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Nutritional recovery with a soybean diet impaired the glucagon response but did not alter liver gluconeogenesis in the adult offspring of rats deprived of protein during pregnancy and lactation

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Abstract

Nutritional recovery of early malnutrition with soybean diet reduces liver glycogen stores in the fed state and produces liver insulin resistance. We investigated whether nutritional recovery on a soybean flour diet alters hepatic gluconeogenesis in the adult offspring of rats deprived of protein during pregnancy and lactation. Male rats from mothers that were fed either 17% (C) or 6% (L) protein during pregnancy and lactation were maintained on a 17% casein (CC, n=16 and LC, n=17), 17% soybean flour (CS, n=10 and LS, n=10) or 6% casein (LL, n=10) diet after weaning. The soybean diet reduced basal serum glucose (soybean diet = 5.6±0.6 mmol/L vs casein diet = 6.2±0.6 mmol/L; p<0.05), but increased alanine aminotransferase mRNA/GAPDH (soybean diet = 0.062 ±0.038 vs casein diet = 0.024 ±0.011; p<0.01) and phosphoenolpyruvate carboxykinase mRNA/GAPDH (soybean diet =1.53 ±0.52 vs casein diet = 0.95 ±0.43; p<0.05), and the glycerokinase protein content (soybean diet =0.86 ±0.08 vs casein diet = 0.75 ±0.11; p<0.05). The serum glucose concentration (recovered groups=5.6±0.5 mmol/L vs control groups=6.2±0.7 mmol/L, p<0.05) and phosphoenolpyruvate carboxykinase activity (recovered groups=2.8±0.6 µU/mg vs control groups=3.6±0.6 µU/mg; p<0.05) were decreased in rats subjected to protein restriction in early life. The glucose area under the curve during the pyruvate tolerance test did not differ among the groups, whereas glucose area under the curve after glucagon infusion was reduced by early malnutrition (recovered groups=4210±572 mg/dL.40min vs control groups=4493±688 mg/dL.40min; p<0.001); and by the soybean diet (soybean diet = 3995±500 mg/dL.40min vs casein diet = 4686±576 mg/dL.40min,
Thus, soybean diet impaired the response to glucagon, but did not alter gluconeogenesis.

**Key words:** gluconeogenesis, early malnutrition, nutritional recovery, soybean diet, liver
Introduction

Animal models examining the association between early malnutrition and diabetes mellitus in adulthood have revealed permanent morphological and functional alterations in pancreatic beta cells and insulin target tissues, such as liver, muscle and adipose tissue (de Oliveira et al. 2016, Zheng et al. 2017, da Silva Aragão et al. 2014). A low activity of liver glucokinase, which participates in glucose oxidation, and high activity of liver phosphoenolpyruvate carboxykinase (PEPCK), which determines the rate of gluconeogenesis, have been observed in adult offspring of rat dams fed a low protein diet during pregnancy and lactation (Burns et al. 1997). The expression of PEPCK and glucose-6-phosphatase (G6Pase), in addition to peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), the transcription factor that regulates the expression of those enzymes, remains high even after nutritional recovery with a chow diet (Liu et al. 2009). Therefore, these animals are prone to developing glucose intolerance through increased hepatic glucose production.

