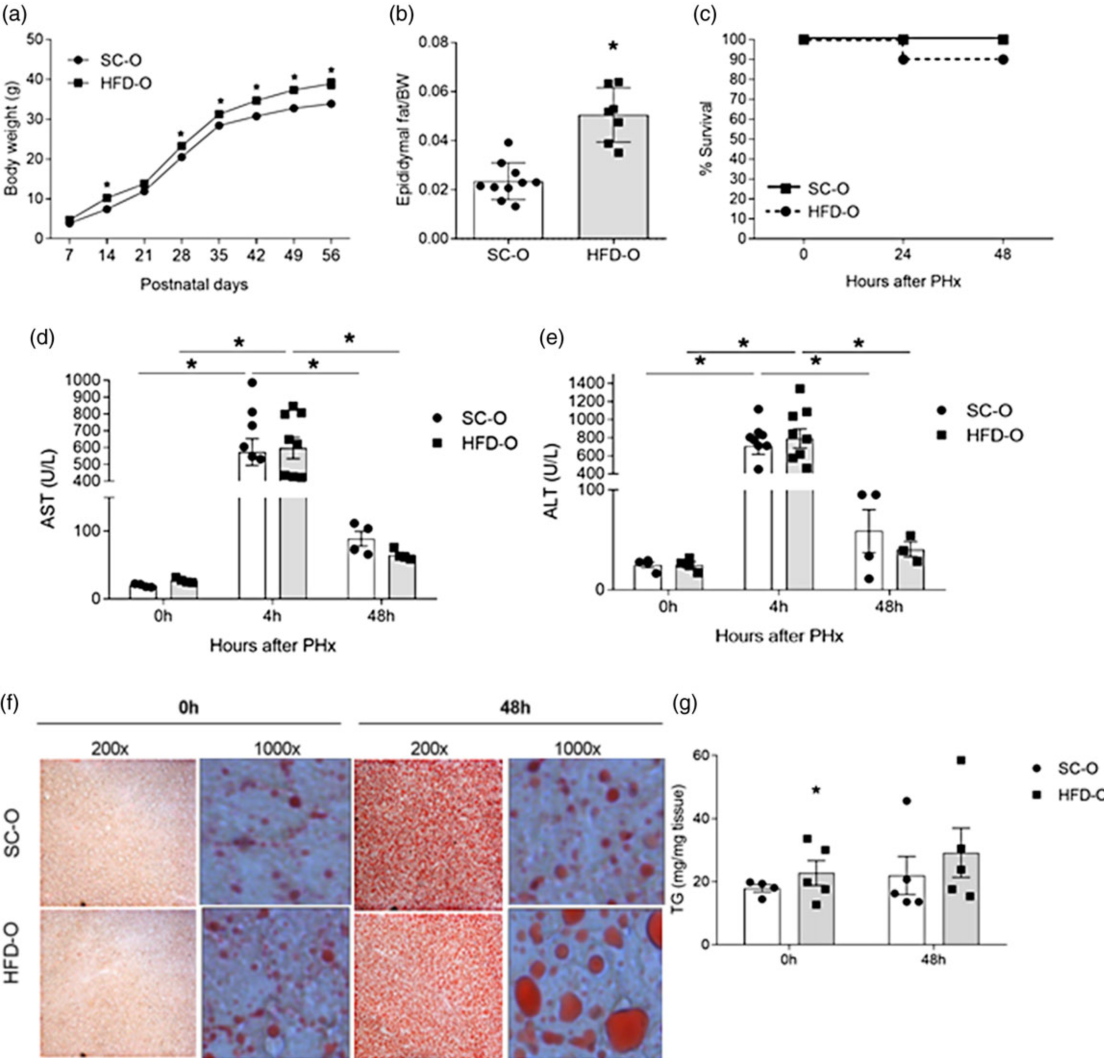

Fig. 2. Metabolic characterization of offspring from SC and HFD dams before hepatectomy and during the postoperative period. Body weight from day 7 to day 56 ($n = 10$ per group) (**a**) and fat pad mass at day 56 ($n = 10$ per group) (**b**), survival rate before hepatectomy and at 24 and 48 h postoperation ($n = 10$ per group) (**c**), serum AST levels ($n = 9$ per group) (**d**) and serum ALT levels ($n = 9$ per group) (**e**) before hepatectomy and at 4 and 48 h postoperation and Liver fat content by oil red staining ($n = 6$ per group, AF = 200× and 1000×), and by colorimetric analysis ($n = 5$ per group, normalized to liver weight) before hepatectomy and on 48 h postoperation. Data are shown as means±SEM. In all graphs, at least three different litters were considered. Repeated measures two-way ANOVA (**a**), *t*-test (**b**) or factorial ANOVA (**c**, **d**, **e**, **f,** and **g**) was used to compare the groups, $^*p < 0.05$.

Effects on LR of offspring directly exposure to an HFD in adulthood

In order to evaluate whether a second insult, a rechallenge to HFD in adulthood, could lead to the compromising of regenerative process in offspring of obese dams, part of the SC-O and HFD-O groups were fed HFD for 3 days before PHx (from p53), and this diet was maintained for 7 days during the recovery procedure.

