Dietary Fat Quality in Normolipidic Diets Affects Hepatocyte's Nuclear Phenotypes

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ABSTRACT: Dietary fat quality affects overall systemic parameters and produce hepatic accumulation of fat and inflammation (steatohepatitis). In this communication we have assessed how mouse liver nuclear phenotypes are influenced by diets containing 7% lipid prepared with lard, linseed oil or soybean oil for 32 weeks. Liver specimens were imprinted on glass slides, fixed and stained with DAPI. 3D confocal images were obtained and employed for the calculation of nuclear thickness, nuclear volume and DAPI-DNA intensity. Hepatocytes' nuclei could be classified as diploid A, diploid B, tetraploid and higher ploidy levels. Linseed oil in the diet resulted in increased frequency of diploid A (more compact) and less polyploidy, while lard caused increased volume and more polyploidy. Soybean oil produced intermediate nuclear sizes. The results suggest a high demand on liver physiology promoted by lard, which has a predominance of saturated fatty acids, while linseed oil promoted the opposite effect.

KEYWORDS: Fatty acids, linseed oil, hepatocytes, nuclear phenotypes, ploidy levels

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Introduction

Nuclear morphology is intrinsically correlated with cell activity/differentiation states, as well as cell type. Liver nuclear morphology, phenotypes and ploidy levels have been associated with diverse physiological situations, including animal growth/size.[1-7] The polyploid state is found in 90% of rodent and 40% human hepatocytes, and is achieved via cytokinesis failure, more specifically by an arrest at the G2/M check point, due to activation of the p53/p21 signalling pathway.[8,9] Interestingly, hepatocyte polyploidy is inhibited by premature weaning,[10] in the E2F8 knockout mouse[10] and in the liver specific E2F7/E2F8 knockout mouse.[11] These latter studies also showed that the polyploid state functions as a growth suppressor for restricting proliferation of hepatocytes under diverse circumstances, including a carcinogenesis challenge.[10,11]

Changes in ploidy levels were reported in the CETP (cholesteryl ester transfer protein) KO and for the LDL-receptor null allele (CETP$^{+/-}$ LDLr$^{+/-}$) mice, suggesting that lipid handling affects liver physiology and demand on nuclear activity.[12]

Recently, we have studied the effect of lipid quality in normolipidic (7% fat) on mouse systemic parameters[13] as well as on the prostate gland histology and physiology.[13,14] We observed that different growth indices as well as liver inflammation and lipid accumulation occur in the animals fed on linseed oil (LO), soybean oil (SO) or lard (pork fat, PF), which vary in the amount of saturated fatty acid (14.5%, 20.1% and 45%, respectively) and in the ω3:ω6 ratios (3.7, 0.11 and 0.07, respectively). Lard in the diet for 32 weeks resulted in steatohepatitis.[13]

In this context, we have examined the livers of mice fed on 7% lipid, normolipidic diets based on lard, linseed oil or soybean oil for 32 weeks, imaged hepatocyte nuclei using confocal microscopy and determined ploidy levels, by image analyses. The results demonstrated that nuclear compaction and ploidy levels vary in response to the different diets, suggesting different demands on hepatocyte function.

Materials and Methods

Animal samples and ethical approval

Liver samples were collected from male C57/BL6 and subsequently fixed or used for imprinting. Experimental procedures and mice lineages utilized were approved by the State University of Campinas Committee on the Use of Experimental Animal, under protocol numbers 3346-1 and 4223-1. All procedures followed the recommendations of the Brazilian Council for the Control of Animal Experimentation (CONCEA). Mice were acquired from the Multidisciplinary Center for Biological Investigation (CEMIB) at the State University of Campinas (UNICAMP).