Due to its low cost, elevated nutritional value and metabolic and/or physiological effects that prevent diabetes mellitus, we have used the soybean flour in the nutritional recovery of rodents submitted to intrauterine and lactation protein restriction, evaluating the implications of its use on mechanisms related to insulin secretion and action. This inexpensive protein source could be an alternative feed to prevent or treat disorders of carbohydrate metabolism in low-income populations. Curiously, we observed that nutritional recovery with a soybean diet increased insulin secretion, reduced liver glycogen stores in the fed state and maintained normal serum glucose levels (de Arruda Oliveira et al. 2016).
Many of the metabolic effects of insulin are mediated by a signaling pathway involving insulin receptor (IR) and insulin receptor substrate (IRS) proteins, activation of phosphatidylinositol 3-kinase (PI3-K) and phosphorylation of Akt (also known as protein kinase B) (Cheatham et al. 1994, Shepherd et al. 1995). Several studies have documented the association of insulin resistance (liver and adipose tissue) with inflammation (Asrih and Jornayvaz 2013). Soybean diet promoted TNF-α-mediated inflammation, decreased IR and IRS-1 levels, reduced IRS-1/PI3-kinase association as well as the Akt phosphorylation stimulated by insulin in hepatic tissue (Feres et al. 2010, Reis et al. 2015). It is known that glycogen synthesis and suppression of hepatic gluconeogenesis in response to insulin are mediated by Akt (Michael et al. 2000). Therefore, in the nutritional recovery with a soybean diet the upregulation of insulin secretion appears to have compensated for insulin resistance so that normal glucose tolerance was maintained. The rapid depletion of hepatic glycogen during the post absorptive and early fasting period underscores the importance of gluconeogenesis from non-glucose precursors (lactate, pyruvate, glycerol and alanine) for the maintenance of glucose homeostasis (Wahren and Ekberg 2007). Thus, we evaluated hepatic gluconeogenesis in adult offspring of rats deprived of protein during pregnancy and lactation and that were maintained with soybean flour diet or casein after weaning.

**Materials and methods**

**Animals and diets**
The experimental procedures involving rats were performed in accordance with the guidelines of the Brazilian Society of Science in Animals of Laboratory (SBCAL) and were approved by the ethics committee at the Federal University of Mato Grosso.

Male and virgin female Wistar rats (85–90 days old) were obtained from the university’s own breeding colony. Mating was performed by housing males with females overnight (one male and four females), and pregnancy was confirmed by the examination of vaginal smears for the presence of sperm. Pregnant females were separated at random and maintained from the first day of pregnancy until the end of lactation on isocaloric diets containing 17% (control [C] diet, n=10) or 6% (low protein [L] diet, n=10) casein as the source of protein. Spontaneous delivery occurred at day 22 of pregnancy, and at 3 d of age, large litters were reduced to eight pups to ensure a standard litter size per mother.

After weaning (week 4), the males were divided into five groups: CC, consisting of the offspring born to and suckled by mothers fed a C diet and subsequently fed the same diet after weaning, n=16; CS, consisting of the offspring born to and suckled by mothers fed a C diet and subsequently fed a soybean flour diet with 17% protein after weaning, n=17; LL, consisting of the offspring of mothers fed an LP diet and subsequently fed the same diet after weaning, n=10; LC, consisting of the offspring of mothers fed an LP diet, but fed a C diet after weaning, n=10; and LS, consisting of the offspring of mothers fed an LP diet, but fed a soybean flour diet containing 17% protein after weaning, n=10. In the soybean diet, adjustments, which included removing soybean oil and fiber, were made to equalize the content of carbohydrate, lipid, fiber and the energy value contained in the casein diet. Whole, inactivated soybean flour was obtained by
industrial processing (thermal treatment, peeling, grinding, and micronization), which reduced the enzymatic and antitrypsin factor contents and contained 80% of the nutritional value of animal casein. The diets are described in Table 1. Throughout the experimental period, the rats were given free access to food and water and were kept under standard lighting conditions (12-h light/dark cycle) at a temperature of 24°C. Dietary intake was measured three times per week, and the animals were weighed weekly until they were sacrificed at day 90 of life.

**Pyruvate tolerance test**

For the pyruvate tolerance test, rats were fasted for 12 h with free access to water before intraperitoneal injection of 2 g/kg sodium pyruvate (Sigma, Missouri, USA). Blood samples were obtained from the cut tip of the tail before (0 minute) and at 15, 30, 60, 90, 120 and 150 minutes after sodium pyruvate injection. Blood glucose was determined using a glucometer (Accu-Chek®, Roche Diagnostics, Germany). The glucose response during the pyruvate tolerance test was calculated by estimating the total area under the glucose curve (ΔG) using the trapezoidal method (Matthews et al. 1990).

