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BIOMEDICAL SCIENCES

Protein-caloric-restriction diet during lactation programs lean phenotype and improves the antioxidative system in adult female rat offspring

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Abstract: Nutritional insults early in life, such as during the suckling phase, are associated with phenotypic alterations and promote adverse permanent effects that impair the capacity to maintain energy balance in adulthood. This study aimed to evaluate the long-term effects of a low-protein (LP) diet during lactation on the metabolism and antioxidant systems of adult female rat offspring. Dams were fed a low-protein diet (4% protein) during the first two weeks of lactation or a normal-protein (NP) diet (20% protein) during the entire lactation period. The female offspring received a standard diet throughout the experiment. At 90 days of age, female LP offspring exhibited decreased body weight, feeding efficiency, and fat pad stores. The adult LP female offspring displayed brown adipose tissue hyperplasia without alterations in glucose homeostasis. The LP diet decreased liver triglyceride content and improved the antioxidant system compared to the NP group. The LP diet during the suckling phase promotes a lean phenotype and improves the hepatocyte antioxidant system in adult female offspring. Thus, the LP diet may play an important role in homeostasis and the prevention of metabolic damage.

Key words: metabolic programming, protein restriction, suckling phase, nutrition, metabolism.

INTRODUCTION

A recent United Nations report presented updates on the state of food and nutritional security worldwide. It includes the latest estimates of the cost and affordability of healthy food, indicating that the number of people affected by hunger globally rose to approximately 828 million in 2021 (The State of Food Security and Nutrition in the World 2022 2022). In the poorest populations, a transition from energy and protein malnutrition to a gradual increase in dietary lipid content was observed (Popkin et al. 2002). Proteins are the most expensive food components in the human diet. These data have become increasingly concerning owing to the COVID-19 pandemic (The State of Food Security and Nutrition in the World 2022 2022). Studies in Brazil and around the world showed that the human population was looking for high-calorie and ultra-processed food owing to the high prices of healthy food in this period, such as those rich in protein (Alves Durães et al. 2021, Huber et al. 2021,

Jafri et al. 2021). However, the harmful effects of these choices on humans are incalculable. Nevertheless, epidemiological and experimental studies have shown impaired metabolism and an increase in noncommunicable diseases when this dietary mismatch occurs.

The Development Origins in Health and Disease (DOHaD) concept suggests that maternal malnutrition in developmental vulnerability windows, such as the suckling phase, programs offspring metabolism, resulting in different phenotypes and promoting adverse permanent effects that impair the capacity to maintain energy balance in adulthood (Barker 2007, Hales & Barker 2001, Cissé et al. 2019, Guzmán-Quevedo et al. 2013). A low-protein diet (LP) is an experimental model widely used to investigate maternal malnutrition during the suckling phase because the maturation of the endocrine and central nervous systems occurs in the first few weeks after birth (Morimoto et al. 2012, Bautista et al. 2019, Zambrano et al. 2005, Martins et al. 2018, Ferreira et al. 2022). Breast milk is considered the best and most complete food for newborns and protects against various metabolic disorders (Martins et al. 2023). In fact, the effects of an LP diet on offspring can be beneficial for some metabolic parameters, such as obesity (Martins et al. 2018), and the thrifty phenotype hypothesis suggests it may lead to changes in food habits throughout life.

In male rat offspring, our research group and others have shown that maternal LP during lactation results in lower body weight, food intake, fat pad stores, hypoinsulinemia, glucose intolerance, higher insulin sensitivity, reduced insulin secretion (Martins et al. 2018, Zambrano et al. 2005) and beta-cell mass, downregulation of key genes regulating beta-cell development (Rodríguez-Trejo et al. 2012), altered autonomic nervous system function (Martins et al. 2018), impaired hypothalamic development, (Guzmán-Quevedo et al. 2013) and hepatic damage (Bertasso et al. 2022) in adulthood. These metabolic dysfunctions can be transmitted to the next generation of cells (Peixoto-Silva et al. 2011, Frantz et al. 2011).

Although several reports have demonstrated the effects of an LP diet on male metabolism, few studies have explored the metabolic effects of this diet during lactation in female rats. Moreover, understanding scientific findings in the context of sex is important for correctly applying research-derived knowledge (Clayton 2016). Thus, in the present study, we evaluated the long-term effects of an LP diet during lactation on body composition, lipid profile, glucose homeostasis, hepatic metabolism, and the antioxidant system in adult female rat offspring.