Animals (n = 12) *were* maintained under constant temperature (22 ± 2°C) and light/dark cycles (12 hours:12 hours) and received water and the specified diets *ad libitum*. C57/BL6 animals were fed isocaloric diets containing 7% fat made with either pork fat (lard), linseed oil or soybean oil for the 32 weeks post weaning, as described.[13] Pelleted food was obtained commercially and kept in dark (aluminized wraps) at −20°C in

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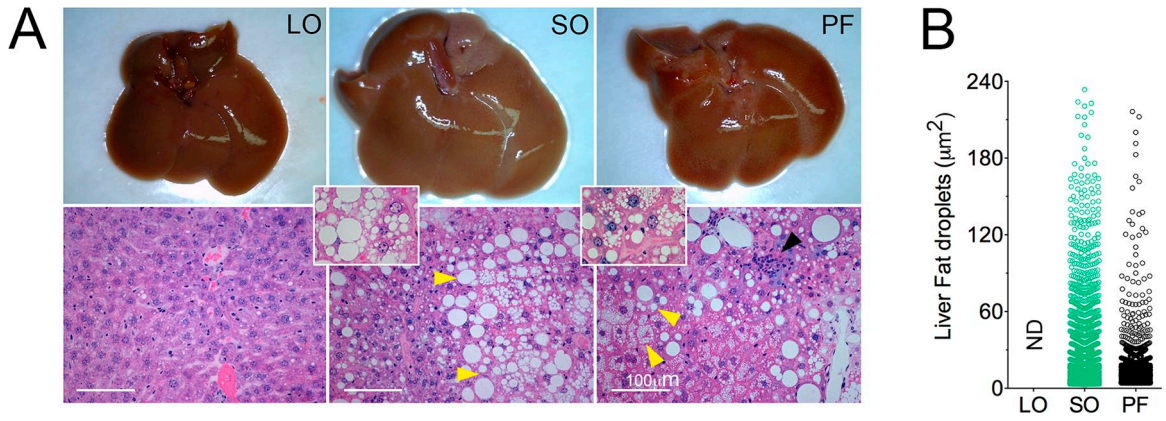

Figure 1. Liver histology: (A) general aspects of liver histology, presenting different levels of lipid accumulation and inflammatory infiltrates in response to the diets. The yellow arrowheads point to lipid droplets. The black arrowhead point to an inflammatory infiltrate and (B) quantification of lipid droplet size variation in response to the different diets.
Reproduced from Ferrucci et al,[13] with permission.
LO, linseed oil; SO, soybean oil; PF, lard.

small packs, under N2 atmosphere to avoid premature oxidation of lipidic components. Livers were dissected out and processed for routine histology or used for the imprints.

Imprintings

Liver freshly cut surfaces were gently pressed against a clean glass slide. Imprintings were fixed in ethanol-acetic acid 3:1 (v:v) solution for 5 minutes and subsequently air dried before stained.

DAPI staining

Imprintings were washed with PBS for 5 minutes before imprinted cells were permeabilized with PBST (PBS 0,1% Tween 20) for 5 minutes. Slides were washed for 3 times of 5 minutes in PBS. Nuclei was stained with 4′,6-diamidino-2-phenylindole (DAPI) diluted 1:1000 from a 2 mg/mL stock solution in PBS for 10 minutes. Slides were mounted in a 90% glycerol, 10% 20 mM Tris pH 8 and 0.5% N-propylgalate solution and stored at 4°C in the dark.

Imaging

Samples were examined in the National Institute of Science and Technology on Photonics Applied to Cell Biology (INFABIC) at the State University of Campinas. For the visualization of Feulgen and toluidine blue stained imprintings we used a Zeiss Axioskop (Carl Zeiss AG, Germany), and images were collected using an AxioCam Mrc. For the visualization of DAPI-stained nuclei we used a Zeiss LSM 780-NLO confocal on an Axio Observer Z.1 microscope.