**Glucagon sensitivity test**

For the glucagon sensitivity test, the rats were fasted for 12 h with free access to water before intraperitoneal injection of 0.2 mg/kg human glucagon (GlucaGen®, Novo Nordisk Farmacêutica do Brasil Ltda, Paraná, Brazil). Blood samples were obtained from the cut tip of the tail before (0 minute) and at 5, 10, 20 and 40 minutes after glucagon injection. Blood glucose was determined using a glucometer (Accu-Chek®, Roche Diagnostics, Germany). The glucose response during the glucagon sensitivity test was calculated by estimating the
total area under the glucose curve (ΔG) using the trapezoidal method (Matthews et al. 1990).

Sample collection

The rats were decapitated during a period of fasting, blood samples were collected, serum was obtained by centrifugation, and aliquots were used to measure free fatty acids (FFAs) using commercial kits (Waco Pure Chemical Industries, Ltd., Chuo-Ku, Japan). After medial laparotomy, the liver, skeletal muscle (Musculus gastrocnemius) and epididymal and retroperitoneal white adipose tissues (EWAT and RWAT) were quickly removed and weighed; aliquots were frozen immediately in liquid nitrogen and stored at -80°C.

Measurement of enzyme activities

Assay of alanine aminotransferase (ALT) activity: A piece of liver was homogenized on ice after the addition of 0.1 M phosphate buffer, pH 7.4 (5 mL/g of liver). The homogenate was centrifuged (15 min at 10000 x g) at 4°C (Walterova et al. 1981). The supernatant was diluted 100x with 0.1 M phosphate buffer, pH 7.4. The enzyme assays were performed using commercial kits (Labtest Diagnostica, Minas Gerais, Brazil). The absorbance was measured using a spectrophotometer at 505 nm.

Assay of lactate dehydrogenase (LDH) activity: A piece of liver was homogenized on ice in isotonic homogenization buffer (5 mL/g of tissue) containing 5 mM MOPS, 250 mM sucrose, 1 mM EDTA, 0.1% ethanol and protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride and 0.2 mM dithiothreitol). The homogenate was centrifuged (20 min at 10000 x g) at 4°C.
(Baumgart et al. 1996). The supernatant was diluted 1000x with saline. The enzyme assays were performed using commercial kits (Labtest Diagnostica, Minas Gerais, Brazil). The absorbance was measured using a spectrophotometer at 500 nm.

Assay of arginase (ARG1) activity: A piece of liver was homogenized with 10 mM Tris-HCl (pH 7.4) containing aprotinin and 0.4% (w/v) Triton X-100 (5 mL/g). The samples were centrifuged at 20000 x g at 4°C for 10 min. The supernatant was diluted 6000x for the arginase assay using the commercial Quantitative Colorimetric Arginase Determination kit (BioAssay Systems-Arginase Assay Kit). The absorbance was measured at 540 nm.

Assay of phosphoenolpyruvate carboxykinase (PEPCK) activity: The homogenization medium consisted of 0.25 M sucrose containing 3.4 mM Tris and 1.0 mM EDTA, pH 7.5 (10 mL/g tissue). The homogenates were centrifuged at 10000 x g, and the supernatant was used for enzyme and protein measurements (Hahn 1984). The continuous spectrophotometric assay was used as previously described (Opie and Newsholme 1967). The assay medium consisted of 50 mM HEPES, pH 7.5, 17 mM NaHCO₃, 1 mM MnCl₂, 2 mM phosphoenolpyruvate (sodium salt), 0.25 mM-NADH (sodium salt), 1.54 mM IDP (sodium salt), 1 mM dithiothreitol and 7.5 units of malate dehydrogenase (Duff and Snell 1982). Briefly, the assay measures the activity of the enzyme producing oxaloacetate (OAA) from the substrate phosphoenolpyruvate (PEP), subsequently converting OAA to malate through NADH and malate dehydrogenase. The oxidation of NADH is proportional to the amount of the
converted substrate and is measured spectrophotometrically at 340 nm. One microunit of PEPCK activity is defined as the amount of enzyme activity required to catalyze the formation of 1 mmol PEP/min at 37°C in this assay system.