METHODS

Ethical approval

All experiments were conducted according to the ARRIVE guidelines (Kilkenny et al. 2010) and the Brazilian Association for Animal Experimentation (COBEA) standards. The protocols were approved by the Ethics Committee for Animal Research of the State University of Maringa (protocol number 8625310521) and were performed in the sectorial animal facility of the Secretion Cell Biology Laboratory of the State University of Maringa.

Nutritional insult and animal groups

After 1 week of acclimatization, female and male Wistar rats (70 and 80 days old, respectively) were mated at a ratio of three females to each male, and the pregnant females were transferred to individual cages and fed a standard diet. At birth, the litter was standardized to eight pups per dam in a 1:1 sex ratio and was fed either a normal-protein diet (NP; 23% protein; Nuvital; Curitiba/PR, Brazil; n = 6) or an isocaloric LP diet

(de Oliveira et al. 2011) (4% protein; n = 6) from delivery until day 14 of lactation and a normal diet for the remaining third part of the lactation period. Due to the intense caloric restriction caused by the LP diet, it was not possible to offer this diet throughout lactation. On postnatal day 21, female offspring were weaned, housed four per cage, and fed a standard diet throughout the experimental period. The male offspring were euthanized because they had been previously evaluated by our research group (Martins et al. 2018). The experimental procedures were conducted at 90 d of age. Throughout the experimental period, the animals were maintained under controlled temperature (23 C ± 2 C) and photoperiod (7:00 am to 7:00 pm, light cycle) conditions. Animals received water and food *ad libitum*.

Biometric parameters and food intake

Body weight (BW) and food intake (FI) were assessed weekly (n = 5–10 rats from 4–6 litter per group) from weaning until 90 d of age to the area under curve (AUC) measurement. The FI of the rats was calculated using the formula [FI(g) = (Df –Di)/7] which is the difference between the amount of diet remaining (Df) and the amount presented previously (Di), divided by the number of days. Feeding efficiency [food consumption (g)/body weight gain (g) was calculated. At 90 d, the rats were anesthetized (thiopental, 45 mg/kg bw), decapitated, and laparotomized to remove their liver, pancreas, retroperitoneal, mesenteric, uterine, ovarian, and brown fat pad stores**.** The weight of the collected tissues was expressed relative to the BW (g/100 g of BW).

Glucose metabolism assessment

At 90 d, a batch of female rats (n = 5–10 rats from 4–6 litter per group) was subjected to fasting for 6 h to perform an intraperitoneal insulin tolerance test (ipITT). Glycemia was determined from the collection of blood from the caudal vein and was measured using a glucometer at five different times (0, 15, 30, 45, and 60 min), once before and four times after the application of intraperitoneal injection of insulin (1 U/kg of BW). The rate constant for the disappearance of plasma glucose (K_{itt}) was calculated (BONORA et al. 1989). Additionally, after two days, surgery was performed for cannula implantation to execute an intravenous glucose tolerance test (ivGTT), as previously described (de Oliveira et al. 2011). After a 12-h fast, blood samples were collected before the injection of glucose (1 g/kg BW) (0 min) and 5, 15, 30, and 45 min later. The glucose response during the test was calculated using the AUC.

Biochemical parameters

Glucose concentrations were measured by the glucose oxidase method using a commercial kit (GoldAnalisa®; Belo Horizonte, MG, Brazil) (n = 5–10 rats from 4–6 litter per group). Fasting insulin levels were measured by ELISA. Total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) enzymes were measured in plasma samples by colorimetric method using commercial kits (Gold Analisa®; Belo Horizonte, MG, Brazil). The absorbance reading was performed in a spectrophotometer (Bioplus®, Barueri/SP, Brazil). Very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) cholesterol levels were calculated using the Friedewald formula (Simões et al. 2007).