Both groups presented the same survival rate after the experimental period (80% after 48 h) (Fig. 5a), although the HFD-O group continued to present higher adiposity (60,3%, $p < 0.05$) (Fig. 5b). Regarding the relative liver weight, there was no significant difference between groups (Fig. 5c). This was also evidenced in macroscopic observation of the liver, shown in a representative image of the regenerated organ after 7 days (Fig. 5d).

Discussion

It is known that offspring of obese dams presented increased adiposity and hepatic TG accumulation.[18–20] However, the relationship between hepatic lipid accumulation and the regenerative process is still controversial. The present study explored the effect of maternal HFD consumption and obesity development on the liver regenerative process in male offspring. Our results demonstrate that NAFLD developed by offspring of HFD dams delays the regenerative process after PHx but allows the offspring to reach success in regenerating the liver and survive even when acutely re-exposed to HFD in adulthood.

It is known that after PHx, even animals that were fed SC develop a state of transient steatosis as a physiological and necessary condition to provide energy during the regeneration process.[29] TG are mainly accumulated in the regenerating liver as a result of lipolysis in peripheral adipose tissue.[30] Interestingly, some studies have shown that steatosis post PHx promotes regeneration, but the process is impaired in steatosis models resulting from previous obesity and HFD consumption conditions.[31,32] In this context, a clinical study showed that individuals with severe steatosis presented reduction in regeneration index compared to individuals without steatosis or with mild steatosis.[33] Additionally, Amini

