

Research Article

A Low Maternal Protein Diet During Pregnancy and Lactation Induce Liver Offspring Dysfunction, in the Rat

Podaza E, Echarte EM and Chisari AN*

Institute of Biological Research- National University of Mar del Plata, Argentina

***Corresponding author:** Chisari AN, Institute of Biological Research- National University of Mar del Plata, CONICET, Funes 3250 4° nivel, CP 7600 Mar del Plata, Argentina, Tel: +54-2234753030; Fax: +54-2234724143; Email: achisari@mdp.edu.ar

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Abstract

Pregnancy and fetal development are periods of rapid growth and cell differentiation when mother and offspring are vulnerable to changes. Adverse events during development can be linked to an increased risk in developing metabolic diseases.

The aim of this work is to study the effect of protein malnutrition during gestation and lactation on liver morphology and physiology.

Pregnant Wistar rats of three months of age who were fed a diet containing 8% of proteins, malnourished group (M) or 20% control group (C). The male offspring of mothers M, or male offspring from mothers C were euthanized. Blood was drawn and liver were dissected.

Body weights and liver were lower in the M group. Liver dysfunction was observed by increased serum transaminase, cholesterol and triglycerides in liver.

At weaning glucose in M rats was significantly lower relative to controls. Serum proteins, albumin and triglycerides, significantly decreased compared to the control. Both glycogen as proteins liver content decreased in the group M with respect to C.

We observed inflammation by increasing TNF- α and IL-6. Histology showed a significant liver injury, cellular swelling was observed, hydropic degeneration, characterized by pale cytoplasm as a result of an increased volume of water stored in the cells.

Conclusion: The lack of protein during development compromises the integrity of the structural and functional liver, manifested in adulthood.

Keywords: Fetal programming; Thrifty phenotype; Low protein diet; Metabolic syndrome; Liver damage; Non-alcoholic fatty liver

Introduction

Fetal intrauterine growth restriction (IUGR) occurs in humans as a consequence of poor maternal nutrition. IUGR has been associated with the development of adult diseases; this phenomenon is called 'fetal programming'. The association of maladaptive programming with adult disease has been termed the 'Barker hypothesis'. In general, the Barker hypothesis contends that the malnourished fetus is programmed to exhibit a 'thrifty phenotype' with increased food intake and fat deposition and possibly decreased energy output [1]. Faced with ample available calories, such individuals develop obesity and other manifestations of the metabolic syndrome as adults due to alterations in homeostatic regulatory mechanisms. IUGR leads to alterations in numerous fetal organs. Obesity is potentiated by alterations in appetite regulation and by increased adipogenesis. Hypertension is made more likely by alterations in renal and blood vessel development, whereas diabetes is associated with alterations in cellular insulin signaling and decreased beta cell function. These programmed alterations in function, together, induce the full metabolic syndrome in the adult. Although the specifics of fetal programming are likely to differ depending on the cause of the IUGR,

this issue has not been well studied, and the discussion below does not attempt to differentiate the various maternal manipulations leading to offspring IUGR [2].

The concept of 'developmental programming' proposes that challenges during an organism's development evoke a persistent physiological response in the offspring [3]. Epidemiological investigations such as those conducted on the children conceived during the Dutch famine of 1944-1945 have highlighted the association between poor maternal nutrition, lowered birth weight and subsequent adult disease [4,5].

Several different experimental animal protocols have been used for the evaluation of developmental programming of metabolism: global nutrient restriction [6,7]; or, maternal exposure to an isocaloric low protein diet [8,9].

However there is little research relating to the lack of protein in pregnant women with metabolic disturbances in a vital organ such as the liver of the offspring.

Individuals who suffered severe malnutrition during the development stage, have a high predisposition to suffer metabolic

diseases during adulthood, such as type II diabetes, dyslipidemia, fibrous liver, fatty liver, among others [10].

Given the difficulty and complexity of the regulation of maternal and fetal physiology the physiologist's researchers uses experimental models. These experimental models used are trying to reproduce the form of supply of certain disadvantaged sectors of developing countries, whose own lack protein in their diet.

