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NAYRA THAIS DELATORRE BRANQUINHO

EFEITOS DA RESTRIÇÃO CALÓRICA E REALIMENTAÇÃO SOBRE O FÍGADO E INTESTINO DELGADO DE RATOS ADULTOS OBESOS: METABOLISMO E MORFOLOGIA

Maringá
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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas
(área de concentração Biologia Celular e Molecular)
da Universidade Estadual de Maringá
para obtenção do grau de doutora em
Ciências Biológicas

Orientadora: Profª Dra. Maria Raquel Marçal Natali
Coorientadora: Profª Dra. Vilma Aparecida Ferreira de Godoi

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Aos meus pais

BIOGRAFIA

Nayra Thais Delatorre Branquinho nasceu em Loanda/PR em 21/09/1989. Possui graduação em Ciências Biológicas pela Universidade Estadual de Maringá (2013) e mestrado em Ciências Biológicas (área de concentração Biologia Celular e Molecular), obtido em junho de 2016 pela Universidade Estadual de Maringá - PBC/UEM. Atualmente é aluna de Doutorado no mesmo Programa. Tem desenvolvido trabalhos nas áreas de fisiologia e histologia; atuando nos seguintes temas: obesidade, restrição calórica; sistema nervoso entérico, metabolismo energético.

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O beija-flor

Voa um colibri faceiro
com plumas de várias cores
beijando e sugando as flores
voando muito ligeiro
passarinho feiticeiro
porque vais tão rápido assim?
é que ninguém vai por mim
faço vezes de maluco
vou colher o doce suco
das rosas lá de um jardim

Em certas horas eu vejo
um colibri benfazejo
pousar por ser desejo
num velho pé de jasmim
depois voa sem malícias
fazendo doces carícias
aproveitando as delícias
das rosas lá de um jardim

Rubi-topázio voando
com suas asas maneiras
as flores das laranjeiras
beijava de quando em quando
lepidamente passando
parando em volta de mim
com lindo tom de cetim
vi-o voando fagueiro
colhendo o suco e o cheiro
das rosas lá de um jardim

De manhã muito cedinho
ele abandona o seu ninho
deixa dormindo o filhinho
porque lhe convém assim
deixando o filho ao relento
vai em busca de alimento
colhendo o doce sustento
das rosas lá de um jardim

Brasiliano Soares Branquinho

APRESENTAÇÃO

Esta tese é composta por dois artigos científicos.

O primeiro artigo já publicado intitulado: "***Responses of the Adult Rat Glucose Metabolism to Early Life Feeding, Caloric Restriction and Refeeding***" (2018) teve o objetivo de avaliar o efeito da redução de ninhada e realimentação pós restrição calórica moderada sobre o metabolismo hepático de glicose em hepatócitos isolados, adiposidade, homeostase glicêmica e adaptação hepática sob as condições alimentares. Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas, este artigo foi redigido de acordo com as normas do periódico (Journal of Pharmacy & Pharmacology) (ISSN 2328-2150, fator de impacto: 3,16) e Qualis (Ciências Biológicas I): B1. doi: 10.17265/2328-2150/2018.

O segundo artigo intitulado: "***Redução de ninhada, restrição calórica e realimentação em ratos alteram a inervação intrínseca entérica, morfologia hepática e intestinal***", teve como objetivo avaliar aspectos morfométricos do fígado e do intestino delgado e inervação intrínseca jejunal de ratos machos com 150 dias provenientes de ninhadas reduzidas, submetidos à restrição calórica com ou sem realimentação posterior. Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas, este artigo foi redigido de acordo com as normas do periódico (Neurogastroenterology and Motility) (ISSN 1350-1925, Fator de impacto: 3,803) e Qualis (Ciências Biológicas I): A1.

RESUMO GERAL

INTRODUÇÃO: A obesidade é uma doença crônica relacionada ao surgimento e agravamento de diversas desordens metabólicas. A restrição calórica (RC), em níveis controlados e moderados, vem sendo defendida como forma eficaz de combate a esse quadro e suas comorbidades. O ambiente nutricional pós-natal pode contribuir significativamente para o excesso de peso, e em roedores a redução do número de filhotes durante a lactação é um modelo clássico de indução precoce de obesidade, com reflexos morfológicos, metabólicos e funcionais que se prolongam até a idade adulta. Apesar dos muitos estudos utilizando este modelo experimental, poucos abordam especificamente o trato gastrintestinal e o metabolismo hepático. O intestino delgado é o órgão responsável pela digestão e absorção dos nutrientes da dieta; o fígado, por sua vez, é o órgão essencial na metabolização dos nutrientes absorvidos e na regulação de seus níveis circulantes.

OBJETIVO: Avaliar os efeitos da restrição calórica de 30%, seguida ou não por alimentação livre, em ratos de ninhadas reduzidas sobre parâmetros biométricos e plasmáticos. No fígado foi avaliado a área, número e percentual de glicogênio e lipídio em hepatócitos e metabolismo da glicose *in vivo* e *in vitro* em hepatócitos isolados. No jejuno foi avaliado histomorfometria (espessura das túnicas) e quantificação celular (índice de células caliciformes e número de células imunes) na lâmina própria e inervação intrínseca jejunal (neurônios mioentéricos HuC/D$^+$ e gliócitos S100$^+$).

MATERIAIS E MÉTODOS: 40 ratos machos Wistar, foram distribuídos em 4 grupos: G9 fornecido água e ração *ad libitum* até a idade de 150 dias; G3L submetido a redução de ninhada e alimentação livre até os 150 dias; G3R redução de ninhada e alimentação livre até os 60 dias, com RC de 30% em relação ao consumo de G3L dos 60 aos 150 dias; G3RL animais que tinham o seu fornecimento de comida reduzida em 30% em relação ao consumo do G3L, em seguida realimentados livremente até completarem os 150 dias de idade. Foi registrado: peso corporal e comprimento naso-anal, peso de gorduras e do fígado e parâmetros plasmáticos. Amostras de fígado e jejuno foram destinadas a realização de técnicas histológicas e histoquímicas. Amostras do jejuno foram submetidos a elaboração de preparados de membrana e posterior imunohistoquímica HuC/D$^+$ e S100$^+$ para avaliação da densidade e morfometria (perfil celular) dos neurônios mioentéricos e gliócitos, respectivamente. A investigação do metabolismo da glicose ocorreu por teste de tolerância à glicose (ivGTT), hipoglicemia induzida por insulina (HII), e incubação de hepatócitos isolados com substratos gliconeogênicos. Os conjuntos de dados foram submetidos a análise de normalidade pelo teste de Shapiro-Wilk e

Kolmogorov-Smirnov. Dados paramétricos foram comparados por One-way ANOVA/Tukey e dados não paramétricos por Kruskal-Wallis/Dunn´s. O nível de significância adotado foi de 5%.

RESULTADOS E DISCUSSÃO: Aos 150 dias, ratos provenientes de redução de ninhada e alimentação livre, não apresentaram diferenças com animas de ninhadas controle (G9) nos parâmetros biométricos, plasmáticos e metabolismo de glicose hepática sob condições basais ou gliconeogênicas, porém constatou-se efeitos sobre a morfologia do fígado (redução na área de hepatócitos) e sobre a morfologia jejunal (redução no índice de células caliciformes e profundidade de criptas e aumento na densidade e perfil de gliócitos do plexo mioentérico). Além da redução do peso corporal e peso de gorduras, a restrição calórica promoveu diversos efeitos no fígado: redução no peso do órgão, redução do decaimento de glicose após a injeção de insulina e a menor liberação de glicose pela via gliconeogênica em hepatócitos isolados, redução na área e aumento no número de hepatócitos. Apesar do aumento do comprimento do intestino delgado, o jejuno mostrou-se mais adaptado a condição imposta, mantendo as características morfométricas e de inervação intrínseca, entretanto, registramos aumento no número de gliócitos. A realimentação após a restrição calórica recuperou os parâmetros biométricos, plasmáticos aos valores do grupo controle, exceto o nível da transaminase glutâmico pirúvica. Esta recuperação também atingiu peso do fígado, área de hepatócitos e inclusão lipídica no tecido e o metabolismo de glicose em hepatócitos isolados. No jejuno a espessura de túnicas e índice de células caliciformes foi mantida. Os neurônios HuC/D^{+} neste grupo apresentaram maior perfil celular quando comparado aos demais grupos.

CONCLUSÃO: Estes resultados sugerem que aos 150 dias o metabolismo da glicose no fígado, perfil de hepatócito e morfometria intestinal de ratos Wistar não sofrem efeitos da programação metabólica no modelo de redução de ninhada, mas é sensível à condição nutricional vigente.

Palavras-chave: homeostase de glicose, programação metabólica, redução de ninhada, restrição calórica, realimentação, morfometria hepática, morfometria jejunal, neurônios mioentéricos.

GENERAL ABSTRACT

INTRODUCTION: Obesity is a chronic disease related to the onset and aggravation of various metabolic disorders. Caloric restriction (CR), at controlled and moderate levels, has been defended as an effective way of controlling this condition and its comorbidities. The postnatal nutritional environment may contribute significantly to overweight, and in rodents the reduction of litter during lactation is a classic model of early obesity induction with morphological, metabolic and functional reflexes that extend into adult age. Despite the many studies using this experimental model, few specifically address the gastrointestinal tract and the hepatic metabolism. The small intestine is directly responsible for the digestion and absorption of nutrients from the diet; the liver, in turn, is an essential organ in the metabolization of the absorbed nutrients and in the regulation of their circulating levels.

OBJECTIVE: To evaluate the effects of caloric restriction of 30%, followed or not by free feed, in rats of reduced litters on biometric and plasma parameters. In the liver was evaluated the area, number and percentage of glycogen and lipid in hepatocytes and glucose metabolism *in vivo* and *in vitro* in isolated hepatocyte. In jejunum was evaluated histomorphometry (tunic thickness) and cell quantification (goblet cell index and number of immune cells) in the cell itself and intrinsic jejunal innervation (myenteric neurons HuC/D $^{+}$ and S100^{+} gliocytes).

MATERIALS AND METHODS: 40 male Wistar rats were, distributed in 4 groups: G9 supplied with water and chow ad libitum; G3L submitted to litter reduction and feed *ad libitum*; G3R litter reduction and free feeding up to 60 days, with CR of 30% relative to G3L from 60 to 150 days; G3RL litter reduction and free feeding up to 60 days, with CR of 30% relative to G3L from 60 to 90 days, then refed freely until 150 days. It was recorded: body weight and naso-anal length, fat and liver weight and plasma parameters. Liver and jejunum samples were used to perform histological and histochemical techniques. Wholemounts of samples of jejunum were submitted to immunohistochemistry HuC/D^{+} and S100^{+} to evaluate the density and morphometry (cell profile) of the myenteric neurons and gliocytes, respectively.The investigation of glucose metabolism was carried out by glucose tolerance test (IvGTT), insulin-induced hypoglycemia (IIH) and incubation of isolated hepatocytes with gluconeogenic substrates. The data submitted to normality analysis by the Shapiro-Wilk and Kolmogorov-Smirnov test. Parametric data were compared using One-way ANOVA/Tukey and non-parametric data by Kruskal-Wallis/Dunn´s. The level of significance adopted was 5%.

RESULTS AND DISCUSSION: At 150 days, rats from reduced litter and free feeding

(G3L), showed no differences from the control group (G9) in biometric, plasma parameters and hepatic glucose metabolism under baseline or gluconeogenic conditions, but effects on liver morphology (reduction in hepatocyte area) were found and on the jejunal morphology (reduction in the index of goblet cells and depth of crypts and increase in the density and profile of myoenteric plexus gliocytes). In addition to the reduction in body weight and fat weight, caloric restriction promoted several effects on the liver: reduced organ weight, reduced glucose decay after insulin injection and reduced glucose release via gluconeogenesis in isolated hepatocytes, reduced area and increase in the number of hepatocytes. Despite the increase in the length of the small intestine, the jejunum was more adapted to the imposed condition, maintaining the characteristic morphometry and intrinsic innervation, however, we registered an increase in the number of gliocytes. The refeeding after caloric restriction (G3RL) recovers the biometric and plasmatic parameters to the control values, except for the level of pyruvic glutamic transaminase. This recovery also affecteds the weight of the liver, area of hepatocytes and lipid inclusion and glucose metabolism in isolated hepatocytes. In the jejunum the tunic thickness and goblet cell index were maintained. The HuC/D^{+} neurons in this group had a larger cellular profile when compared to the other groups.