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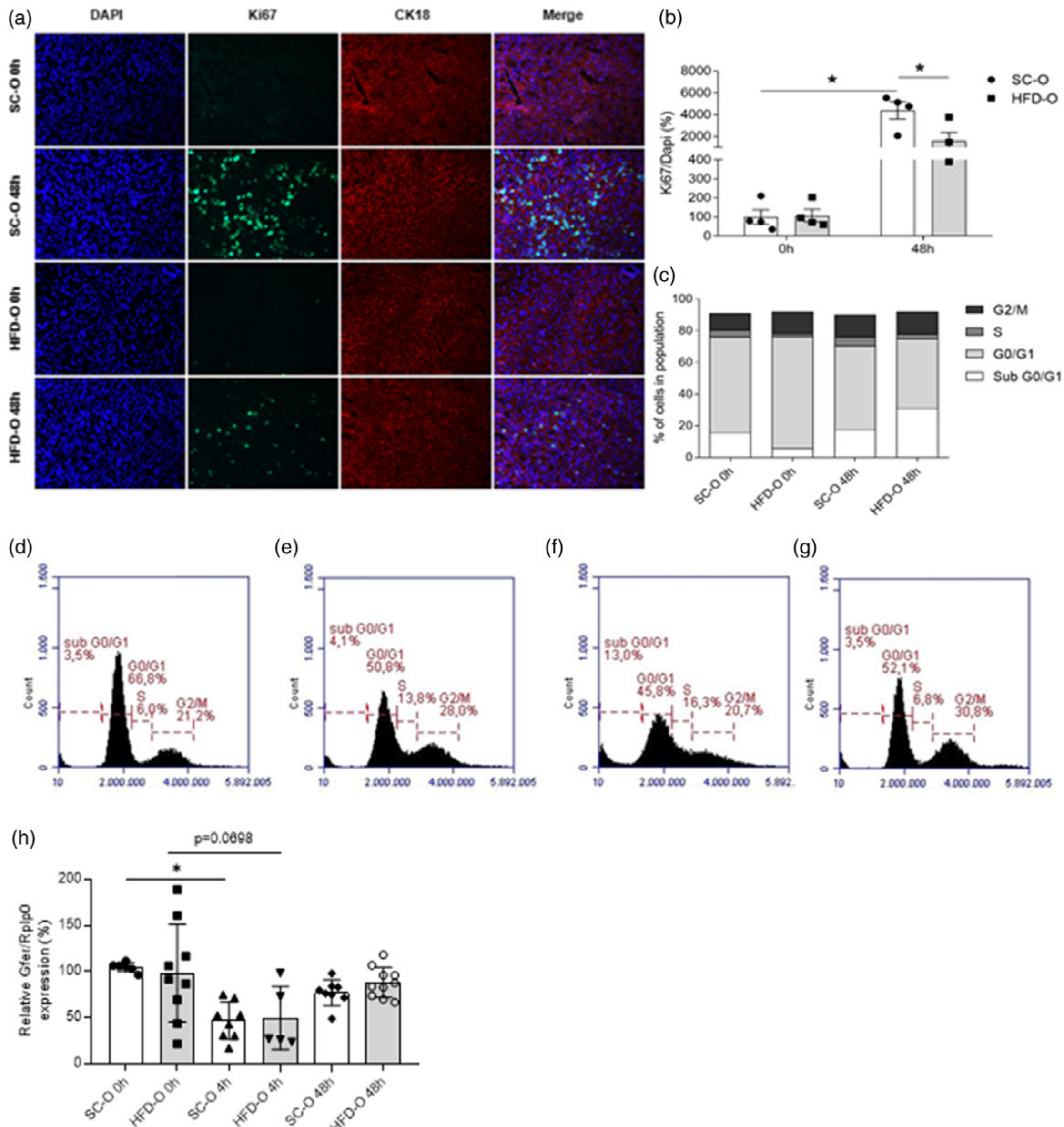

Fig. 3. Hepatic cell cycle analysis of offspring from SC and HFD dams before hepatectomy and during the postoperative period. Representative images of liver sections (before and 48 h after PHx) costained with anti-Ki67 and anti-CK18 antibodies ($n = 4$ per group) analyzed by immunofluorescence (**a**), Ki67+ cells were counted and normalized to total DAPI + cells ($n = 4$ per group) (**b**), distribution of hepatocytes in the cell cycle (pool of three pups per group) analyzed by flow citometry (**c**). Representative histogram of cell cycle distribution (pool of three pups per group) by flow cytometry – SC-O (**d**) and HFD-O (**e**) before hepatectomy and SC-O (**f**) and HFD-O (**g**) at 48 h postoperation. Relative gene expression of *Gfer* by qPCR and normalized to *RPLP0* of offspring from SC and HFD dams before hepatectomy and on 4 and 48 h postoperative ($n = 10$ per group) (**h**). Data are shown as means±SEM. In all graphs, at least three different litters were considered. Factorial ANOVA, $^{*}p < 0.05$.

et al.[34] showed that the risk of a major complication after hepatectomy was greatest among obese patients compared to normal weight patients.