Oxidative stress assessment

Right-lobe hepatic samples (approximately 100 mg, n = 7–12 rats from 4–6 litter per group) were weighed and homogenized in potassium phosphate buffer (200 mM, pH 6.5). A total of 60 µL of the homogenate was transferred to

polypropylene tubes to determine the reduced glutathione (GSH) levels (Borges et al. 2018, Rissato et al. 2020). The other part of the homogenate was centrifuged for (20 min at 9000 × g), and part of the supernatant was used to determine the lipid hydroperoxide (LOOH) levels according to a previously described methodology (Jiang et al. 1991). The supernatants were collected for biochemical assays to determine the enzymatic activities of superoxide dismutase (SOD) and glutathione S-transferase (GST). The GSH levels were measured according to a previously described method (Sedlak & Lindsay 1968). For the GSH reaction, trichloroacetic acid (TCA) was added to the sample for protein precipitation and centrifuged for (15 min at 9700 × g). Then, the supernatant was mixed with 5,5'-dithiobis-2-nitrobenzoic acid and tris (hydroxymethyl) aminomethane hydrochloride 1 (TRIS-HCl) buffer (0.4 M; pH 7.0) and read at 412 nm. Individual values were interpolated based on the GSH standard curve and expressed as µg of GSH/g of liver.

The total LOOH was determined according to a previously described method (Jiang et al. 1991). The supernatant was diluted with 30% methanol and centrifuged for (30 min at 10000 × g). A solution of xylenol orange, sulfuric acid 25 mM, butylated hydroxytoluene 4 mM, FeSO4NH4 250 mM, and methanol was added to the supernatant. The plates were incubated in the dark. The absorbance was measured at 560 nm using a spectrophotometer. LOOH concentrations were determined using an extinction coefficient of 4.3 mmolar 1/cm, and the results are expressed as mmol/mg of tissue.

The enzymatic SOD assay is based on the ability of SOD to inhibit pyrogallol autoxidation (Marklund & Marklund 1974). TRIS-HCL EDTA buffer was added to the supernatant. The reaction was initiated with pyrogallol and stopped after 20 minutes by adding HCL 1M. The readings were obtained at 405 nm using a spectrophotometer. The results are expressed as U of SOD/mg of protein.

The supernatant was used to measure GST enzymatic activity. GST activity was measured based on the capacity of a former conjugate of glutathione and 1- chloro-2, 4-dinitrobenzene (CDNB) (Warholm et al. 1985). Samples were diluted in potassium phosphate buffer (0.2 M). Subsequently, CDNB and GSH were added. The absorbance was read at 340 nm using a spectrophotometer. An extinction coefficient of 9.6 mmolar 1/cm was used, and the results were expressed as µmol/min/mg of protein.

Hepatic levels of cholesterol and triglycerides

Left-lobe hepatic samples of approximately 100 mg were removed (n = 5–10 rats from 4–6 litter per group) to determine the total lipid content using the Folch method (Folch et al. 1957). The extract was then evaporated and diluted with isopropanol. Cholesterol and triglyceride contents were measured using a commercial kit, in accordance with the manufacturer's instructions (GoldAnalisa, Belo Horizonte, MG, Brazil).

Pancreas, brown and white fat histology

After 90 d, the fat pad stores were collected for histological analysis. Retroperitoneal white adipose tissue and interscapular brown adipose tissue samples were removed (n = 6 rats from six litters per group), fixed in 4% paraformaldehyde for 24 h, dehydrated in alcohol of increasing concentrations, and embedded in histological paraffin after diaphanization in xylene. Slices of 5-µm thickness were prepared for staining with hematoxylin and eosin (H&E). The histological sections had followed the interval of 30 µm between the slices. After processing and fixation on histological slides, the sections were examined using capture light microscopy (20

optic zones per animal, 40x). ImageJ for Windows (Open Source) was used for the analysis.

Statistical analysis

Data are presented as the mean ± SEM and were analyzed using GraphPad Prism, version 8.0, for iOS (GraphPad Software, Inc. San Diego, CA, USA). The statistical test used was the Student's t-test, and values of P <0.05 were considered statistically significant.

RESULTS

Biometric parameters, food intake, and morphometric analysis

Protein restriction caused 22% lower body weight gain in the LP group (Figure 1a; P <0.0001) than in the NP group, as shown by the AUC. The LP group showed increased food intake (+16.35%; P <0.001; Figure 1b) and decreased feeding efficiency (-14%; P <0.001; Figure 1c) compared to the NP group.