By contrast, those nutritional circumstances that predispose to Intrauterine Growth Restriction (IUGR) might be due to early childhood exposure. Whereas in some developing countries hunger and inadequate nutritional resources are constant, history has provided many circumstances in which short-term phenomena, whether famine or war, have deprived a cohort of fetuses in utero, only to be followed by a return to normal nutritional status. Therefore we not only have animal models to separate the effect of these constraints, but also natural history epidemiologic work [11].

Animal models clearly demonstrate the effect of poor maternal nutrition on fetal development.

However, difficulties with socio-economic confounds and the inability to include precise contemporaneous controls in epidemiological studies demonstrate the need for carefully controlled animal investigations to address the association between maternal nutrient intake and subsequent health of the offspring. There are five main protocols that have been used for the evaluation of developmental programming of metabolism: (1) exposure of the mother to an isocaloric low protein diet [8,9,12] (2) global nutrient restriction [6]; (3) experimentally induced maternal diabetes [13,14]; (4) restriction of uterine blood flow [15]; and (5) over-exposure of the fetus to glucocorticoids [16]. Extensive epidemiological and laboratory evidence indicates that a suboptimal environment during fetal and neonatal development in both humans and experimental animals impacts on offspring susceptibility to later development of altered carbohydrate metabolism [17-20].

Actually, there are a few researches relating to the lack of protein in the diet with metabolic disturbances in a vital organ to the body such as the liver.

The aim of this work was studying the effect of low protein diet in pregnant mothers during pregnancy and lactation in structural and functional liver offspring.

Materials and Methods

Care and maintenance of animals

All procedures were performed in accordance with the guidelines set by the Faculty of Exact and Natural Sciences of Mar del Plata National University Council of Animal Care and upon approval of the Animal Care Committee OCA 2555/14 and 1499/12.

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. Controlled-pregnant, determined by the presence of sperm in the vaginal smears after mating with an adult male for 24 h, were individually housed in plastic cages with free access to water and food.

At random to one or two groups to fed either 23% casein

(CTRL=control diet, AIN93G) or an 8% casein isocaloric diet (LPD= low protein diet group). Food was provided in the form of a large flat biscuit which was retained behind a grill through which the rats nibbled. A weighed excess of feed was provided each day. The day of delivery was considered as day 0 of post natal life.

To ensure homogeneity of study subjects, litters of over 12 pups or less than 7 were not included in the study.

On day 24 (between 8 am and 9 am), pups per mother were counted, sex was determined, and body weights of male pups were recorded.

For consistency, only male offspring were used for the study because early-life programming is known to occur in a sexually dimorphic manner [21], which was not the focus of this study.

The animals were euthanized on d 24. Blood was collected and processed to obtain serum, which was individually kept at -80°C until assay. Liver was dissected out.

Metabolites Determinations

Serum Total protein, albumin, glucose and cholesterol concentration were determinate by enzymatic assays from Wiener Laboratories, Argentina. Alkaline phosphatase (AP), Serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) were measured by using a commercial kit (WienerLab, Rosario, Argentina) and performed according to the appended protocol. Protein concentration liver was measured with bicinchoninic acid protein assay [22].