Quantitative parameter determination

Nuclear volume (V) was calculated based on the assumption that imprinted nuclei were discs, and on the measurement of disc height on nuclei stained with DAPI as visualized by the confocal microscopy. Volume was calculated using the equation $V = A \cdot h$, in which A represents nuclear area and h represents disc height. Disc height was measured using the BoneJ plugin version 1.4.2[15] in the ImageJ software version 1.51n.[16]

Results and Discussion

Figure 1 shows the gross inspection of representative livers from animals fed on the different diets, the histological appearance after H&E staining and the quantification of lipid droplets' area. The presence of fat is visible in the liver of animals from both SO and PF, but not in the LO groups. Inflammatory infiltrates are easily found in the PF group.[13]

Liver squashes had very flat nuclei, suggesting a distortion of nuclear geometry caused by the procedure. Nuclei thickness, as observed by confocal microscopy, corresponded to about 1/40 of the nuclear diameter (Figure 2A). Nuclear volume was then calculated as the product of the nuclear area by the height, which allowed the identification of nuclear volume classes (Figure 2B). The nuclei of non-hepatocyte cell (NHC), including endothelial cells, macrophages and other immune cell types were excluded from this characterization. Comparison of the nuclear volume classes and the DAPI-DNA intensity classes resulted in the identification of 2 populations of diploid cells (diploid A and diploid B), which had different volumes but pertained to the same DAPI-DNA content class, tetraploid and higher ploidy nuclear volume classes. The microscopic view of these different nuclei is shown in the inset for Figure 2B.

Plotting the measured DAPI-DNA intensity against the calculated nuclear volume permitted the classification of nuclear phenotypes according to the corresponding lipid diet (Figure 2C). Hepatocytes from animals fed on lard showed the highest dispersion of nuclear volumes, reaching 3000 µm^3. As a matter of fact, there was a graded distribution of nuclear sizes, linseed oil occupied the other extreme, with smaller nuclei and soybean, showing intermediate values. Considering the exponential trend lines, we can assume that animals fed on linseed oil had more compact chromatin, as compared to soybean and lard, in this sequence.