At 90 days old, metabolic programming by protein restriction decreased body weight (-15.81%; P <0.0001; Figure 1d), liver (-14.73%; P <0.01; Figure 1e), ovarian (-31.2%; P <0.001; Figure 1f), mesenteric (-16.6%; P <0.01; Figure 1g), retroperitoneal (-36.1%; P <0.0001; Figure 1h), and uterine (-44.4%; P <0.0001; Figure 1i) fat pad compared to NP group.

As shown in Figure 1l-m, LP rats had a higher number (+35.45%; P <0.01) and area (+2%; P <0.05) of brown adipocytes than NP rats (Figure 1p-q). No differences were observed in the number (Figure 1j) or area (Figure 1k) of white adipocytes (Figure 2n-o) between the groups.

Glucose metabolism

In adulthood, the LP group demonstrated normal glucose levels in the ivGTT (Figure 2a), no difference in insulin sensitivity as demonstrated by K_{itt} (Figure 2b), and normal fasting glycemia (Figure 2c) compared to the NP group. Protein restriction did not alter fasting insulinemia (Figure 2d), pancreatic weight (Figure 2e), or islet area (Figure 2f-h).

Biochemical parameters

Regarding the lipid profile, the LP group displayed lower serum total cholesterol (-16.6%; P <0.05; Figure 3a) and HDL-C (-23.6%; P <0.01; Figure 3c) levels than the NP group. No differences were observed in serum triglyceride (Figure 3b), LDL-C (Figure 3d), VLDL-C (Figure 3e), AST (Figure 3f), or ALT (Figure 3g) levels between the groups.

In the liver tissue, LP increased SOD (+42%; P <0.01; Figure 3h) and GST (20.43%; P <0.05; Figure 3k) activity in female offspring. No difference was observed in liver catalase (CAT) activity (Figure 3i) or GSH content (Figure 3j). The liver triglyceride content was lower (-26.6%; P <0.05; Figure 3n) in the LP group. Protein restriction increased the liver LOOH (+20.29%; P <0.05; Figure 3l) and CHOL (+11.63%; P <0.05; Figure 3m) levels compared to those in the NP group.

DISCUSSION

In the present study, we evaluated metabolic programming by protein caloric restriction during the first 14 days of lactation using biometric and biochemical parameters of adult female rat offspring. At 90 d of age, the LP diet decreased body weight and fat pad stores, and food intake and brown adipose tissue area increased. In addition, these results suggested a lean phenotype without significant alterations in glucose homeostasis. Metabolic alterations induced by diet decreased serum cholesterol and increased cholesterol deposition within hepatocytes, thereby improving the liver antioxidant system.