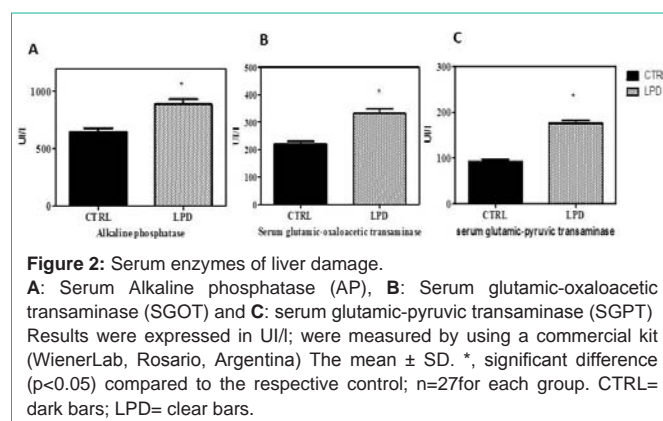
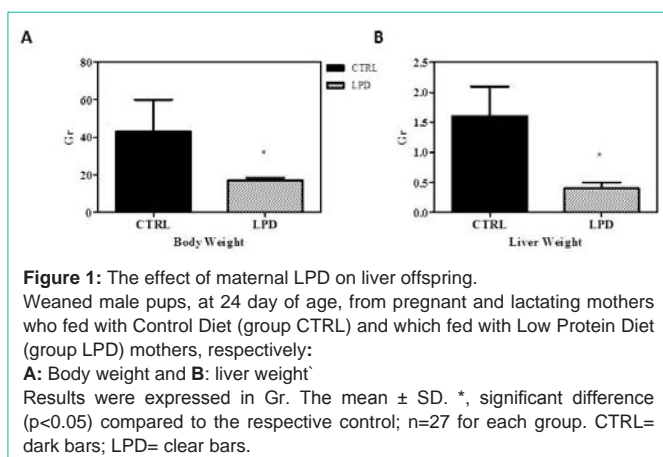
Serum, IL-6 and TNF- α levels were quantified by enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences PharMingen) following the manufacturer's directions. The minimum detectable concentration is 4.5 pg/ml for TNF- α and 10 pg/ml for IL-6.

Tissue lipids: Tissue samples of approximately 0.2 g, together with 2 mL of chloroform-methanol (2:1) solution and were homogenized. The tubes were then centrifuged at 3,000 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 μ L isopropyl alcohol, after which cholesterol and triglyceride levels in the isopropyl alcohol were measured by conventional enzymatic methods (WienerLab, 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 minutes. 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 3,000 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.

Histological Studies

For morphological observation by light microscopy, liver tissues

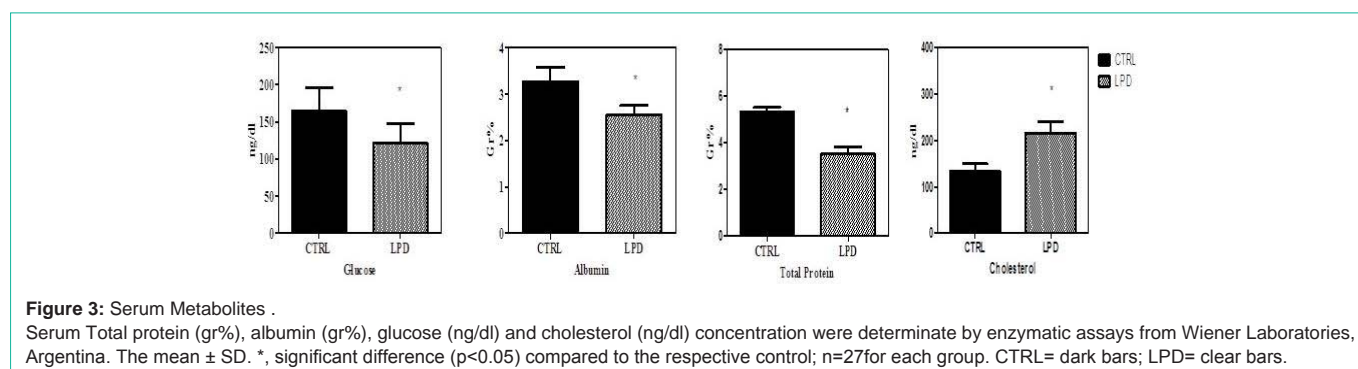


were fixed in 10% neutral formaldehyde solution, dehydrated in an ascending ethanol series and embedded in paraffin. Sections were stained with haematoxylin and eosin (H&E) and reticulin. The structures of liver lobules, hepatocytes, portal tracts and sinusoidal and perisinusoidal lacini were analyzed.

Statistical Analysis

All data are presented as means \pm SD. Data comparison was performed using unpaired two tailed Student's t test (InStat, Graph Pad software). P values lower than 0.05 were considered significant.

Data analyzed include at least three separate experiments. In each experiment three male offspring from 3 different mothers was separated; finally total N = 27 for each experimental group.