LR after PHx induces hyperplasia by replication of preexisting adult hepatocytes without activation of progenitor cells.[22] Previous *in vivo* studies have shown that TG accumulation within hepatocytes can induce failure in the regeneration stages. Data from the present study provide evidence that offspring steatosis as a result of maternal HFD consumption affects the initial stages of LR. The priming phase trigger the regeneration when hepatocytes switch from the G0 to the G1 state mediated by IL-6 signaling. However, the hepatocytes become trapped in G1,[22,35] not reaching the progression phase, when hepatocytes are committed for the G1-to-S transition.[35] The priming phase seems to have occurred in HFD-O, considering that STAT3 phosphorylation was observed at 4h after PHx. However, we observed a consistent increase of IL-6 levels only in SC-O mice after PHx. The increase in IL-6 was expected since it is the main cytokine mediating acute response after hepatic injury, inducing cytoprotective and mitogenic functions.[36] In addition to IL-6, many humoral factors could activate STAT3 pathway, as well as effectors released by nerve terminals like acetylcholine that mediate the cholinergic anti-inflammatory pathway through efferent vagus nerve.[37]

ERK is phosphorylated at threonine and tyrosine residues and translocated to the nucleus, contributing to the cell cycle progression and regenerative mechanisms through G1-to-S phase transition stimulation.[38,39] Difference in its activation could explain the delay in hepatic regeneration observed in our model. However,

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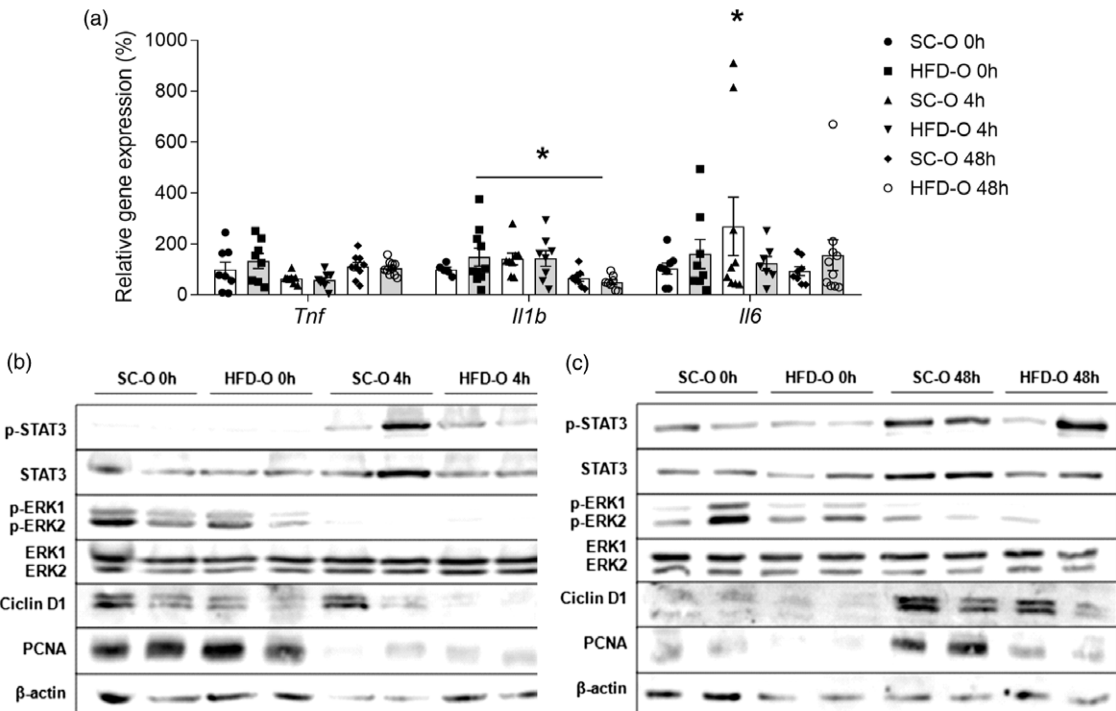