CONCLUSION: These results suggest that at 150 days, glucose metabolism in the liver, hepatocyte profile and intestinal morphometry of Wistar rats do not suffer from effects of metabolic programming in the litter reduction model, but are sensitive to the current nutritional condition.

Key words: glucose homeostasis, metabolic programming, brood reduction, caloric restriction, feedback, liver morphometry, jejunal morphometry, myenteric neurons.

SUMÁRIO

Artigo Científico 1:

Responses of the Adult Rat Glucose Metabolism to Early Life Feeding, Caloric Restriction and Refeeding

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Responses of the Adult Rat Glucose Metabolism to Early Life Feeding, Caloric Restriction and Refeeding

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Abstract: Early life overfeeding in the rat can be experimentally induced by reducing litter size. This investigation assessed the consequences of this manipulation on glucose metabolism *in vivo* and in isolated hepatocytes in 150-day old rats. Additionally, after body growth, the effects of caloric restriction and refeeding were tested. Adult rats from control (G9) and reduced litters (G3L) did not differ in body and fat weights, glucose tolerance or insulin resistance (insulin-induced hypoglycemia), or hepatocyte glucose release under basal or gluconeogenic conditions. Caloric restriction (G3R) reduced body and fat weights, decreased glucose decay after insulin injection and decreased hepatocyte gluconeogenic glucose release. Refeeding after caloric restriction reversed these parameters to those of the freely-fed groups (G9 and G3L). Taken together, these results suggest that the liver glucose metabolism is not programmed by lactational overfeeding, but rather is responsive to the current nutritional condition of the animal.

Key words: Glucose homeostasis, metabolic programming, reduced litter, caloric restriction, refeeding.

1. Introduction

In the rat, reducing litter size during lactation is a classical model leading to adult obesity [1-4]. It is reported that overfeeding in early life programs later life obesity by modulating the hypothalamic circuits that control food ingestion, energy expenditure and metabolism, which in the rat are susceptible to environmental conditions during late gestation and lactation [1, 2]. As a central organ of energy metabolism, the liver is a potential target of these early life events. Glucose homeostasis is reported to be compromised in adult life after perinatal overfeeding [3, 4] and a role for the liver should be expected.

Moderate caloric restriction may overcome the negative effects of obesity and its comorbidities [5-7]. In previous investigations, reduced-litter rats were subject to caloric restriction soon after weaning, and it had the expected result on body growth and composition. However, in those young adult rats (up to 90 days old), litter size did not overtly influence liver metabolism or whole-body glucose homeostasis, while caloric restriction had much more pronounced effects.

As in these previous studies caloric restriction was imposed during the period of body development, the alterations could have been the consequence of blunted growth rather than from decreased food intake *per se*. Therefore, this investigation was designed to complement those other investigations and answer the following questions: Is liver glucose metabolism in the grown adult rat programmed by early life nutritional conditions? To what point systemic glucose changes, if they appear, can be ascribed to the liver? After body growth is complete, can caloric restriction reverse any changes caused by early life overfeeding? If so, is this

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reversal resistant to refeeding?

2. Method and Materials

2.1 Ethical Approval

The international ethical guidelines on animal care and experimentation were followed. All the experimental procedures were approved by the Ethics Commission of the Institution (certificate 1720290116).

2.2 Experimental Groups

Wistar *Rattus norvegicus* albino rats were used. Pregnant females were placed in individual cages in an animal house of controlled temperature (22 ± 2 ^{o}C) and light/dark cycles (12 h light/12 h dark). Water and rodent chow were supplied *ad libitum*.

One day after birth, the litters were organized so that each dam had either nine or three male pups, thus establishing the initial experimental groups, control-litter (G9) and reduced-litter (G3) groups, respectively. Control litter size was based on the average number of pups born per litter of the original rat colony.

At weaning (21 days old), the pups were placed in plastic boxes (three rats/box). Group G9 (n = 20) was given water and chow *ad libitum* until the age of 150 days. Group G3 was fed at will until 60 days of age, when it was subdivided into the following groups:

G3L (n = 20): animals that were fed at will until the age of 150 days;

G3R (n = 25): animals that had their supply of chow reduced in 30% relative to the intake of the G9L, corrected for body weight, from 60 to 150 days of age;

G3RL (n = 24): animals that had their supply of chow reduced in 30% relative to the intake of the G9L, corrected for body weight, until the age of 90 days; refeeding at will was then allowed until these rats were 150 days old;

Body weight and naso-anal length were measured at the ages of 60, 90 and 150 days. These were used to calculate the body mass index for rats (BMI, g/cm^2) [8, 9].

At the age of 150 days, some of the rats of each group were used for the *in vivo* procedures described below. Immediately after each test, the animals were returned to their boxes and given water and chow. Euthanasia was carried out a few days later. Other rats of each group were used for *in vitro* protocols (hepatocyte isolation and incubation). All the procedures were carried out after overnight fasting (approx. 14 h).

Euthanasia was performed by intraperitoneal injection of excess anesthetic (thionembutal 120 mg/kg body weight plus lidocaine 5 mg/kg). Blood was rapidly collected, centrifuged and stored at -80 ºC for further analytical determinations. Fats and liver were removed and weighed.

2.3 Intravenous Glucose Tolerance Test (ivGTT)

Under anesthesia (thionembutal 40 mg/kg body weight plus lidocaine 5 mg/kg, ip), a heparinized cannula (50 IU heparin; 1 mL NaCl 0.9%) was implanted into the right jugular vein and attached to the dorsal cervical region. After overnight fasting (about 12 hours) a bolus of glucose (1 g/kg body weight, dissolved in saline) was infused through the cannula. Samples of blood were collected immediately before glucose infusion (0 min) and 5, 15, 30, 45 and 60 min after glucose infusion. Blood glucose was determined with test strips and glucometer (Optium Exceed®; Abbott, São Paulo-SP, Brazil). The AUC (area under curve) of blood glucose variation was calculated using blood glucose at 0 min as baseline [10].

2.4 IIH (Insulin-Induced Hypoglycemia)

The rats were ip injected with regular insulin (1 U/kg body weight; Novolin®; Novo Nordisk, Montes Claros-MG, Brazil). Blood samples were collected from the tail just before insulin injection (0 min) and at 5, 10, 15, 20, 25, 30, 60, 120, 180, 240 and 300 min. Blood glucose was determined with test strips and glucometer (Optium Exceed®). The index of glucose decay (kITT, %/min) was calculated for the first 30 min

of the IIH [11].

2.5 Determination of Plasma Insulin

Plasma insulin was measured by radioimmunoassay in gamma counter (Wizard2 Automatic Gamma Counter®, TM-2470, PerkinElmer, Shelton-CT, USA). Human insulin was used as standard along with an anti-rat insulin antibody (Sigma-Aldrich, St. Louis-MO, USA) and ^{125}I-labeled recombinant human insulin (PerkinElmer). The intra-assay coefficients of variation were in the range of 8-10%.

2.6 Incubation of Hepatocytes

After overnight fasting, the rats were anesthetized with thionembutal (40 mg/kg body weight plus 5 mg/kg lidocaine, ip). The liver was perfused through the portal vein for 15 min with non-recirculating aerated calcium-free KH (Krebs-Henseleit) buffer (pH 7.4, 37 ºC). Next, KH containing calcium and collagenase (700 U/dL, pH 7.4) was perfused in a recirculating system for 5-7 min. The liver was removed, manually fractioned, filtered and centrifuged three times (4 ºC, 530 rpm) with albumin-containing KH (0.2 g/dL) [9, 11-13]. Samples of 10^6 cells/mL were incubated with a gluconeogenic precursor (glycerol or lactate or alanine or glutamine) at the concentration of 5 mM each [9, 11] in a water bath for 60 min under constant agitation and aeration. Glucose and urea release under these conditions were termed gluconeogenic release. Additional flasks containing only the KH buffer were used to determine the control (basal) release of the products analyzed. After incubation, the samples were centrifuged for 10 min at 3,000 rpm (room temperature) and the supernatant stored for biochemical assays.

2.7 Biochemical Assays

Commercial kits (Gold Analisa®; Belo Horizonte-MG, Brazil) were used for the biochemical determinations from the plasma samples (total and HDL cholesterol, triglycerides and glucose) and from the supernatants of the hepatocyte incubation (glucose and urea).

2.8 Statistical Analyses

For the sake of consistency, all data sets in tables and figures are shown as mean ± SD. The number of replicates for each data set is indicated.

Shapiro-Wilk and Kolgomorov-Smirnoff normality tests were applied. Student's test t was used for comparisons of data sets from two groups, or two time-points within the same group. One-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test was employed to compare three or more data sets. The significance level adopted was 5%. The statistical analyses and the construction of the figures were made on Prism® 5.0 (GraphPad, San Diego-CA, EUA).

3. Results and Analysis

Table 1 brings the body weight, BMI and blood glucose of the groups at the ages of 60, 90 and 150 days. Tissue weights at 150 days are also shown. Group G3L had higher body weight, BMI and blood glucose than the G9 at 60 days of age, but not at later ages. Food restriction from 60 to 150 days of age (G3R) decreased body weight, BMI and blood glucose, while refeeding from 90 to 150 days of age (G3RL) restored these values to the G9 and G3L levels.

Food restriction (G3R) markedly decreased the relative weight of all body fats and increased the relative liver weight. Abdominal fats and liver of the refed group (G3RL) were statistically similar to those of groups G9 and G3L, but mean values were higher (from 6% for epidydimal fat to 24% for mesenteric fat; 15% for liver). Subcutaneous fat weight of group G3RL, on the other hand, was four times higher than in group G3R, but 50% lower than in groups G9 and G3L.

The plasmatic profile of the groups at the age of 150 days is given in Table 2. Group G3L had higher TGL and VLDL than group G9. In the food-restricted rats (G3R), the lipid values were similar to those of the control, except for VLDL, which was even lower. On

Table 1 Biometric parameters, fasting blood glucose and tissue weights of rats from control (G9) or reduced litters (G3) fed at will (G3L), food-restricted in 30% (G3R) or refed after food restriction (G3RL), at the ages of 60, 90 and 150 days.

60 days	G9 (n = 11)		G3L (n = 13)	
Body weight (g)	230.3 ± 11.17		284.6 ± 17.62[a]	
BMI (g/cm^2)	0.52 ± 0.02		0.62 ± 0.04[a]	
Glucose (mg/dL)	76.55 ± 4.63		83.92 ± 9.82[a]	
90 days	G9 (n = 6)	G3L (n = 8)	G3R (n = 9)	
Body weight (g)	349.2 ± 13.00	345.9 ± 15.47	272.4 ± 23.37[a,b]	
BMI (g/cm^2)	0.61 ± 0.02	0.63 ± 0.03	0.59 ± 0.04[b]	
Glucose (mg/dL)	77.17 ± 5.57	71.38 ± 6.30	69.22 ± 4.92[a]	
150 days	G9 (n = 12)	G3L (n = 14)	G3R (n = 17)	G3RL (n = 16)
Body weight (g)	440.2 ± 34.16	444.2 ± 34.89	215.9 ± 31.66[a,b]	432.6 ± 33.45[c]
BMI (g/cm^2)	0.69 ± 0.03	0.70 ± 0.02	0.45 ± 0.07[a,b]	0.71 ± 0.06[c]
Glucose (mg/dL)	91.83 ± 8.44	96.35 ± 11.48	85.71 ± 8.84[b]	90.38 ± 6.04
Retroperitoneal fat (g%)	1.46 ± 0.19	1.56 ± 0.35	0.04 ± 0.03[a,b]	1.73 ± 0.46[c]
Epidydimal fat (g%)	1.41 ± 0.24	1.49 ± 0.14	0.14 ± 0.09[a,b]	1.56 ± 0.28[c]
Mesenteric fat (g%)	0.77 ± 0.19	0.86 ± 0.22	0.12 ± 0.06[a,b]	1.07 ± 0.40[c]
Visceral fat (g%)	3.73 ± 0.63	3.97 ± 0.60	0.37 ± 0.10[a,b]	4.04 ± 0.67[c]
Subcutaneous fat (g%)	1.50 ± 0.20	1.39 ± 0.20	0.20 ± 0.06[a,b]	0.80 ± 0.12[a,b,c]
Liver (g%)	3.36 ± 0.28	3.22 ± 0.24	4.00 ± 0.55[a,b]	3.72 ± 0.16[c]

Data shown as mean ± SD; [a] $p < 0.05$ *vs.* G9; [b] $p < 0.05$ *vs.* G3L; [c] $p < 0.05$ *vs*. G3R; Student's t-test for groups at 60 days of age; ANOVA-Tukey for groups at 90 and 150 days of age.