The feeding behavior is regulated by the hypothalamus through orexigenic and

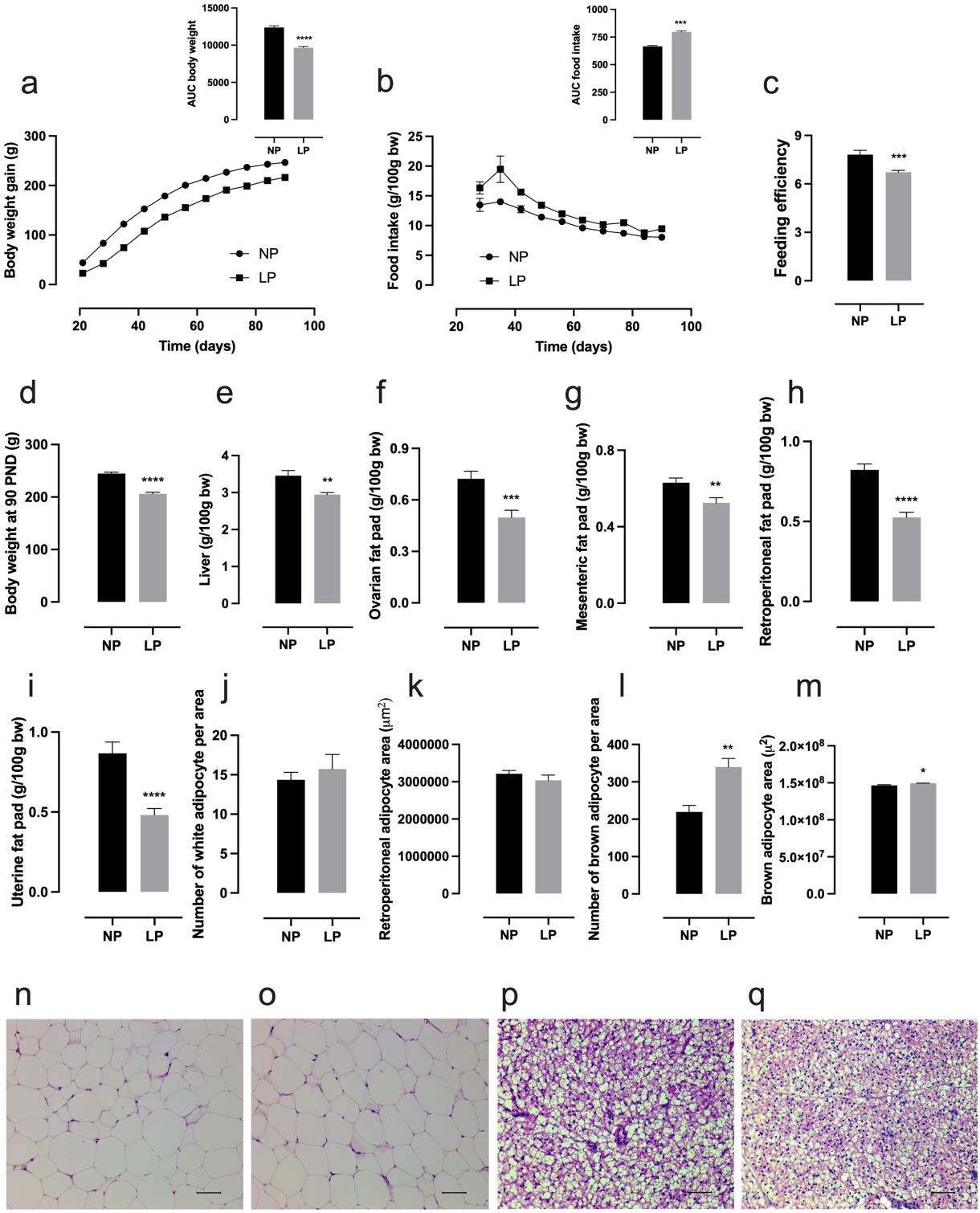

Figure 1. Biometric parameters and fat pad morphometry. Body weight gain (a), food intake (b), feeding efficiency (c), body weight at 90 days old (d), liver (e), ovarian (f), mesenteric (g), retroperitoneal (h) and uterine fat pad store (i), number of white adipocyte (j), retroperitoneal adipocyte area (k), number of brown adipocyte (l), brown adipocyte area (m), NP WAT (n), LP WAT (o), NP BAT (p) and LP BAT (q). Hematoxylin & Eosin-stained sections. Magnification: 200x. Scale bar = 50µm. The data are expressed as the mean ± S.E.M. and were obtained from 8-12 rats of each group (from 3-6 different litter). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

anorexigenic neuropeptides (Orozco-Solís et al. 2010). The brain is fully developed during the first few weeks of the suckling phase in rats (Martin Agnoux et al. 2018). The LP diet during lactation decreased pup milk intake and protein content in breast milk (Bautista et al. 2019), suggesting lower production of these neuropeptides and provoking malformation of the hypothalamus (da Silva et al. 2016). Moreover, poor maternal nutrition can impair behavioral outcomes, such as anxiety and reward, due to damage to the neuronal development of the frontal cortex and limbic system (Reyes-Castro et al. 2012). In the present study, we showed for the first time that