**Western blotting**

A fraction of liver from each experimental group was homogenized by sonication in an anti-protease cocktail. After sonication, an aliquot of the extract was collected, and the total protein content was determined by the dye-binding protein assay kit (Bio-Rad Laboratories, Hercules, CA). Samples of crude membrane preparations from each experimental group were incubated for 5 min at 100°C with 5x concentrated Laemmli sample buffer (1 mmol/L sodium phosphate, pH 7.8, 0.1% bromophenol blue, 50% glycerol, 10% SDS and 2% mercaptoethanol) (4:1, v/v). The samples were then separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, SP, Brazil) for 2:30 h at 120 V (constant) in buffer containing methanol and SDS. After checking the efficiency of the transfer by Ponceau S staining, the membranes were blocked with 50 g/L dry skimmed milk in TTBS (10 mmol/L Tris, 150 mmol/L NaCl and 0.5% Tween 20) overnight at 4°C. PEPCK, ARG1, ALT, LDH and GK were detected on the membrane after a 4-h incubation at room temperature with their respective antibodies (diluted 1:500 in TTBS) (Santa Cruz Biotechnology, CA, USA). The membranes were then incubated with a secondary antibody (diluted 1:5000 in TTBS) for 2 h at room temperature. Radiolabeled protein bound to the antibody was detected by autoradiography.
The intensity of the bands was evaluated by densitometry using Scion Image software (Scion Corp., Frederick, MD).

**RNA preparation and real-time RT-PCR**

Total RNA was separated from the frozen liver sample using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the supplier's instruction. Three micrograms of total RNA was transcribed into cDNA with a high-capacity reverse transcriptase (Applied Biosystems, CA, USA). Primers specific for rat PEPCK (RN01529008_G1), LDH (RN00820751_G1), ALT (RN00578989_G1), GK (RN00577740_M1), ARG1 (RN00567522_M1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Applied Biosystems, CA, USA. GAPDH was used as an endogenous control. PCR was carried out in duplicate using a Step One instrument with the Taqman Gene Expression Master Mix (Applied Biosystems, CA, USA). cDNA was amplified under the following conditions: 95 °C for 10 min for denaturation and subjected to 40 cycles of 95 °C for 15s, 60 °C for 20 s, and 72 °C for 15 s, followed by extension at 72 °C for 10 min. The real-time data were analyzed using the Step One System (Applied Biosystems, CA, USA). Quantitative values were obtained from the threshold cycle value (Ct), which is the point where a significant increase in fluorescence is first detected. The results were calculated according to the $2^{-\Delta \Delta CT}$ method.

**Statistical analyses**

The results are expressed as the mean ± standard deviation. Levene’s test for the homogeneity of variances was initially used to check the normality of the
data before testing with parametric ANOVA. When necessary, the data were log-transformed to correct for variance in heterogeneity or non-normality (Sokal and Rohlf 1995). A two-way ANOVA analysis of variance (effects of nutritional status of mother and effect of diet of offspring after weaning) was used to compare the data from the CC, CS, LC, and LS groups. One-way ANOVA was used to verify differences among LC, LS and LL groups. When it was observed an interaction effect in the two-way ANOVA or significant difference in the one-way ANOVA, these analyses were complemented by the least significant difference test to determine the significance of individual differences. \( P < 0.05 \) indicated statistical significance. All the statistical comparisons were performed using the Statistic Software package (Statsoft, Tulsa, OK, USA).