Result

Effect of protein restriction diet on the body weight in pregnant mothers and body weight of weaned pups

The evolution of body weight LPD mothers was characterized by time-related increased. This way was similar than CTRL mothers. But, the LPD mothers intake more food after 12 days of gestation. One hundred and two pups (of both sexes) were born from 10 CD mothers and 100 were born from 14 LPD mothers. Interestingly, the number of female pups per mother was similar in the two groups.

The body weight of weaned pups were analyzed, we found a significantly ($p < 0.05$) lower body weight in male pups from LPD (17 ± 1.5) than from CTRL (43 ± 1.2) mothers (Figure 1A).

The weight of the livers of the offspring of mothers fed LPD was also significantly lower than control group (0.4 ± 0.1 vs 1.6 ± 0.5), see Figure 1B.

Biochemical determinations

At weaning glucose in LPD rats was significantly lower relative to controls. Serum proteins and albumin significantly decrease compared to the control, while the levels of cholesterol, AP and transaminase (SGOT and SGPT) were increased (Figure 2 and 3).

Hepatic content of triglycerides and cholesterol were significantly higher in malnourished (group LPD) compared to the control group (CTRL). Both glycogen and proteins content decrease in the group M with respect to C (Figure 4).

The values of serum cytokines, TNF- α and IL-6 were more elevated in rats who mothers fed with low proteins diet compared with control group (Figure 5).

Liver histology

The liver of CTRL rats showed the typical lobular structure with normal central veins and portal tract (Figure 6A). The hepatocytes showed normal cytological structures with well-preserved staining and morphological characteristics of cytoplasm and nucleus. In LPD liver sections showed that although the lobular organization was partially preserved, the hepatocytes displayed a vacuolated cytoplasm. Also, nuclei were absent in some areas (Figure 6B). In addition hepatocytes from these rats showed a decreased nuclear size with indented nuclear membrane and an increase of the perinuclear aggregation of heterochromatin. Exhibit cellular swelling hepatocytes, are not visible sinusoids.

Figure 6 C and D showed reticulin stain, in C we observed

Experimental Group	Glycogen	Triglyceride	Cholesterol	Protein
CTRL	5.38 ± 0.50	4.31 ± 0.21	1.23 ± 0.12	340,8 ± 42
LPD	0.700 ± 0.06*	12.5 ± 1.65 *	3.28 ± 0.71*	179,2 ± 22*

Figure 4: Hepatic metabolites content.

Tissue lipids (cholesterol and triglycerides), glycogen and total protein: Cho and Tri, after homogenized and chloroform-methanol (2:1) extracted, were determined by conventional enzymatic methods WienerLab, Rosario, Argentina. Glycogen, following the protocol described in methodology and after determined by the technique of Anthrone reagent. Results were expressed the mean ± SD. *, significant difference (p<0.05) compared to the respective control; n=27 for each group.

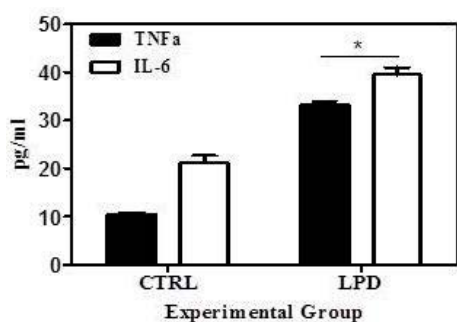


Figure 5: The effect of maternal LPD on Inflammatory response offspring.

Serum, IL-6 (dark bars) and TNF-α (white bars) levels were quantified by enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences PharMingen). Results were expressed the mean ± SD. *, significant difference (p<0.05) compared to the respective control; n=27for each group.

liver of CTRL rats, an orderly and normal position directly related to the provision of the hepatic parenchyma preserved; but in D, we observed the presence of retained fibers with slight alterations in the spatial arrangement because of the increase in the volume of water stored in the cells.

Discussion

During pregnancy, the developing fetus is completely dependent on his mother and maternal environment for nourishment.