Table 2 Plasmatic parameters of 150-day old rats from control (G9) or reduced litters (G3) fed at will (G3L), food-restricted in 30% (G3R) or refed after food restriction (G3RL).

	G9 (n = 5)	G3L (n = 5-8)	G3R (n = 5-7)	G3RL (n = 5-8)
Total Chol (mg/dL)	78.38 ± 8.28	70.63 ± 18.98	67.36 ± 7.39	99.56 ± 9.15[b,c]
HDL-Chol (mg/dL)	39.50 ± 5.12	25.94 ± 10.54[a]	29.71 ± 8.30	36.00 ± 4.33
VLDL (mg/dL)	4.85 ± 0.93	22.75 ± 13.66[a]	4.41 ± 0.89[b]	28.55 ± 8.72[a,c]
LDL (mg/dL)	28.78 ± 7.86	21.94 ± 12.17	33.23 ± 5.65	35.01 ± 7.86[b]
TGL (mg/dL)	24.25 ± 4.63	113.8 ± 68.32[a]	22.07 ± 4.47[b]	142.8 ± 43.60[a,c]
Total proteins (g/dL)	6.42 ± 0.60	6.28 ± 0.05	5.03 ± 0.31[a,b]	5.53 ± 0.66[a]
Insulin (pg/mL)	17.00 ± 1.41	16.5 ± 1.92	28.25 ± 5.06[a,b]	35.00 ± 4.97[a,b]
Atherogenic index	1.85 ± 0.13	3.20 ± 0.97[a]	2.39 ± 0.59	2.79 ± 0.28[a]

Atherogenic index = Total Chol/HDL-Chol. Data shown as mean ± SD; [a] $p < 0.05$ *vs.* G9; [b] $p < 0.05$ *vs.* G3L; [c] $p < 0.05$ *vs.* G3R; ANOVA-Tukey.

the other hand, after refeeding (G3RL), total cholesterol, TGL, VLDL and LDL had values even higher than those of group G3L. Plasma proteins (Table 2) were decreased by food restriction (G3R) and did not increase significantly after refeeding ($p > 0.05$, G3RL *vs.* G3R). Insulin was significantly higher in reduced-litter rats under caloric restriction (G3R) or refed (G3RL). The atherogenic index was higher in groups G3L and G3RL compared to group G9.

The profile and AUC of the ivGTT are in Fig. 1. Five min after glucose infusion, blood glucose reached values of 300 mg/dL or higher in all the groups. At this time, group G3L had the highest blood glucose, while group G3R had the lowest. By the end of the test (60 min), blood glucose returned to basal (0 min) values in all the groups. Although blood glucose at 0 min was lower in groups G3R and G3RL than in group G3L, it was not different from the 60 min value of the group. The AUC did not differ between the groups.

Fig. 2 is the profile and kITT of the insulin-induced hypoglycemia (IIH). At 10 min, blood glucose was significantly lower than at 0 min in every group. The

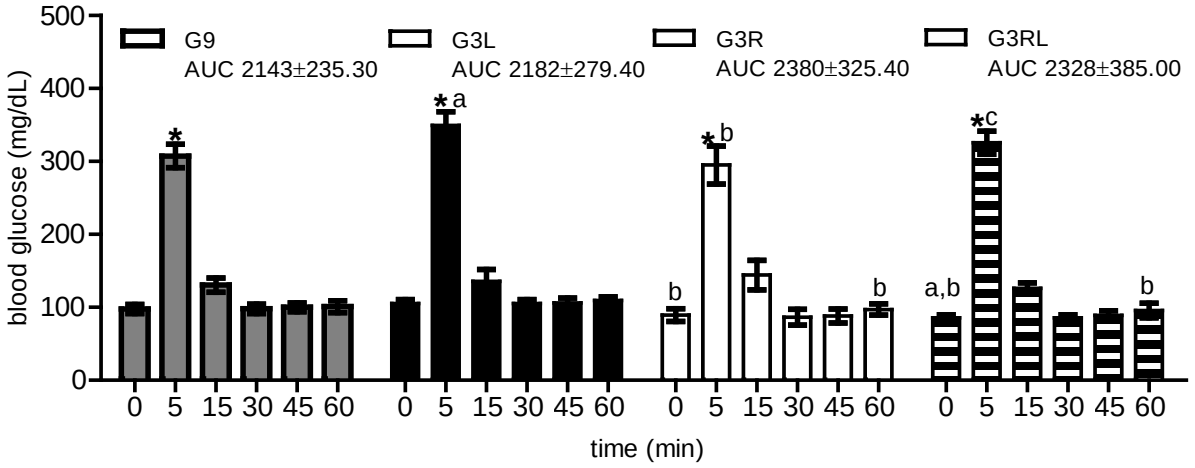

Fig. 1 Profile and AUC of intravenous glucose tolerance test (ivGTT) of 150-day old rats from control (G9) or reduced litters (G3) fed at will (G3L), food-restricted in 30% (G3R) or refed after food restriction (G3RL).
Data shown as mean ± SD; n = 5-7 per group. [a] $p < 0.05$ *vs.* G9; [b] $p < 0.05$ *vs.* G3L; [c] $p < 0.05$ *vs.* G3R; ANOVA-Tukey.* $p < 0.05$ *vs.* time 0 min of the group; Student's t-test.

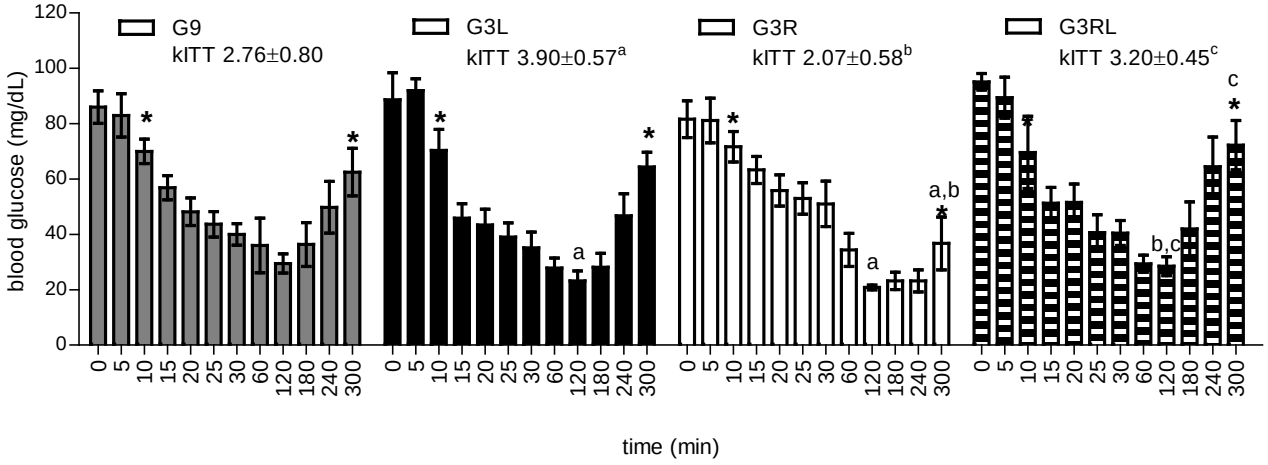

Fig. 2 Profile of insulin-induced hypoglycemia (IIH) and kITT of 150-day old rats from control (G9) or reduced litters (G3) fed at will (G3L), food-restricted in 30% (G3R) or refed after food restriction (G3RL).
Data shown as mean ± SD; n = 6-9 per group. [a] $p < 0.05$ *vs.* G9; [b] $p < 0.05$ *vs.* G3L; [c] $p < 0.05$ *vs.* G3R; ANOVA-Tukey.* $p < 0.05$ *vs.* time 0 min of the group; student's t-test.

lowest blood glucose was recorded 120 min after insulin injection. At this time, blood glucose was lower in groups G3L and G3R than in the control (G9) and refed (G3RL) groups.

None of the groups recovered completely from the hypoglycemic episode at 300 min. Recovery was significantly lower in group G3R, where blood glucose at 300 min was less than 40 mg/dL. At this moment, blood glucose of the other groups was at least 60 mg/dL.

Blood glucose decay was faster in group G3L (as indicated by the higher kITT), but it was markedly slower in group G3R.

Fig. 3 is the glucose release by isolated hepatocytes incubated under basal condition or with the gluconeogenic precursors glycerol (gly), lactate (lac), alanine (ala) or glutamine (gln). Basal glucose release was significantly higher in the G3R and lower in the G3RL when compared with groups G9 and G3L. Glucose release under gluconeogenic conditions, on

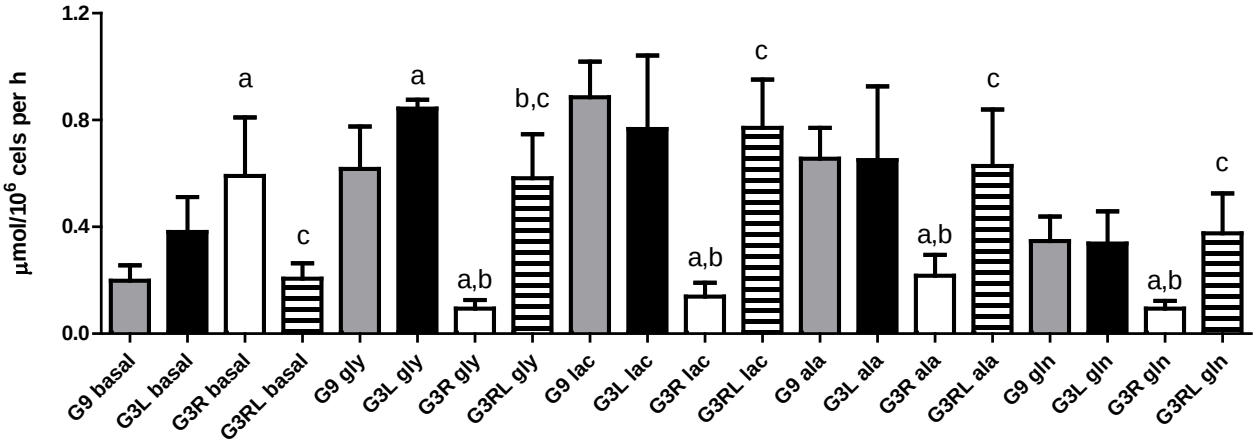

Fig. 3 Glucose release by incubated hepatocytes of 150-day old rats from control (G9) or reduced litters (G3) fed at will (G3L), food-restricted in 30% (G3R) or refed after food restriction (G3RL).
Data shown as mean ± SD; n = 5-7 per group. [a] $p < 0.05$ *vs.* G9; [b] $p < 0.05$ *vs.* G3L; [c] $p < 0.05$ *vs.* G3R. ANOVA-Tukey.
gly: glycerol; lac: lactate; ala: alanine; gln: glutamine.