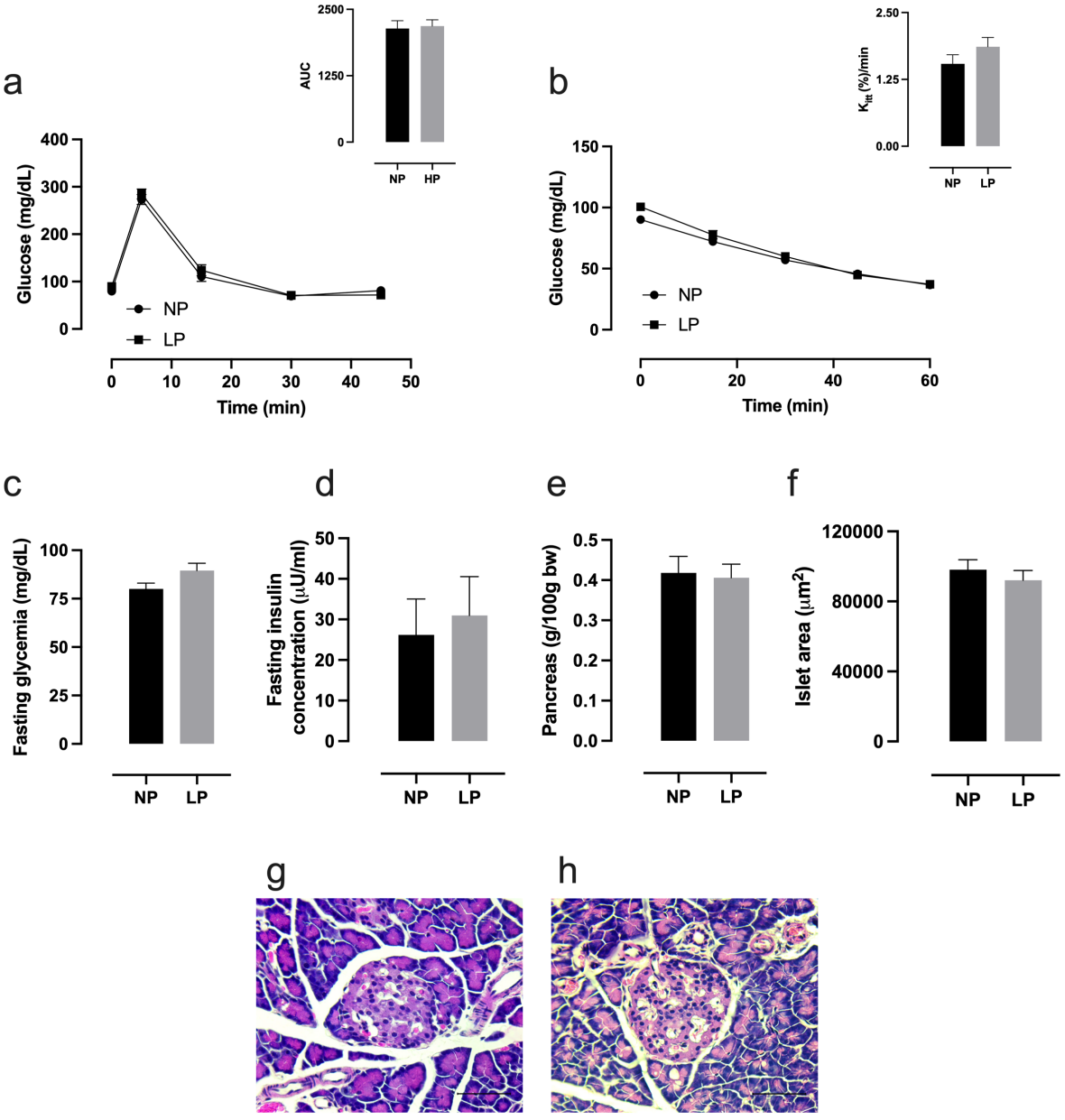

Figure 2. Glucose homeostasis. Plasma glucose during ivGTT (a), insulin tolerance test and K_{itt} (b), fasting glycemia (c), fasting insulin concentration (d), pancreas weight (e), islet area (f), NP islet (g) and LP islet (h). Hematoxylin & Eosin-stained sections. Magnification: 400x. Scale bar = 50µm. The data are expressed as the mean ± S.E.M. and were obtained from 6-12 rats of each group (from 3-6 different litter).

an LP diet increased food intake and decreased feeding efficiency in adult female offspring, while male offspring had normophagia at the same age, as we have shown previously (Martins et al. 2018). However, as previously observed in males (Martins et al. 2018), female offspring had lower body weights and fat pad stores, which has also been demonstrated by Bertasso et al. (2022) (8% protein content in the diet). In the LP high-carbohydrate murine model, lower body weight in male rats is associated with an increase in brown adipose tissue thermogenesis due to higher expression of the mitochondrial electron transport chain uncoupling protein 1 (UCP1) (de França et al. 2016). Here, we show for the first time that an LP diet provokes BAT hyperplasia in female offspring, suggesting a higher activity of the sympathetic nervous system, as we have previously shown in LP male offspring (Martins et al. 2018).