Fig. 4. Hepatic transcript levels of Inflammatory markers, IL6 signaling pathway and cell cycle related protein of offspring from SC and HFD dams before hepatectomy and during the postoperative period. Relative gene expression of *Tnfα*, *Il1β* and *Il6* normalized to *RPLP0* by qPCR before hepatectomy and at 4 and 48 h postoperation ($n = 10$ per group) (**a**). Representative western blot of liver samples prepared from offspring from SC and HFD dams before and 4 h after PHx ($n = 4$ per group) and immunoblotted with anti-phospho-STAT3, anti-phospho-ERK1/2, Cyclin D1 and PCNA antibodies (**b**). Representative western blot of liver samples prepared from offspring from SC and HFD dams before and 48 h after PHx ($n = 4$ per group) and immunoblotted with anti-phospho-STAT3, anti-phospho-ERK1/2, Cyclin D1 and PCNA antibodies (**c**). Data are means±SEM. In all graphs, at least three different litters were considered. Factorial ANOVA, *$p < 0.05$.

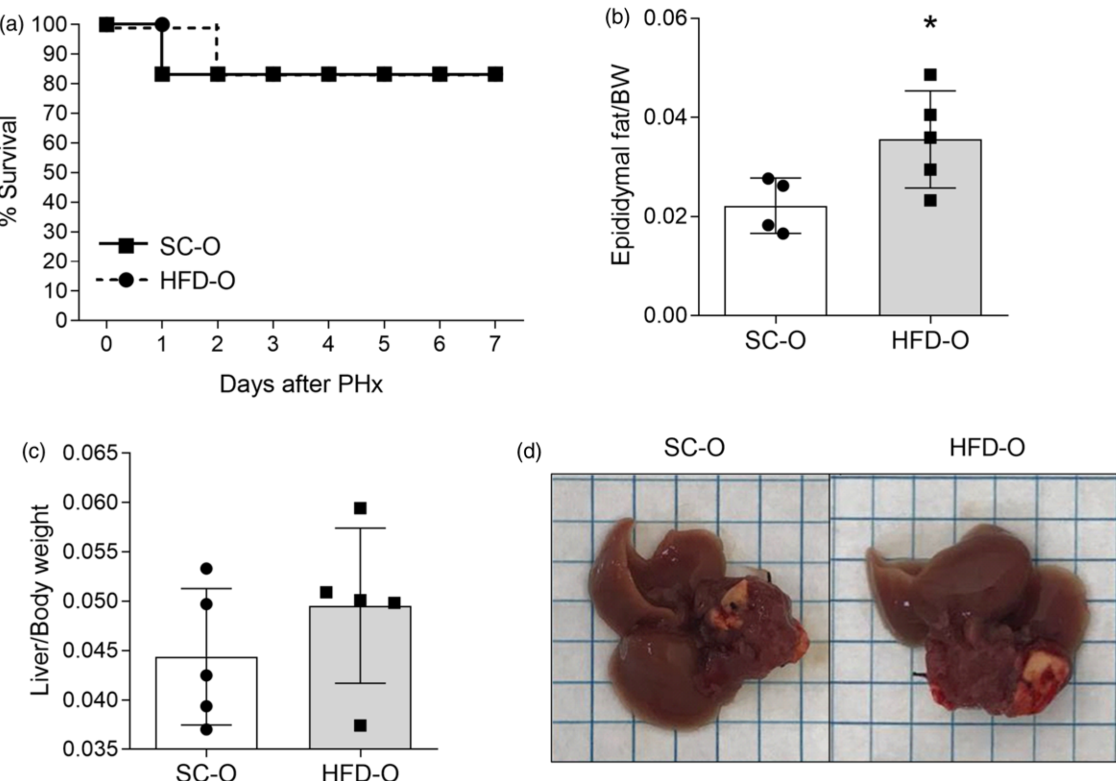

Fig. 5. Liver regeneration 7 days after hepatectomy of offspring from SC and HFD dams rechallenged with HFD in later life. Survival rate 7 days after PHx ($n = 6$ per group) (**a**), fat pad mass at day 63 ($n = 5$ per group) (**b**), Liver to body weight ratio ($n = 5$ per group) (**c**), Representative regenerative liver image 7 days after PHx (**d**). Data are shown as means ±SEM. In all graphs, at least three different litters were considered. Student's *t*-test, two-tailed, *$p < 0.05$.