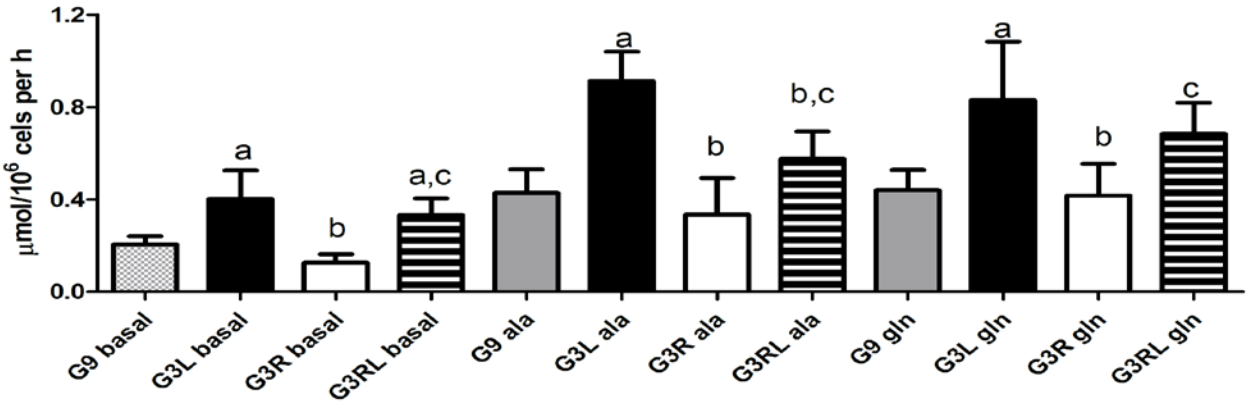

Fig. 4 Urea release by incubated hepatocytes of 150-day old rats from control (G9) or reduced litters (G3) fed at will (G3L), food-restricted in 30% (G3R) or refed after food restriction (G3RL).
Data shown as mean ± SD; n = 5-7 per group. [a] $p < 0.05$ *vs.* G9; [b] $p < 0.05$ *vs.* G3L; [c] $p < 0.05$ *vs.* G3R. ANOVA-Tukey.
ala: alanine; gln: glutamine.

the other hand, was much lower in group G3R in the presence of every precursor, while the other groups had similar gluconeogenic glucose release.

Urea release by incubated hepatocytes was determined in the presence of alanine and glutamine, as shown in Fig. 4. It was higher in group G3L than in group G9 for all three conditions, and lower in group G3R than in group G3L. Refeeding after caloric restriction (G3RL) tended to restore urea release to either G9 or G3L levels.

4. Discussion

A brief survey of the results of this work shows that 60-day old reduced-litter rats (G3L) had body weight and BMI greater than control litters (G9), but did not differ from them at later ages. Nevertheless, plasma triglycerides and VLDL were higher at 150 days of age. Food restriction of 30% from 60 to 150 days of age (group G3R) decreased body and fat weights and the lipid profile. Upon refeeding (group G3RL), these parameters were restored to those of the freely-fed (G9

and G3L) groups. In fact, visceral fat and triglycerides were higher than in group G3L.

During the insulin challenge (IIH), systemic glucose homeostasis was changed significantly in group G3R: blood glucose decay was slow, and recovery was impaired. Similarly, only this group had an overt change of glucose handling by the liver, with higher basal and lower gluconeogenic glucose release.

The effects of early life and adult feeding on hepatocyte glucose metabolism were the primary targets of this investigation. Although body weight, BMI and fat weights were not different between G9 and G3L rats at the age of 150 days, the possibility remained that litter size could have changed liver glucose metabolism. Caloric restriction increased basal and decreased gluconeogenic glucose release from incubated hepatocytes, in a fashion very similar to that seen in younger rats [9, 11]. In addition, glucose release from hepatocytes of refed animals (group G3RL) recovered the profile of rats from control or reduced litters that were fed at will since weaning (groups G9 and G3L, respectively). These two groups, in turn, did not differ significantly from each other in their basal or gluconeogenic glucose release. Therefore, litter size reduction, and the consequent early life overfeeding, did not program liver glucose metabolism in adult life. Instead, the liver seems to have a pattern of basal and gluconeogenic glucose release that is dependent on the current nutritional condition of the animal.

Increased glucose release under basal incubation conditions in the food-restricted group (G3R) suggests glucose release from endogenous stores, that is, glycogen. Although glycogen content was not measured in this investigation, the higher liver weight is an indirect indicative of this supposition. Accordingly, a previous work on younger rats under similar feeding conditions showed increased liver weight and glycogen content [11]. The higher plasma insulin levels of group G3R could have enhanced this glycogen storage, as discussed later.

Gluconeogenesis is the primary response of the liver to maintain blood glucose homeostasis, especially when glycogenolysis decreases as fasting gets longer [14-16]. The incubation of hepatocytes was made in a glucose-free medium, and the cells were obtained from overnight-fasted animals, both of which would favor gluconeogenesis, given appropriate substrates were present. Certainly, all the groups showed gluconeogenic glucose release, but the magnitude was much lower in hepatocytes from G3R rats. The supposed presence of glycogen discussed above could have decreased gluconeogenic flux in this group, or some of the early intermediates of gluconeogenesis, such as pyruvate, could have been diverted to replenish glycogen stores. This would imply that inhibition of glycogen synthesis—or stimulation of glycogen breakdown—by the counter-regulatory hormones is incomplete in the calorically-restricted rat. Alternatively, the lower urea release of group G3R in the presence of alanine or glutamine may indicate that these hepatocytes had a reduced capacity of either uptaking or metabolizing gluconeogenic precursors. Decreased urea release is also a consistent finding in this group, as it was also reported in younger G3R rats [9, 11]. In addition, lactate measurements were higher in the G3R flasks incubated with this gluconeogenic precursor (data not shown), suggesting that lactate uptake might be decreased in this group, thus leading to lower gluconeogenesis from lactate.

Glucose intolerance, assessed by the ivGTT, was not seen in any group of this investigation. However, insulin resistance, indicated by the lower kITT, was present in group G3R, and can be correlated, at least partially, with hepatocyte glucose metabolism: if glycogen stores were still present (as assumed by the high hepatocyte basal glucose release), the liver would have a decreased capacity of responding to the insulin challenge—which it would do by enhancing glucose phosphorylation and glycogen synthesis—thus delaying the glucose drop during the IIH. A similarly diminished contribution of skeletal muscle can be

speculated, as G3R animals have lower body weight, a large proportion of which is represented by this tissue.

In the food-restricted rats (group G3R), the slow and poor recovery of blood glucose during the last three hours of the IIH contrasts with the slow decay of blood glucose during the first 120 min. Exogenous insulin virtually disappears after 120 min, and counter-regulatory hormones take place to restore blood glucose to normal levels [17, 18]. Their actions are mainly liver-based, as they stimulate glycogenolysis and gluconeogenesis, thus increasing liver glucose output. As blood glucose in group G3R at time 300 min was about 50% lower than in the other groups, it seems that counter-regulatory effects on the liver were blunted. This supposition is further supported by the lower rate of gluconeogenesis in this group.

Plasma insulin did not show a consistent relation with lactational or immediate feeding conditions. First, it did not differ between groups G9 and G3L, so that lactation did not interfere with adult plasma insulin. However, this similarity in plasma insulin could be one factor why groups G9 and G3L had similar *in vivo* glucose profiles and hepatocyte glucose metabolism. Second, insulin was increased in the animals subjected to caloric restriction either prior to (G3RL) or at the time of measurement (G3R); thus, insulin was not reflecting the momentary feeding condition. Instead, the link between these groups (G3R and G3RL) and their higher plasma insulin was caloric restriction.

Certainly, the higher plasma insulin of group G3R could account for some of the metabolic changes of these rats. Higher basal insulin would decrease the buffering capacity of the liver during the insulin challenge (IIH) by continuously stimulating liver glycogen synthesis. In addition, it would inhibit gluconeogenesis [16]. These possibilities were suggested before [11]. However, *in vivo* and *in vitro* glucose metabolism in group G3RL was similar to groups G9 and G3L. Therefore, in group G3RL any effect of the hyperinsulinemia was overcome by refeeding.

In the rat colony from which the animals of this work were taken, body weight reaches a plateau by the age of 60 days. Therefore, food restriction was implemented only at this age so as not to compromise development. The 30%-caloric restriction (group G3R) decreased body weight and fat mass, and restored VLDL and triglyceride levels to those of G9 (control) animals. Although the reduced-litter rats (G3L) were not heavier or fatter than the G9 rats at the age of 150 days, they had higher VLDL, triglycerides, and atherogenic index, and the decrease of these values by caloric restriction deserves attention, as high lipid profiles and visceral fat are risk factors for cardiovascular disease and metabolic impairments [19, 20]. Unfortunately, refeeding (group G3RL) not only reversed these changes but worsened the scenario: triglycerides were even higher than in group G3L, and all visceral fats were increased. Although, statistically speaking, abdominal fats (retroperitoneal, epididymal and mesenteric) were not different between G3L and G3RL, the percentage increases (up to 24% for the mesenteric fat) cannot be regarded as biologically insignificant. In fact, a relevant role for mesenteric fat in the etiology of obesity-related metabolic syndrome was suggested [19, 20]. The same reasoning applies to the subcutaneous fat weight, which was 50% lower after refeeding (G3RL) than under continuous free feeding (G3L). These two fat deposits are very different from the metabolic point of view, with visceral fat correlating with metabolic disturbances, while subcutaneous fat is reported as having a protective role [19, 20]. Therefore, as far as fat is concerned, refeeding after caloric restriction would be increasing the metabolic risk and decreasing metabolic protection.

In summary, taking into account the proposed hypotheses of this investigation, the results indicated that the adult rat liver is not programmed or altered whatsoever by the lactational condition the animals had. On the other hand, glucose homeostasis and liver metabolism were responsive to caloric restriction *per*

se, that is to the current nutritional condition. In support of this, glucose metabolism (1) was shown to be similar between rats raised in control (G9) and reduced litters (G3L), indicating that it was not programmed by early life (lactational) feeding; (2) showed changes, both *in vivo* (ITT) and especially *in vitro* (basal and gluconeogenic liver glucose release) in reduced-litter rats under food restriction (G3R); (3) reversed to control patterns upon refeeding (G3RL).

5. Conclusions

Reducing litter size during lactation in the rat aims at mimicking human perinatal overfeeding. In both humans and rodents, this has been linked to later, adult life metabolic disturbances, a phenomenon termed metabolic programming. However, liver metabolism was largely unexplored in the reduced-litter rat, despite its central role in energy homeostasis. The results of this investigation, together with the other reports in younger rats, suggests that the liver glucose metabolism is not affected by early (perinatal) nutrition, but is responsive to the current amount of food ingested. Metabolic disturbances recorded in adult life after perinatal overnutrition possibly involve a complex interplay between organs and tissues and are related to adult adiposity. However, as long as adult weight and adiposity are kept within certain acceptable limits, the liver seems capable of maintaining glucose homeostasis. Therefore, liver glucose metabolism seems to be quite adaptable to the feeding conditions. Other changes in the intermediary metabolism, such as lipid metabolism, cannot be discarded and would deserve attention, especially considering that the major goal of human caloric restriction is to decrease adipose mass and control overweight and obesity.

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Artigo Científico 2:

Redução de ninhada, restrição calórica e realimentação em ratos alteram a inervação intrínseca entérica, morfologia hepática e intestinal

Redução de ninhada, restrição calórica e realimentação em ratos alteram a inervação intrínseca entérica, morfologia hepática e intestinal

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Abstract

Background: Caloric restriction (CR), at controlled and moderate levels, has been defended as an effective way to combat obesity and its comorbidities. Reducing litter during lactation is a classic model of early obesity induction in rodents. The small intestine is the organ responsible for the digestion and absorption of nutrients from the diet; the liver, in turn, is the essential organ in the metabolism of the absorbed nutrients and in the regulation of its circulating levels. This work evaluated morphometric aspects of the liver and small intestine and intrinsic jejunal innervation in rats from small litter.

Methods: Male Wistar rats were divided into 4 groups: G9 provided water and food *ad libitum* until 150 days; G3L submitted to litter size reduction and free feeding until 150 days; G3R litter size reduction and free feeding up to 60 days, with a CR of 30% relative to the ingestion of G3L, from 60 to 150 days; G3RL CR of 30% then fed freely until 150 days of age. It was recorded: body weight and naso-anal length and plasma parameters. Liver and jejunum samples were submitted to histological, histochemical and imunofluorescence techniques. Morphometric and quantitative analysis were performed with HuC/D$^+$ myoenteric plexus neurons and S100$^+$ gliocytes.

Key results: In addition to reducing body weight and fat weight, caloric restriction promoted several effects on the liver: reduction in the area and increase in the number of hepatocytes. Despite the increase in the length of the small intestine, the jejunum

was more adapted to the imposed condition, maintaining the characteristic morphometry and intrinsic innervation, however, we registered an increase in the number of gliocytes. The refeeding after CR recovereds the biometric parameters to the control values. This recovery also affecteds the weight of the liver, area of hepatocytes and lipid inclusion in the tissue and glucose metabolism in isolated hepatocytes. In the jejunum the tunic thickness and goblet cell index were maintained. The HuC/D^{+} neurons in this group had a higher cellular profile when compared to the other groups.