A hypermetabolic status can alter glucose homeostasis through hormonal influences on key enzymes. Glucokinase (GCK) is the first rate-limiting step in glycolysis in the liver and pancreas (Sternisha & Miller 2019). Phosphoenolpyruvate carboxykinase (PEPCK-1) is the main enzyme that catalyzes the first committed step in hepatic gluconeogenesis (Quinn & Yeagley 2005). In adult female LP offspring (8% protein content), GCK and PEPCK-1 expression levels were higher (Bertasso et al. 2022). Here, we demonstrated that female offspring have normal glucose tolerance during GTT. Furthermore, higher levels of PEPCK-1 reflected the formation of new glucose molecules and increased fasting glucose levels.

Maternal malnutrition caused by protein restriction alters the lipid profiles of male and female offspring (Bertasso et al. 2022, Martins et al. 2018). The diet used in this study was isocaloric and had very low protein content (4%) (de Oliveira et al. 2011). The replacement of protein with carbohydrates in this diet leads to a change in the lipid profile, with lower HDL-C levels in both sexes (Flynn et al. 1999, Nowacka-Woszuk et al. 2017) and in the next generation (Vargas et al. 2023). Here, we show that female offspring displayed decreased CHOL levels in the plasma and increased levels in the liver. A decrease in plasma CHOL levels suggests an increase in tissue uptake of steroid hormones. In addition, the CHOL levels in the plasma and liver, as shown in this study, suggest increased hepatic LDL-C uptake. However, we did not observe a decrease in plasma LDL-C levels.

In male offspring, an LP diet (8% protein content) promotes fatty free acid (FFA) uptake by hepatocytes by increasing white adipose tissue lipolysis (Bertasso et al. 2022). Studies have shown that estrogen protects against hepatic steatosis (Grossmann et al. 2019). In fact, steatosis was not observed in the female rats. Interestingly, in ovariectomized rats, estrogen replacement reversed hepatic steatosis and reduced the hepatic lipogenic protein expression of acetyl-CoA carboxylase and fatty acid synthase in a high-fat high-fructose model (Buniam et al. 2019). Moreover, we showed that the liver TG content was lower in female offspring. A reduction in the liver TG content can occur because of an increase in TG utilization, suggesting an increase in the sympathetic nervous system, as observed in adult male offspring (Martins et al. 2018). In addition, female offspring show increased β-oxidative status by higher carnitine palmitoyl-transferase 1α (CPT-1α) content (Bertasso et al. 2022), suggesting a hepatic compensatory mechanism. In ovariectomized rats, fatty acid oxidation decreases, suggesting an estrogen-dependent pathway (Paquette et al. 2009). Moreover, liver lipid metabolism is essential for neutralizing the impact of FFA on hepatocyte membrane integrity. Here, we show for the first time that