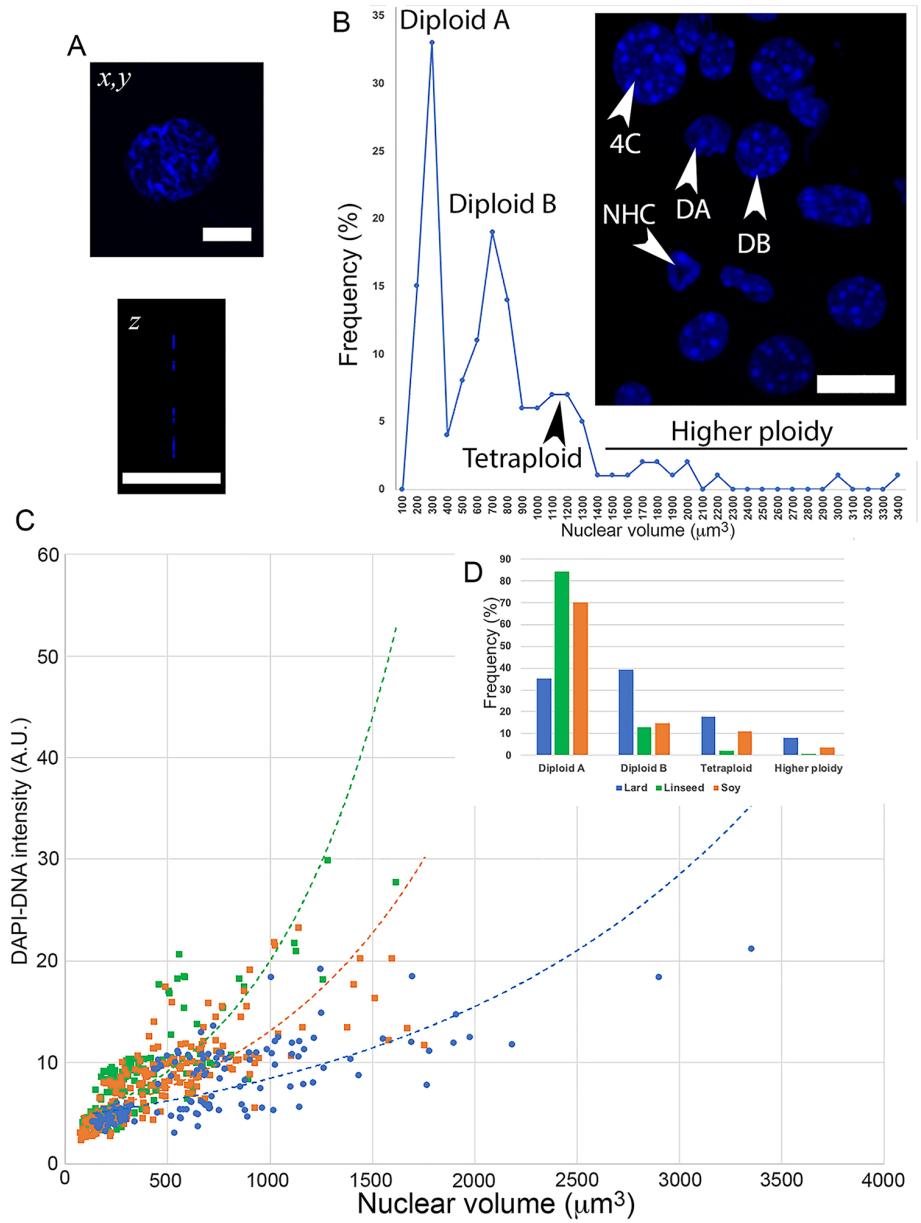

Figure 2. Characterization of hepatocytes' nuclear phenotypes and ploidy levels in animals fed on normolipidic diets (7% fat) based on lard, linseed oil or soybean oil: (A) confocal imaging of a single hepatocyte nucleus observed in the x,y and z directions. Scale bars = 10 μm. (B) nuclear volume classes and nuclear phenotypes. Diploid A (DA), diploid B (DB), tetraploid (4C) and higher ploidy nuclei were identified. Non-hepatocyte cell (NHC) were omitted from the analyses, (C) dispersion plots of DAPI-DNA intensity (in arbitrary units, A.U.) versus nuclear volume (μm³) obtained for the animals fed on linseed oil (green), soybean oil (orange) or lard (blue), and the corresponding exponential trend lines, and (D) percentage distribution of the different hepatocytes' nuclear phenotypes in the animals fed on the different diets.

Finally, when classified according to the nuclear phenotypes shown in Figure 1B, diploid A nuclei predominated in both linseed oil and soybean, while tetraploid and higher ploidy nuclei predominated in lard (Figure 2D).

This study has investigated whether different lipid quality in normolipidic diets affect hepatocyte's nuclear phenotypes and ploidy levels. Hepatocyte binuclearity and ploidy levels has been associated with different systemic parameters, such as growth and animal size.[2,6,7]

Mice fed on isocaloric diets containing 7% fat from either lard (pork fat; PF), linseed oil (LO) or soybean oil (SO) for 32 weeks showed distinct systemic parameters.[13] PF group had a biphasic growth pattern: growth had a similar rate to SO group until 10 to 12 weeks of diet, when it began to show growth patterns similar to the LO group. Furthermore, circulating inflammatory mediators indicated that SO and PF had increased systemic TNF-α, a pro-inflammatory cytokine. Accordingly, their livers showed different levels of lipid accumulation and inflammatory infiltrates. As a matter of fact, lard resulted in steatohepatitis, that is, overt inflammatory infiltrates and extensive lipid accumulation (Figure 1).[13]

The present results classify hepatocyte's nuclei in 3 different classes. LO showed a predominance of diploid A nuclei, which

are diploid more compact nuclei. Feeding on PF resulted in less compact nuclei and higher ploidy (tetraploids and higher ploidy levels). Animals fed on SO showed intermediate sizes and ploidies. It was interesting to note that the smaller animals., i.e. those fed on LO,[13] showed increased chromatin compaction and decreased frequency in polyploid nuclei, in contrast to those animals fed on PF or SO, which showed increase growth indices.

In conclusion, different lipid quality in normolipidic diets, varying with respect to the content of saturated fatty acid and in ω3:ω6 ratios, affects hepatocyte's nuclear phenotype and ploidy levels, possibly reflecting different demands on liver physiology.

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Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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