Conclusions and inferences: The diet promoted a reduction in body weight, promoted the profile of hepatocytes and gliocytes in the jejunal myenteric plexus and defense cells, but these parameters were attenuated with the return of free feeding.

Key words: reduced litter, caloric restriction, refeeding, myenteric neurons, jejunum, liver.

1. INTRODUÇÃO

A obesidade é uma doença crônica, geralmente associada a um balanço energético positivo, relacionada a fatores genéticos e ambientais e ao surgimento e agravamento de diversas desordens metabólicas[1]. A origem de alguns distúrbios alimentares e metabólicos manifestados na vida adulta pode ter suas raízes nas condições nutricionais do organismo durante o desenvolvimento[2].

O modelo experimental que altera o tamanho da ninhada em animais, tanto a expansão[3,4], quanto a redução de ninhada[5-10] implica em modificações nos parâmetros corporais em períodos iniciais de vida, durante a lactação por alterarem o ambiente nutricional pós-natal, sendo que a redução de ninhada promove modificações da adiposidade em ratos[5-8,10].

A restrição calórica (RC) moderada (redução de 10-30% do consumo livre), não causa desnutrição[8,9,11] e tem sido defendida como profilaxia contra obesidade e

distúrbios metabólicos [12-15], aumento da longevidade e expectativa de vida saudável [16-18].

O período de administração da RC, quando prolongada, ou seja, desde o período de lactação até o período adulto, causou atraso no desenvolvimento corporal, resultando em órgãos menores (rins, fígado, gorduras) e alterando sua função[5,6,9]. Considera-se período não prolongado quando a RC é iniciada na fase adulta do animal. Segundo Branquinho e colaboradores[8], ocorrem alterações em parâmetros biométricos durante o período de 30 e 90 dias de RC (peso corporal, índice de massa corporal (IMC), peso de gorduras e fígado). Quanto ao metabolismo hepático, animais que permaneceram recebendo dieta de RC (G3R) apresentaram resistência à insulina, estímulo à glicogenólise, maior liberação basal de glicose, menor taxa de gliconeogênese. Porém, quando realimentados após 30 dias de restrição calórica, os animais retornaram aos valores dos animais controle[8].

O trato gastrointestinal é porta de entrada dos nutrientes da alimentação[19], que são absorvidos em maior quantidade na porção jejunal do intestino delgado[15]. Organizada em mucosa, submucosa, muscular externa e serosa ou adventícia. A mucosa apresenta especializações para esta função como vilosidades e microvilosidades que aumentam a área de absorção e cujos enterócitos dispõem de mecanismos apropriados para realizar esse processo[18,20].

A função intestinal envolve o controle da motilidade, das secreções e da função circulatória local, por meio do Sistema Nervoso Entérico (SNE), organizado em dois plexos ganglionados principais, os plexos submucoso e mioentérico[21]. Pela circulação portal hepática os nutrientes são transportados para o fígado, órgão responsável pela homeostase energética e glicêmica do organismo.

O objetivo deste trabalho foi avaliar aspectos morfométricos do fígado e do intestino delgado de ratos machos com 150 dias provenientes de ninhadas reduzidas, submetidos à restrição calórica com ou sem realimentação posterior.

2. MATERIAIS E MÉTODOS

Os procedimentos experimentais foram aprovados pela Comissão de Ética no Uso de Animais da Universidade Estadual de Maringá (UEM) (certificado 1720290116).

2.1 Estabelecimento dos grupos experimentais

Foram utilizados *Rattus novergicus* da linhagem *Wistar*. As matrizes foram fornecidas pelo Biotério Central da UEM; mantidas, bem como sua prole, no Biotério do Departamento de Ciências Fisiológicas sob ciclos regulares de iluminação (12 h claro:12 h escuro) e temperatura controlada (22 ± 2^{o}C), com livre acesso à água e à ração (Nuvilab CR1®; Nuvital, Curitiba-PR, Brasil) durante a gestação e a lactação.

No primeiro dia após o nascimento, a prole foi organizada em ninhadas de 9 (G9) ou 3 filhotes (G3) machos, estabelecendo-se assim o modelo experimental de redução de ninhada[5]. Ambos os grupos, G9 e G3, receberam ração padrão para roedores *ad libitum* até os 60 dias de idade; nesse momento, os animais foram reorganizados conforme o regime alimentar a que foram submetidos (n=8 animais/grupo), da seguinte forma:

G9 (ninhada controle) e G3L: continuaram recebendo ração padrão *ad libitum* até os 150 dias de idade;

G3R: submetidos a restrição calórica, RC (redução de 30%) dos 60 aos 150 dias de idade;

G3RL: submetidos a RC de 30% dos 60 aos 90 dias de idade e realimentados *ad libitum* até os 150 dias.

O consumo alimentar relativo dos animais do G3L (g/10 g de peso corporal por animal por dia) foi usado para calcular o alimento fornecido aos grupos G3R (para o período de 60 a 150 dias) e G3RL (para o período de 60 a 90 dias). O peso corporal foi aferido no mesmo dia.

2.2 Coleta do material biológico

Os animais de 150 dias foram eutanasiados após jejum noturno por aprofundamento de anestesia com Tiopental sódico (Thionembutal® 120 mg/kg de peso corporal associado a lidocaína 5 mg/kg, intraperitoneal) e foi mensurado o comprimento nasoanal para o cálculo do Índice de Lee[22]. Por meio de punção cardíaca foram coletados 4 mL de sangue para análises da transaminase glutâmica oxalacética (TGO) e transaminase glutâmica pirúvica (TGP), usando kits comerciais (Gold Analisa® Belo Horizonte, Brasil), para o cálculo da razão TGO/TGP. Posteriormente, os animais foram laparatomizados, para coleta do fígado e intestino delgado, o qual foi mensurado em seu comprimento.

2.3 Fígado

Amostras do fígado foram fixadas em solução de Bouin por 24 horas para análise morfoquantitativa de hepatócitos e avaliação do percentual de glicogênio intracelular. Após procedimento histológico de rotina foram obtidos cortes semi-

seriados de 6µm de espessura (Micrótomo Rotativo Leica RM2145 Microsystems, Wetzlar, Alemanha), corados pela técnica de Hematoxilina-Eosina (HE) para avaliação do número e área de hepatócitos e também submetidos a reação histoquímica com Ácido Periódico de Schiff (PAS) para evidenciação do glicogênio intracelular.

Amostras do fígado também foram fixadas em nitrogênio líquido ou armazenadas em freezer -80°C para avaliação do percentual de lipídeos no tecido hepático por intensidade de cor. Posteriormente, foram embebidos em OCT (Optimum Cotting Temperature, Tissue-Tek, Sakura Finetek, Torrance, EUA), realizados cortes com 12µm de espessura (Criostato Leica CM1850 Microsystems, Wetzlar, Alemanha) e submetidos a reação histoquímica com Sudan III (*red oil*).

Imagens dos cortes foram capturadas com objetiva de 40x em microscópio óptico (Olympus BX41, Olympus America Inc., Nova Iorque, EUA) acoplado a câmera de alta resolução (Olympus Q Color 3 Olympus America Inc., Nova Iorque, EUA), acoplado a um computador com programa computacional Q-capture® e software Image Pro Plus (version 4.5 Media Cybernetics, EUA). Para análise morfométrica foi mensurada a área celular de 200 hepatócitos/animal e o número de hepatócitos presentes em 50 imagens/animal. A análise foi realizada em uma área de 3,29 mm^2, sendo excluída deste valor a área correspondente a região da veia centro-lobular de 0,25 mm^2.

O percentual de glicogênio intracelular e o percentual de inclusões lipídicas no tecido hepático foram avaliados por análise da intensidade de cor (Image Pro Plus).

2.4 Intestino delgado

Amostras do jejuno foram fixadas em solução de paraformaldeido 4% ou nitrogênio líquido, para avaliação morfométrica da parede intestinal, índice de células caliciformes e evidenciação das células imunes presentes na lâmina própria.

As amostras foram submetidas a processamento histológico e foram realizados cortes semisseriados de 6μm de espessura (Micrótomo Rotativo Leica), corados com Hematoxilina-Eosina (HE) para a mensuração da espessura das túnicas e parede intestinal total. Também foram submetidos a reação histoquímica com Ácido Periódico de Schiff (PAS) para evidenciação das células caliciformes presentes no epitélio intestinal.

Amostras do jejuno foram fixadas em nitrogênio líquido e armazenadas em freezer -80°C. Posteriormente, foram embebidas em OCT, realizados cortes semisseriados com 10 μm de espessura (Criostato Leica CM1850) e submetidos a técnica histoquímica da Peroxidase, para evidenciação das células imunes presentes na lâmina própria.

Imagens foram capturadas em Microscópio Óptico Olympus BX41®, com câmera de alta resolução (Q Color 3 Olympus), acoplado a um computador com programa computacional Q-capture®, e software de análise de imagem Image Pro Plus.

Para análise morfométrica, foi mensurada a espessura da parede total e das túnicas mucosa, submucosa e muscular externa, bem como a altura dos vilos e profundidade das criptas, com objetiva de 10x, em 10 pontos aleatórios por corte, perfazendo 100 mensurações/animal por grupo. O Índice de Células Caliciformes [ICC = (n° de células caliciformes/n° total de células na vilosidade) x 100], foi obtido

a partir da quantificação de 2500 células (enterócitos e caliciformes)[23]. O número de células imunes (NCI) presentes na lâmina própria foi quantificado em 24 campos/animal em objetiva de 40x [24].

2.4.1 Inervação intrínseca – Imunomarcação para neurônios mioentéricos e gliócitos

Amostras do jejuno foram lavadas com tampão fosfato salino (PBS 0.1 M, pH 7.4), fixadas e preenchidas com paraformaldeído 4% (pH 7.4), dissecadas sob estereomicroscópio com trans-iluminação (Olympus SZ61) para a remoção das túnicas mucosa e submucosa, obtendo-se os preparados de membrana. As membranas foram lavadas 3 vezes por 10min em PBS 0,1M (pH 7,4) com Triton X-100 (Sigma-Aldrich) a 0,5 % e incubadas em solução de bloqueio contendo 1% de albumina de soro bovino (BSA) e 10 % de soro de burro. Posteriormente as membranas foram incubadas por 48h em anticorpo primário anti-HuC/D (HuC/D^+, 1:800 Molecular Probes) e anti-S100 ($S100^+$, 1:500 Sigma-Aldrich) em uma solução mãe (PBS, 1% BSA e 10% de soro de burro), lavados três vezes em PBS 0,1M (pH 7,4) com Triton X-100 a 0,5% por 10 minutos e incubadas por duas horas à temperatura ambiente com os anticorpos secundários (1:500, Alexa Fluor 546 e Alexa Fluor 488, Invitrogen, Oregon, EUA). Os preparados foram lavados com PBS 0,1M (pH 7,4) e dispostos entre lâmina e lamínula com ProLong® Gold Antifade Reagent (Invitrogen, EUA).

As lâminas foram examinadas sob microscópio de fluorescência Olympus FSX-100 equipado com filtros específicos para imunofluorescência e acoplado a câmera Moticam 2500. Os neurônios e gliócitos presentes em 40 campos microscópicos sob objetiva de 20X foram quantificados (densidade células/mm^2) em

uma área de 5.89 mm^2, assim como o número de corpos celulares de neurônios HuC/D^+ e gliócitos $S100^+$ em 40 gânglios. Para a análise morfométrica, foram mensuradas as áreas (μm^2) dos corpos celulares de 100 neurônios HuC/D^+ e 100 gliócitos $S100^+$ por animal, utilizando o programa Image Pro Plus.