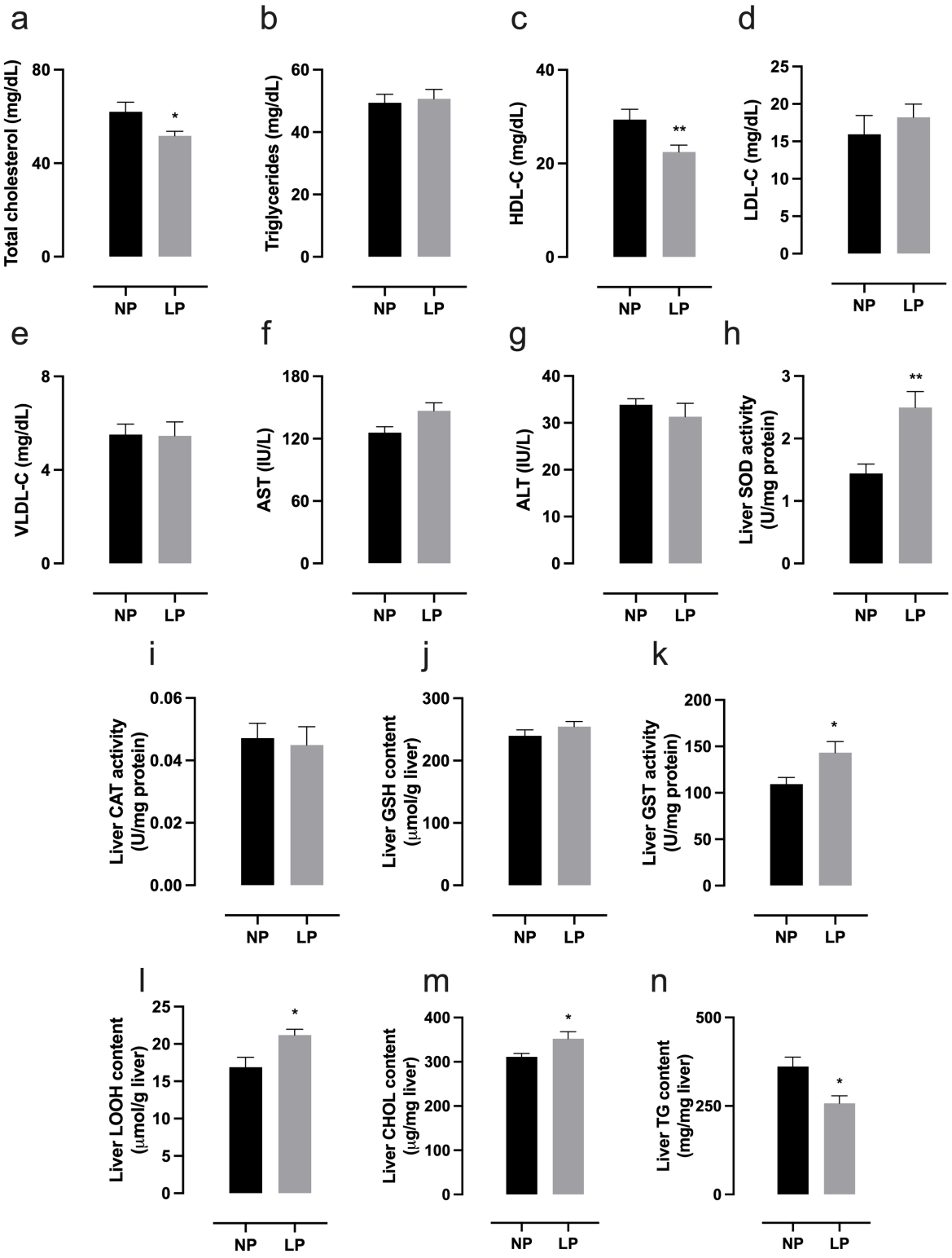

Figure 3. Biochemistry parameters. Total cholesterol (a), triglycerides (b), HDL-C (c), LDL-C (d), VLDL-C (e), AST (f), ALT (g), liver SOD activity (h), liver CAT activity (i), liver GSH content (j), liver GST activity (k), liver LOOH content (l), liver cholesterol content (m) and liver triglycerides content (n). The data are expressed as the mean ± S.E.M. and were obtained from 8-12 rats of each group (from 3-4 different litter). *P<0.05, **P<0.01.

LOOH content, a hallmark of lipid peroxidation, is increased in female offspring with LP.

The increase in liver GST, which is responsible for eliminating lipid peroxidation products (Balogh & Atkins 2011), suggests an effective antioxidant system. Additionally, SOD converts superoxide anions to hydrogen peroxide, which can then be converted, by CAT action, to water and oxygen (Jones 2008). Here, we showed that SOD activity, but not CAT activity, was higher in LP female offspring. It is important to remember that mitochondria are the main organelle producers of reactive oxygen species, and that the mitochondrial genome is inherent to the maternal lineage (Braz et al. 2017). Thus, our data suggest that LP during lactation can positively modulate the antioxidant system and prevent damage to mitochondrial and nuclear DNA.

In conclusion, the LP diet during lactation promotes a lean phenotype and improves the antioxidant system in adult female offspring. These outcomes are essential for understanding the impact of metabolic alterations in the next generation. Further studies are required to elucidate these underlying mechanisms.

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