Nutrition during the perinatal period is of major importance for proper tissue development and functional maturation. Epidemiological studies in humans have recently suggested that complex metabolic dysfunctions during adulthood, such as glucose intolerance or insulin resistance, could originate from in utero malnutrition.

Recent studies demonstrated that the consequences of inadequate nutrition in utero may extend to adulthood body concerned. The first observations of this phenomenon led to the development of a globally known under the name of ‘fetal programming’ [3] hypothesis.

A variety of animal models were used to study what the impact of different diet biomolecules on fetal programming of various pathologies were. The most commonly used involved rodents which were placed on low protein diets with high lipid content (high fat) or deficient in micronutrients such as iron [23].

The observations we made were the offspring showed low birth weight, so had a smaller liver [24].

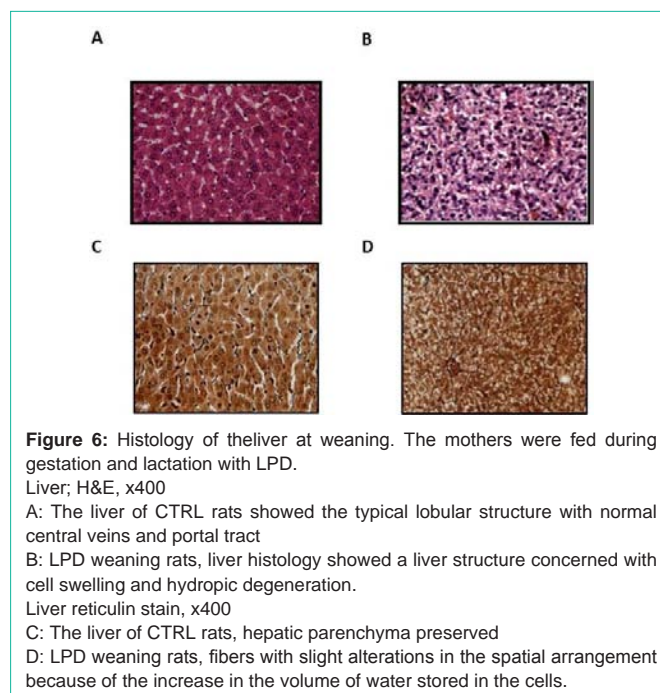


Figure 6: Histology of the liver at weaning. The mothers were fed during gestation and lactation with LPD.

Liver; H&E, x400

A: The liver of CTRL rats showed the typical lobular structure with normal central veins and portal tract

B: LPD weaning rats, liver histology showed a liver structure concerned with cell swelling and hydropic degeneration.

Liver reticulin stain, x400

C: The liver of CTRL rats, hepatic parenchyma preserved

D: LPD weaning rats, fibers with slight alterations in the spatial arrangement because of the increase in the volume of water stored in the cells.

For analysis of liver function results of serum biochemical determinations showed obvious hepatocellular dysfunction reflected in serum levels of the enzymes studied as indicators of the process, GOT, GPT and FA, which would suggest that hepatocytes suffered alterations affecting its integrity. In addition to what is seen by histology. It has been described as important dietary protein requirement, the lack of them in the diet, partial or chronically determined chronic liver disease with hepatocellular deterioration [25,26].

We observed higher TNF-α, TNF-α is a multifunctional cytokine that in the liver acts as a cytotoxic agent in many types of hepatic injury. Kupffer cells, the resident macrophages, which constitute 15% of the liver cell population, are major sources of TNF-α and other inflammatory cytokines, like IL-6 [27].

Conclusion

In short, this work demonstrated that intrauterine malnutrition induced IUGR led to enhanced cholesterol at weaning, through reprogramming of expression of key factors involved in the regulation of cholesterol and bile acid metabolism. The fundamental mechanisms underlying these changes and their influence on the metabolic syndrome remain unclear. Given the plasticity of the liver in fetal and neonatal stages, understanding the effects of intrauterine malnutrition on cholesterol dysregulation may be potentially helpful for developing early life dietary and drug treatment strategies to reduce the incidence of the metabolic syndrome in adulthood.

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