2.5 Análise estatística

Os dados foram submetidos aos testes de Shapiro-Wilk e Kolmogorov-Smirnov para verificação da normalidade. Dados paramétricos foram comparados por *One-way* ANOVA e pós-teste de Tukey [expressos como média ± desvio padrão (DP)] e dados não paramétricos submetidos à análise de Kruskal-Wallis seguido do pós-teste de Dunn´s (expressos como mediana com 95% de intervalo de confiança). O nível de significância para todas as comparações estatísticas foi de 5%. A análise estatística e a construção dos gráficos foram realizadas usando o programa Prisma® versão 7.0 (GraphPad, San Diego-CA, EUA).

3. RESULTADOS

Em ratos *Wistar* aos 150 dias, constatou-se que a redução de ninhada (G3L) não interferiu nos parâmetros biométricos (peso corporal, índice de Lee e comprimento do intestino delgado). No entanto, a associação da redução de ninhada com restrição calórica de 30% durante 3 meses (G3R), promoveu a redução de valores como peso corporal, de gordura e comprimento nasoanal. Neste trabalho, animais realimentados após 30 dias de RC diferiram o peso corporal (G3RL) (Tabela 1), assim como constatado em trabalho anterior, também mostrou diferir o IMC, peso de gorduras e fígado[8]. A razão das enzimas transaminases TGO/TGP mostra diferenças entre os

grupos (Tabela 1). O grupo G3R diferiu dos grupos controles G9 e G3L, sendo seu aumento em proporções de 4:1. A enzima TGP foi marcadamente reduzida no grupo G3R e esse baixo nível se manteve no grupo G3RL, que foi realimentado após a RC.

Tabela 1. Parâmetros biométricos: Peso corporal (g), índice de Lee e comprimento do intestino delgado (CID) (cm) e razão transaminase glutâmico oxalacética e transaminase glutâmico pirúvica (TGO/TGP), de ratos aos 150 dias de idade, provenientes de ninhadas controles (G9), ninhadas reduzidas com alimentação livre (G3L), ninhadas reduzidas com restrição calórica de 30% (G3R) ou realimentados dos 90 aos 150 dias (G3RL).

	G9	G3L	G3R	G3RL
Peso corporal (g)	429.1 ± 31.28	434.4 ± 31.5	210.4 ± 31.96[ab]	430.4 ± 36.23[c]
Índice de Lee*	301.1 (297-305.2)	304.6 (301.9-307.3)	276.1 (259.5-292.7)	305.7 (297.4-313.9)
CID (cm)	105.8 ± 13.5	114.3 ± 6.5	92.4 ± 11.2b	104.4 ± 14.4
TGO/TGP*	1.5 (1.1-1.9)	1.49 (1.19-1.9)	4.43 (3.6-5.3)[ab]	2.04 (1.5-2.7)

One-way ANOVA/Tukey, expressos em média ± DP. * Kruskal-Wallis/Dunn´s, expressos em mediana com 95% de intervalo de confiança. (n=8/grupo). [a]$p < 0.05$ *vs.* G9; [b]$p < 0.05$ *vs.* G3L; [c]$p < 0.05$ *vs.* G3R.

3.1 Fígado

Houve preservação da organização morfológica padrão do fígado e resposta positiva para as reações histoquímicas PAS e Sudan III, que apresentaram distribuição heterogênea e difusa do glicogênio e lipídeos em todos os grupos. Diferenças morfométricas com relação à densidade e área celular dos hepatócitos, percentual de glicogênio intracelular e percentual de inclusão lipídica no tecido hepático foram constatadas (Tabela 2).

Os hepatócitos dos animais submetidos à redução de ninhada e restrição calórica (G3R) apresentaram maior densidade e menor área celular do que os grupos alimentados livremente (G9 e G3L). Comparados ao grupo controle (G9), todos os grupos derivados de ninhadas de 3 filhotes (G3L, G3R e G3RL) apresentaram menor área (perfil celular) de hepatócitos, os quais apresentaram maior densidade celular

(não significante para G3L em comparação com G9). Comparados entre si, o grupo G3R apresentou as menores áreas celulares e a maior densidade celular. A área celular no grupo G3RL igualou a do grupo G3L, mas a densidade celular permaneceu elevada.

O percentual de glicogênio intracelular encontrou-se aumentado no grupo G3RL. O maior percentual de inclusões lipídicas dos grupos G3 foi significante apenas entre G3RL (que apresentou o maior valor) e G9 (que teve o menor valor) (Tabela 2).

Tabela 2. Densidade de hepatócitos (hepatócitos/mm^2), área celular de hepatócitos (μm^2), glicogênio intracelular (%), inclusão lipídica no tecido hepático (%) de ratos aos 150 dias de idade, provenientes de ninhadas controles (G9), ninhadas reduzidas com alimentação livre (G3L), ninhadas reduzidas com restrição calórica de 30% (G3R) ou realimentados dos 90 aos 150 dias (G3RL).

	G9	G3L	G3R	G3RL
Densidade de hepatócitos (cel/mm^2)	984.2 ±25.67	1023 ± 52.76	1390 ± 212.8[ab]	1283 ± 33.71[ab]
Área de hepatócitos (μm^2)	339.8 ± 25.7	308.3 ± 26.3[a]	263.2 ± 25.6[ab]	305.1 ± 28.5[ac]
Glicogênio* (%)	0.38 (0.32-0.41)	0.36 (0.31-0.40)	0.36 (0.32-0.38)	0.74 (0.10-1.43)[abc]
Inclusão lipídica * (%)	2.35 (1.53-2.82)	4.17 (1.32-5.88)	3.06 (2.71-3.50)	4.57 (3.37-5.42)[a]

One-way ANOVA/Tukey, expressos em média ± DP. * Kruskal-Wallis/Dunns, expressos em mediana com 95% de intervalo de confiança. (n=5/grupo). [a]$p < 0.05$ *vs.* G9; [b]$p < 0.05$ *vs.* G3L; [c]$p < 0.05$ *vs.* G3R.

3.2 Intestino delgado (Jejuno)

O jejuno apresentou características teciduais padrão independente do grupo no que se refere a organização das túnicas intestinais. Os parâmetros morfométricos avaliados são apresentados na Tabela 3. A redução no tamanho da ninhada (G3L) promoveu o aumento da altura das vilosidades, redução na profundidade das criptas e redução do índice de células caliciformes, em relação ao grupo de ninhada controle

(G9). Essas diferenças foram em grande parte observadas também nos outros grupos de ninhadas reduzidas (G3R e G3RL).

A realimentação por um período de 3 meses após restrição calórica de curta duração (G3RL) manteve os parâmetros morfométricos semelhantes ao grupo G3R, com exceção da túnica muscular e vilosidades intestinais que se mostraram reduzidas.

A redução de ninhada não interferiu no comportamento morfométrico das células caliciformes (ICC) e das células imunes (NCI) na mucosa intestinal (Tabela 3). Destaca-se o aumento significativo do número de células imunes quantificados na lâmina própria no grupo G3R em comparação com os outros grupos.

Tabela 3. Morfometria da parede jejunal: espessura da parede total, túnicas mucosa, submucosa, muscular externa, altura de vilos e profundidade de criptas, índice de células caliciformes (ICC) e número de células imunes/campo (NCI) na mucosa intestinal de ratos aos 150 dias de idade, provenientes de ninhadas controles (G9), ninhadas reduzidas com alimentação livre (G3L), ninhadas reduzidas com restrição calórica de 30% (G3R) ou realimentados dos 90 aos 150 dias (G3RL).

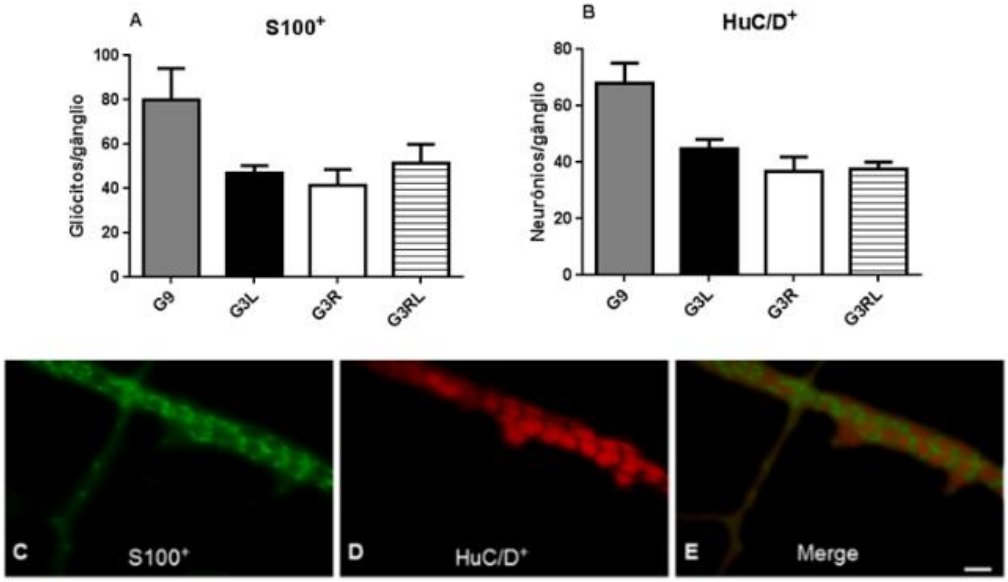
	G9	G3L	G3R	G3RL
Parede total (µm)	683 ± 69.65	647.2 ± 74.7	640.1 ± 61.28	672 ± 69.05
Mucosa (µm)	583.2 ± 48.03	547.9 ± 67.6	528.3 ± 50.24	572.2 ± 56.96
Muscular (µm)	77.6 ± 6.8	75.8 ± 4.7	86.5 ± 5.7[ab]	75.85 ± 6.2[c]
Altura do vilo*(µm)	378.4 (375.2-382.7)	401.3 (395.8-403.6)[a]	447.7 (450.5-471.4)[ab]	390.3 (385.8-393.6)[abc]
Profundidade da cripta(µm)	196.9±11.6	188.5 ± 11.5[a]	176.2 ± 11.5[ab]	177.1 ± 8.9[ab]
ICC	15.83 ± 1.6	13.3 ± 1.4[a]	13.3 ± 1.01[a]	14.8 ± 1.3
NCI	13.32 ± 1.96	16.20 ± 4.40	18.71 ± 2.35[a]	16.57 ± 2.30

One-way ANOVA/Tukey, expressos em média ± DP. * Kruskal-Wallis/Dunns, expressos em mediana com 95% de intervalo de confiança. (n=5/grupo). [a]$p < 0.05$ *vs.* G9; [b]$p < 0.05$ *vs.* G3L; [c]$p < 0.05$ *vs.* G3R.

3.2.1 Inervação intrínseca

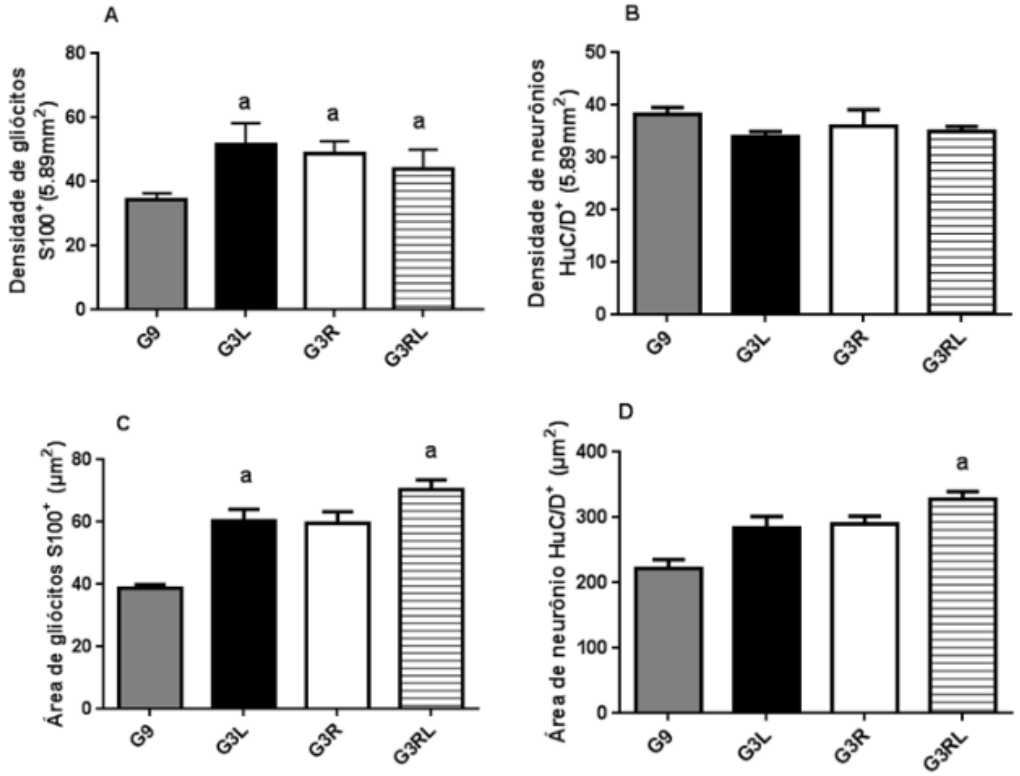
A Figura 2 apresenta a imunomarcação do plexo mioentérico. A quantificação de células gliócitos $S100^+$ por gânglio (2A) e de neurônios HuC/D^+ por gânglio (2B) não apresentaram diferenças significativas, mas existe uma tendência a redução destes em animais submetidos à redução de ninhada. A fotomicrografia do plexo mioentérico apresenta gliócitos $S100^+$ (2C), população geral neuronal HuC/D^+ (2D) e dupla imunomarcação (2E). Independente do grupo experimental, os gânglios mioentéricos apresentaram formato predominantemente alongado e não diferiram significativamente quanto ao número de gliócitos e neurônios avaliados em 40 gânglios por animal.