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SC-O and HFD-O mice similar increase in ERK1/2 phosphorylation before PHx, that was diminished after 4 h-PHx in both groups evaluated, suggesting that a defect in ERK activation is not responsible for the transient arrest in cell cycle observed for HFD-O group.

The delay observed in hepatic regeneration was associated with decreased markers of cell progression (Ki67+ cells and percentage of cells in S phase) 48 h after PHx in HFD-O compared to SC-O. Balance between hepatic proliferation and cell death may be critical for liver homeostasis maintenance during regenerative process.[40] Therefore, a higher percentage of cells in sub G0/ and diminished cell number in S phase G1 observed in HFD-O group are markers of delay on the proliferative process.

Additionally, by using immunoblotting analysis, we identified reduced levels of proteins involved in the cell cycle checkpoint (cyclin D1 and PCNA) 48 h after PHx in HFD-O. Cyclins participate in the control of the cell cycle progression by activating CDKs. Cyclin D1 forms a complex with CDK4/6 and promotes the switch from G0/G1-to-S phase.[32] Furthermore, Cyclin D1 expression could be regulated by hepatopoietin, a growth factor enconded by *Gfer* gene.[41] Although we did not evaluate CDKs and *Gfer* mRNA content has not been different between groups in our study, the results of cyclin D1 were confirmed by those of PCNA.

Although there was no difference in survival rate after PHx of mice exposed to a HFD during perinatal period, we tested if maternal HFD would produce long-lasting effects on the liver of offspring. Nowadays it is accepted the "multiple-hits model" theory to explain the interaction of genetic and environmental factors leading to liver disease progress.[42–45] Maternal exposure to HFD during developmental stages would represent the first hit and a new challenge in adulthood could represent a second insult with more harmful consequences for the liver, when compared to offspring who had no contact in perinatal life.

Considering our previous studies,[19,20] showing that HFD re-exposure in adult life aggravated metabolic impairment of HFD-O compared to SC-O and additionally, mice subjected to 3 days of HFD feeding presented alteration in liver metabolic pathways,[46] we acutely switched HFD-O and SC-O from the control diet to HFD for 3 days before and kept it after 7 days of surgery. HFD-O mice showed to be protected from mechanical injury, given they did not show differences from SC-O in survival, hepatic regenerative index (LW:BW ratio) and regenerated liver area after the re-exposition to HFD 3 days prior PHx until liver mass re-establishment. According to the literature, it is estimated that 7 days after surgical removal in mice the remaining liver has regenerated in size equivalent to its original mass, and so this time was used in our experimental protocol.[22] Although offspring from obese dams rechallenged to HFD in adulthood presented pronounced damage to insulin signaling in metabolically active tissues, as previously described, liver shown to be the last affected tissue.[20]

Our results demonstrate for the first time the effect of maternal HFD consumption on offspring LR after PHx. Altogether, the data reinforce that the development of steatosis is related to liver regenerative capability but repair mechanisms in offspring programmed by maternal overnutrition overlap to metabolic damage, in order to promote tissue regeneration, even with some delay, to ensure the survival.

Supplementary materials. For supplementary material for this article, please visit https://doi.org/10.1017/S2040174421000659

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals "Brazilian College for Animal Experimentation" (COBEA) and have been approved by the institutional committee "Ethical Committee for Animal Use" (CEUA), Protocol 3964-1, State University of Campinas – UNICAMP (Campinas, São Paulo, Brazil).

Author contribution statements. **AST**: Conceptualization, Data curation, Project administration, Resources, and Writing – original draft; **TF, LAPS, MFF, VAC, AR, and TGR**: Formal analysis, Investigation, and Methodology. **LAPS, MAT, LMIS, MM, EGM, and PCL**: Writing – review & editing. All authors approved the final version.

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