Figura 2. Fotomicrografia de gânglio mioentérico do jejuno de ratos do grupo ninhada controle (G9). A. Número de gliócitos mioentéricos $S100^+$ por gânglio; B. Número de neurônios mioentéricos HuC/D^+ por gânglio; C. Imunomarcação de gliócios mioentéricos $S100^+$ (verde); D. Imunomarcação de neurônios HuC/D^+ (vermelho); E. Dupla imunomarcação de gliócitos $S100^+$ e neurônios HuC/D^+. Barra de escala=100µm.



A Figura 3 apresenta a densidade e perfil celular de gliócitos $S100^+$ e neurônios HuC/D^+. A densidade glial $S100^+$ não diferiu entre os grupos de ninhadas reduzidas, porém nesses grupos, a densidade foi elevada com relação ao grupo de ninhada controle (3A). Também houve aumento do perfil celular dos gliócitos dos grupos provenientes de redução de G3L, G3R e G3RL (não significante para G3R) quando comparado ao grupo controle (G9) (3C). A densidade neuronal HuC/D^+ (3B) não diferiu entre os grupos, no entanto o perfil celular do grupo G3RL foi significativamente aumentado quando comparado ao grupo controle (3D).

Figura 3. Densidade e perfil celular de neurônios e gliócitos do plexo mioentérico do jejuno de ratos aos 150 dias de idade, provenientes de ninhadas controles (G9), ninhadas reduzidas com alimentação livre (G3L), ninhadas reduzidas com restrição calórica de 30% (G3R) ou realimentados dos 90 aos 150 dias (G3RL). A. Área de corpo celular de gliócitos $S100^+$; B. Área do corpo celular de neurônios HuC/D^+; C. Densidade de gliócitos $S100^+$; D. Densidade de neurônios HuC/D^+.



One-way ANOVA/Tukey, expressos em média ± DP. * Kruskal-Wallis/Dunns, expressos em mediana com 95% de intervalo de confiança. (n=5/grupo). $^a p < 0.05$ *vs.* G9.

4. DISCUSSÃO

No início da restrição calórica (RC), quando os animais estavam com 60 dias de idade (grupos G3R e G3RL), constatou-se que os ratos não consumiam a ração rapidamente, quando fornecido a alimentação livre. Decorridos alguns dias do início da RC, foi observado disputa entre os animais da caixa pelo acesso ao alimento; em consequência a ração era rapidamente consumida. Animais submetidos à RC exibiam comportamento agitado em relação aos de alimentação livre ou realimentados.

O ritmo diário de alimentação e jejum, por manter o balanço entre a absorção de nutrientes e seu consumo, tem efeito importante sobre a fisiologia e o comportamento de um organismo[25]. A RC moderada proposta neste trabalho, quando imposta dentro dos ciclos diários de alimentação e jejum, sem causar subnutrição, promoveu alterações no nosso modelo. Tais alterações podem estar associadas à redução do surgimento de doenças metabólicas[26].

O período de restrição prolongada foi descrito por Nakamura e colaboradores, como sendo menos estressante observando ainda menor nível de cortisol do que o jejum por curto período de tempo[27]. Isto pode ocorrer devido a adaptação dos animais ao tipo de dieta.

Relata-se amplamente na literatura que, em roedores, a redução no tamanho da ninhada, uma forma clássica de induzir o fenômeno de programação metabólica, ocasiona distúrbios que perduram durante toda a vida adulta do animal [2,5,7,10,28].

Em outros estudos conduzidos em nosso laboratório, a redução de ninhada resultou em animais com massa corporal aumentada até a idade de 60 dias[6,8]; a partir dessa idade, a diferença neste parâmetro entre ninhadas de tamanhos diferentes era atenuada em animais alimentados livremente até os 90 dias [5,8].

Aos 150 dias, o modelo proposto não promoveu efeitos sobre os parâmetros biométricos. Porém o tempo da dieta e o estado alimentar do animal foi decisivo para a determinação da massa corporal, sendo que animais realimentados tiveram um retorno aos valores do controle, de forma similar ao encontrado por Branquinho[8]. O comprimento do intestino delgado de ratos do G3R encontrava-se reduzido, resultado frequente diante de uma dieta restritiva, resposta adaptativa a condição nutricional imposta. Essa adaptação pode estar associada ao aumento na espessura da túnica muscular constatada após o período de 3 meses de RC.

Aos 150 dias, os ratos de ninhadas reduzidas com RC dos 60 aos 150 dias (G3R) apresentaram massa corporal reduzida, assim como já descrito[8,29], sugerindo que o prolongamento no tempo da dieta é eficaz para reverter as alterações com origem nos períodos iniciais de vida pós-natal, assim como a melhora de outros parâmetros. Já a RC por curto período de tempo (G3RL) seguido da realimentação não foi suficiente para alterar o comportamento alimentar destes animais, a análise de parâmetros plasmáticos (colesterol total e frações) e pesos de gorduras (retroperitoneal, periepididimal, mesentérica e visceral), onde houve aumento dos valores.

A análise histomorfométrica do fígado dos animais sob RC (G3R) apresentou maior densidade de hepatócitos os quais possuíam menor área celular semelhante ao encontrado por Branquinho e colaboradores[5]. Isto já era esperado, pois os animais RC possuem o peso corporal menor, devido a menor disponibilidade de alimento e o menor ganho energético. Porém, a realimentação (G3RL) reverteu as características adquiridas durante o período de RC, retornando novamente aos valores dos animais controle (G9).

Além do restabelecimento total e parcial das características histomorfométricas. Os grupos apresentaram deposição de glicogênio no fígado, inclusive o G3R, corroborando para a sugestão de que em hepatócitos isolados, existe liberação de glicose via glicogenolítica, em condições basais, trabalho anterior[8]. A quantidade de glicogênio não necessariamente estabelece redução ou aumento na utilização desta reserva como fonte de glicose para o organismo, já que o grupo realimentado (G3RL) apresentou maiores estoques de glicogênio, mas reduzida produção basal de glicose, via glicogenolítica, demonstrando que o estado nutricional do animal determina a via bioquímica a ser utilizada.

A hiperinsulinemia observada em G3R e G3RL mostra animais submetidos à RC apresentando resistência à insulina[8]. Apesar de se tratar de um fator agravante da Síndrome Metabólica, estes animais não apresentaram altos níveis de triglicerídeos plasmáticos e índice aterogênico[8], podendo se tratar de uma adaptação à dieta de RC e melhora deste quadro.

Em animais obesos, a associação entre resistência à insulina, Síndrome Metabólica e diabetes *mellitus* tipo 2, promove liberação de ácidos graxos pelo tecido adiposo que se acumulam no hepatócito contribuindo para a lipogênese de novo e aumento dos triglicerídeos no fígado[30]. Os animais do grupo G3RL mostraram acentuada deposição de glicogênio e também lipídios. Pode-se supor que o animal estabelece uma reprogramação metabólica na realimentação pós-RC (G3RL) que favorece o armazenamento de energia pelo fígado. Assim, o aumento dos estoques de glicose (glicogênio) e de lipídios (gordura) ocorre como compensação pela RC prévia e o estresse ocasionado devido a alteração da alimentação[27].

Outra alteração que pode ocasionar aumento da permeabilidade intestinal são alterações quanto a flora intestinal, podendo levar a passagem de moléculas, por exemplo, lipopolissacarídeos, que alcançam a circulação portal e sistêmica podendo desencadear inflamação crônica de baixo grau e disfunção no tecido adiposo, muscular e hepático[31,32]. Apesar de a permeabilidade intestinal não ser alterada na RC[33], porém o tempo de trânsito gastrointestinal é menor em animais G3R e G3RL devido a menor quantidade de alimento[29].

A razão TGO/TGP apresentou G3R com valores aumentados em quatro vezes em relação aos valores dos grupos controle (G9 e G3L). Consideramos que estes valores não sugerem lesão hepática[34]. Quando essa acontece, é geralmente seguida por extravasamento dessas enzimas na corrente sanguínea. Sendo a TGP geralmente, apresentando valores maiores do que TGO, porém em G3R temos menores valores de TGP e maior de TGO, indicando que a RC é um modelo protetivo para o fígado[5], com redução da permeabilidade de TGP nos hepatócitos e consequentemente, menores níveis no sangue.

A redução de ninhada (G3L) maximizou a função absortiva, com aumento na altura dos vilos. Porém, a profundidade da cripta e Índice de células caliciformes produtoras de mucinas neutras foram reduzidas (G3L). Estas alterações na funcionalidade podem estar relacionadas ao maior consumo destes animais durante as primeiras semanas de vida, tendo em vista seu peso corporal aumentado, pelo menos até os 60 dias.

Apesar dos animais submetidos a 60 dias de RC (G3R) terem apresentado aumento na altura dos vilos, e consequente aumento da superfície em contato com o alimento, a mucosa intestinal mostrou espessura semelhante para todos os grupos

indicando ausência de efeitos da RC proposta neste modelo. Resposta divergente foi obtida no intestino grosso de ratos em modelo experimental semelhante (G3R), que apresentaram redução na espessura da mucosa intestinal[29].

O maior número de células imunes presentes na lâmina própria do jejuno de ratos do grupo G3R, é indicativo de maior vulnerabilidade dessa mucosa, e pode ser uma resposta decorrente da redução no índice de células caliciformes, considerando que as mucinas produzidas por essas células representam a primeira linha de defesa da mucosa e que alterações qualitativa ou quantitativa na secreção e/ou nos padrões de expressão das mucinas pode afetar a eficiência da barreira protetora, podendo desencadear importantes implicações fisiológicas ou patológicas[35].

Segundo Schoffen e colaboradores[23] (2014), em ratos provenientes de expansão de ninhada e RC de 50% até os 90 dias, a produção de mucinas neutras estaria reduzida e de mucinas ácidas aumentada no cólon. No nosso trabalho, houve também redução de células caliciformes produtoras de mucinas neutras em animais submetidos à RC, apesar de não ter sido realizada análise de mucinas ácidas.

Sugerimos que se houvesse o aumento de mucinas ácidas, poderia ativar o recrutamento de células imunes na lâmina própria[23], isto explicaria o aumento de células imunes na mucosa jejunal de animais submetidos à RC (G3R). A RC promove a regeneração de células imunes[36], camundongos apresentaram também melhora na proteção contra infecções e tumores quando administrada a redução calórica de 50%[37]. Portanto, a RC seria um modelo protetivo ao animal, aos 150 dias, porém submetido à alimentação livre pós-RC mostrou redução no número de células imunes, retornando aos valores dos grupos controle G9 e G3L.

Os animais submetidos à ninhadas reduzidas, independente do regime alimentar vigente ou anterior (alimentação livre, restrição calórica ou realimentação), mantiveram o número de neurônios e gliócitos por gânglio, bem como a densidade neuronal mioentérica preservada, indicando o não comprometimento da função intestinal, no que se refere ao controle da motilidade e das secreções[21]. Entretanto, os fatores redução de ninhada e diferentes programas de alimentação durante o período de 150 dias influenciaram significativamente a população glial que apresentaram maior densidade e área de corpo celular (perfil) quando comparados a animais controle.

Isso sugere uma programação desenvolvida no período lactacional que, embora não tenha causado mudanças importantes na população neuronal geral (HuC/D^{+}) devido a plasticidade destas células frente a manipulações dietéticas, influenciou a população de gliócitos ($S100^{+}$).

Considerando a importância dos gliócitos na manutenção do ambiente neuronal[37], a ausência de variações na densidade neuronal mioentérica, no número de neurônios e gliócitos/gânglio, bem como na manutenção na espessura da parede intestinal inferimos que o intestino de animais submetidos à redução de ninhada independente do regime alimentar encontrava-se adaptado às condições nutricionais impostas.

O TGI pode se adaptar rapidamente e eficientemente aos desafios dietéticos, e o sistema nervoso entérico é sensível ao estado nutricional, podendo alterar seus processos e se reprogramar, modificando o trânsito intestinal em função da dieta[38]. A RC é considerada como um modelo protetivo neuronal[23], associada ao aumento de neurônios mioentéricos NADH-diaforase[39], em ratos diabéticos, a RC preveniu

alterações no número e área celular de neurônios e gliócitos, principalmente no jejuno[15].

5. CONCLUSÃO

A restrição calórica moderada (30%) por 90 dias administrada a ratos adultos provenientes de ninhada reduzida promoveu redução do peso corporal, do perfil de hepatócitos e de gliócitos no plexo mioentérico jejunal, além de aumentar o número de células imunes da mucosa. Estes parâmetros foram revertidos com a realimentação livre por 60 dias e consequente restabelecimento do animal.

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6. ANEXO I- Parecer da Comissão de Ética no Uso de Animais/UEM

CERTIFICADO

Certificamos que o Projeto intitulado "EFEITOS DA PROGRAMAÇÃO METABÓLICA E DA RESTRIÇÃO CALÓRICA SOBRE A MORFOLOGIA DO INTESTINO DELGADO E O METABOLISMO EM RATOS", protocolado sob o CEUA nº 1720290116, sob a responsabilidade de **Maria Montserrat Diaz Pedrosa** *e equipe; Cristian Lima Borrasca; Elizete Rosa dos Santos Silva; Gabriel Henrique de Paula Cruz; Maria Montserrat Diaz Pedrosa; Márcia Fabrício; Márcia do Nascimento Brito; Nayra Thais Delatorre Branquinho; Valéria Schoffen Romão Carrascoza; Vilma Aparecida Ferreira de Godoi; Maria Raquel Marçal Natali* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Maringá (CEUA/UEM) na reunião de 13/05/2016.

We certify that the proposal "EFFECTS OF METABOLIC PROGRAMMING AND OF CALORIC RESTRICTION ON THE MORPHOLOGY OF THE SMALL INTESTINE AND ON METABOLISM IN RATS", utilizing 50 Heterogenics rats (50 females), protocol number CEUA 1720290116, under the responsibility of **Maria Montserrat Diaz Pedrosa** *and team; Cristian Lima Borrasca; Elizete Rosa dos Santos Silva; Gabriel Henrique de Paula Cruz; Maria Montserrat Diaz Pedrosa; Márcia Fabrício; Márcia do Nascimento Brito; Nayra Thais Delatorre Branquinho; Valéria Schoffen Romão Carrascoza; Vilma Aparecida Ferreira de Godoi; Maria Raquel Marçal Natali* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the State University of Maringá (CEUA/UEM) in the meeting of 05/13/2016.

Vigência da Proposta: de 03/2016 a 12/2018 Área: Ciências Fisiológicas

Procedência:	Biotério Central da UEM						
Espécie:	Ratos heterogênicos	sexo:	Fêmeas	idade:	70 a 70 dias	N:	50
Linhagem:	Wistar			Peso:	0 a 0		

Resumo: A obesidade é uma doença crônica relacionada ao surgimento e agravamento de diversas desordens metabólicas. A restrição calórica, em níveis controlados e moderados, vem sendo defendida como forma eficaz de combate a esse quadro e suas comorbidades. O ambiente nutricional pós-natal pode contribuir significativamente para o excesso de peso, e em roedores a redução do número de filhotes durante a lactação é um modelo clássico de indução precoce de obesidade, com reflexos morfológicos, metabólicos e funcionais que se prolongam até a idade adulta. Apesar dos muitos estudos utilizando este modelo experimental, poucos abordam especificamente o trato gastrintestinal e o metabolismo hepático. O intestino delgado é o responsável direto pela digestão e absorção dos nutrientes da dieta; o fígado, por sua vez, é um órgão essencial na manipulação dos nutrientes absorvidos e na regulação de seus níveis circulantes. A proposta deste trabalho é avaliar os efeitos da restrição calórica de 20%, seguida ou não por alimentação livre, em ratos de ninhadas reduzidas. Os grupos serão avaliados dos 90 aos 150 dias de idade. Serão registrados: peso corporal e comprimento naso-anal, peso de tecidos e parâmetros plasmáticos. Cortes de fígado, tecido adiposo e jejuno serão coradas por Hematoxilina-Eosina para avaliações histomorfométricas. Preparados de membrana do jejuno serão marcados por imunohistoquímica para HuC/HuD para quantificação dos neurônios mioentéricos. Cortes de jejuno serão empregados para imunohistoquímica do índice de proliferação celular e de células serotoninérgicas. Também serão avaliadas as atividades enzimáticas específicas da mucosa jejunal. A investigação do metabolismo da glicose ocorrerá por teste de tolerância à glicose (ivGTT), hipoglicemia induzida por insulina (HII), e incubação de hepatócitos isolados com substratos gliconeogênicos ou agentes glicogenolíticos. Os conjuntos de dados serão expressos como média±desvio padrão (DP) de no mínimo oito repetições e serão submetidos a análise de normalidade pelo teste de Shapiro-Wilk. Os grupos experimentais serão comparados por one-way ANOVA com pós-teste de Tukey ou Kruskall-Wallis com pós-teste de Dunns. O nível de significância para todas as comparações estatísticas será de 5%.

Maringá, 17 de maio de 2016

Profa. Dra. Vilma Aparecida Ferreira de Godoi
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Estadual de Maringá

Profa. Dra. Tatiana Carlesso dos Santos
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Universidade Estadual de Maringá

Maringá, 03 de junho de 2016
CEUA N 1720290116

Ilmo(a). Sr(a).
Responsável: Maria Montserrat Diaz Pedrosa
Área: Ciências Fisiológicas
Maria Raquel Marçal Natali (orientador)

Título do projeto: "EFEITOS DA PROGRAMAÇÃO METABÓLICA E DA RESTRIÇÃO CALÓRICA SOBRE A MORFOLOGIA DO INTESTINO DELGADO E O METABOLISMO EM RATOS".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais UEM

A Comissão de Ética no Uso de Animais da Universidade Estadual de Maringá, no cumprimento das suas atribuições, analisou e **APROVOU** a Emenda (versão de 17/maio/2016) do protocolo de estudo acima referenciado.

Resumo apresentado pelo pesquisador: "A presente solicitação se justifica em função 1) das sugestões feitas pela banca de dissertação de mestrado da aluna Nayra Branquinho, que justificam a mudança nos protocolos de tratamento dos animais (grupos G3R e G3RL), conforme descrito abaixo, sem modificações nos procedimentos experimentais subsequentes, no número de animais empregados ou no cronograma de execução; 2) da inclusão de coleta de material biológico (colon); 3) da inclusão de membro na equipe executora. Essas solicitações são descritas a seguir e foram incluídas no projeto, anexado online junto com esta solicitação de emenda.".

Comentário da CEUA: "A Comissão de Ética no Uso de Animais (CEUA-UEM), na sua reunião de 02/06/2016, APROVOU os procedimentos éticos apresentados neste Protocolo, visto que a metodologia proposta é compatível com a legislação pertinente à ética no uso de animais na experimentação, na forma da Lei no 11.794/08, Decreto 6.899/09, Resolução Normativa nº 01/2010 CONCEA e complementares, Lei Estadual no 14.037/03, Diretriz Brasileira para o cuidado e a utilização de animais para fins científicos e didáticos DBCA (portaria nº 596 CONCEA - de 25/junho/2013, disponível no endereço http://www.ppg.uem.br/index.php/etica-biosseguranca/ceua) e Resolução UEM nº 004/2016-CEP, vez que não se constatam óbices legais para o desenvolvimento dos procedimentos experimentais nos moldes propostos pelo(a) pesquisador(a).".

Profa. Dra. Vilma Aparecida Ferreira de Godoi
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Estadual de Maringá

Profa. Dra. Tatiana Carlesso dos Santos
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Universidade Estadual de Maringá

7. ANEXO II- Normas da revista Neurogastroenterology & Motility

Author Guidelines

Contents

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Authors should kindly note that submission implies that the content has not been published or submitted for publication elsewhere except as a brief abstract in the proceedings of a scientific meeting or symposium.

Once the submission materials have been prepared in accordance with the Author Guidelines, manuscripts should be submitted online at https://mc.manuscriptcentral.com/nmo.

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Over the past few years, *Neurogastroenterology & Motility* has become one of the leading journals in the field of gastroenterology and related areas of physiology. This is reflected by the steadily increasing number of high-quality manuscripts submitted to the Journal. The length of a manuscript needs to be closely adhered to, with any additional material to be published as supporting information. Authors need not pay for the publication of figures in colour.

Manuscripts that do not meet these formal criteria will be returned for reformatting, which will delay the review process and possible acceptance.

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3. MANUSCRIPT CATEGORIES AND REQUIREMENTS

Article types

Original Articles
Original Articles describe the results of basic or clinical studies, clinical trials or significant **Case Reports**. The length of an Original Article should be no longer than **5000** words, excluding acknowledgements and disclosures, references, tables, figures, table legends and figure legends, and to limit the number of figures and tables to a maximum of eight in the regular edition of the Journal (e.g. five figures and three tables) in normal circumstances, with any additional material to be published as supporting information.

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To make it easier for re-review, we encourage authors to make the revisions in their manuscript using a colored font (blue or red) and/or a colored highlighter (yellow). They should also provide a point by point response to the editor of the changes that were made in a letter that describes the requested change and the responses.

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The manuscript should be double-spaced with 30mm margins. Manuscripts must be numbered consecutively in the following sequence: Title Page; Abstract, if required; Main Body of Text; Acknowledgement; Reference List; Tables and Figure caption List.

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ii. A short running title of less than 40 characters;
iii. The full names of the authors;

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v. Acknowledgments;
vi. Abstract and keywords;
vii. Main text;
viii. References;
ix. Tables (each table complete with title and footnotes);
x. Figure legends;
xi. Appendices (if relevant).

Figures and supporting information should be supplied as separate files.

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The abstract must not exceed 250 words. It should summarize the aim of the study and describe the work undertaken, results and conclusions. For Original Articles and Technical Notes, the abstract should be structured under four subheadings: **Background, Methods, Key Results and Conclusions & Inferences**. For Review Articles, the abstract should be structured under **Background and Purpose**. For Mini-review editorials, "Hot Topics" and Case Reports, the abstract should be unstructured, i.e. without the subheadings. In addition, you should list up to six keywords in alphabetical order. For ideas on optimising your abstract, see here.

Keywords
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Original articles should be divided into the following sections and appear in the following order: (1) title page (with short running page heading, title, authors names and affiliations) (2) abstract and keywords, (3) introduction, (4) materials and methods, (5) results, (6) discussion, (7) acknowledgments, funding, and disclosures, (8) references, (9) appendices, (10) supporting information, (11) tables, (12) figure legends, and (13) figures.

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Book:

2. Voet D, Voet JG. Biochemistry. New York: John Wiley & Sons; 1990. 1223 p.

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Endnotes should be placed as a list at the end of the paper only, not at the foot of each page. They should be numbered in the list and referred to in the text with consecutive, superscript Arabic numerals. Keep endnotes brief; they should contain only short comments tangential to the main argument of the paper.

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- GenBank www.ncbi.nlm.nih.gov/genbank

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