**Results**

Adult offspring from mothers that were protein restricted during pregnancy and lactation had lower food intake \( (F_{1,42}=2656.7, \ p<0.0001) \), body weight gain \( (F_{1,49}=132.2, \ p<0.0001) \), EWAT weight \( (F_{1,49}=97.1, \ p<0.0001) \), RWAT weight \( (F_{1,49}=94.3, \ p<0.0001) \), liver weight \( (F_{1,49}=24.7, \ p<0.0001) \) and serum glucose concentration \( (F_{1,15}=4.9, \ p<0.05) \) than those from mothers that were not protein restricted during pregnancy and lactation. In addition, offspring fed soybean diet had lower food intake \( (F_{1,42}=121.1, \ p<0.0001) \), body weight gain \( (F_{1,49}=33.8, \ p<0.0001) \), EWAT weight \( (F_{1,49}=28.3, \ p<0.05) \), RWAT weight \( (F_{1,49}=26.8, \ p<0.0001) \), muscle weight \( (F_{1,49}=5.4, \ p<0.0001) \) and serum glucose concentration \( (F_{1,15}=6.2, \ p<0.05) \) than those fed casein diet. The FFA concentration did not differ in the LS and CS groups, and both were lower in relation to the CC group \( (P<0.001 \text{ and } P<0.0001, \text{ respectively}) \). Free fatty acids
concentration did not differ in LC group and CS groups and in both groups FFA concentration was lower than the LS and CC rats ($P<0.01$ and $P<0.0001$, respectively). The LS rats had lower food intake, body weight gain, muscle and EWAT weights than the LC rats, but these variables were higher in relation to the LL rats ($P<0.05$). The liver weight did not differ in the LS and LC groups, and both were higher than the LL group ($P<0.01$). The RWAT weight did not differ in the LS and LL rats, and both values were lower than those of the LC rats ($P<0.01$). The FFA concentrations were higher in the LS rats in relation to the LC and LL rats ($P<0.01$). Serum glucose concentrations did not differ among the LC, LS and LL groups (Table 2).

After 12 h of fasting, the AUGC value during the glucagon sensitivity test was lower in the adult offspring maintained on soybean diet compared to the adult offspring fed casein diet ($F_{1,15}=17.7$, $p<0.001$). Adult offspring from mothers that were protein restricted during pregnancy and lactation had lower mean total areas under the glucose curves during the test than those from mothers that were not protein restricted during pregnancy and lactation ($F_{1,15}=5.8$, $p<0.05$). The glucagon response was lower in the LS group than in the LL and LC groups ($p<0.01$), which did not differ (Fig. 1A). No difference in the AUCG from the offspring from mothers that were not protein restricted during pregnancy and lactation and those offspring from mothers that were protein restricted during pregnancy and lactation was observed during the pyruvate tolerance test. The mean total areas under the glucose curves during the pyruvate tolerance test in the fasting state were lower in the LL rats than in the LC and LS rats (Fig. 1B).

The LDH mRNA expression in the LS, CS and LC groups was lower than in the CC group ($p<0.05$). Both the LS and LC rats had a higher liver LDH mRNA
content than the LL rats ($p<0.05$) (Fig. 2A). The mRNA expression of both ALT and PEPCK were increased in the liver tissue from the offspring fed soybean diet in relation to those maintained with casein diet ($F_{1,15}=12.4$, $p<0.01$ and $F_{1,15}=6.4$, $p<0.05$, respectively). The LS group showed higher levels of liver ALT and PEPCK mRNA transcript than the LC and LL groups ($p<0.05$). The liver ALT mRNA in the LC group was increased compared to the LL group ($p<0.0001$), whereas PEPCK mRNA expression did not differ in the LC and LL rats (Figs. 2B and 2C). There was no observed significant effect of effect of nutritional status of mother and effect of diet of offspring after weaning or interaction between these effects with regard the ARG1 and GK mRNA expression levels. Although the LC rats tended to have lower ARG1 and GK mRNA levels than the LS rats, this level was not significantly different from the latter group or from the LL rats. However, the levels of ARG1 and GK mRNA in the LL rats were significantly lower than the levels observed in the LS rats ($p<0.05$) (Figs 2D and 2E).

The hepatic LDH content was higher in the CS group than in the CC group ($p<0.01$), and both groups did not differ to the LS and LC groups. The hepatic LDH content did not differ among the LS, LC and LL groups (Fig. 3A); the liver ALT (Fig. 3B) and PEPCK contents (Fig. 3C) did not differ among the groups. The groups fed soybean diet had a higher GK content in relation to the groups maintained with casein diet ($F_{1,16}=5.5$, $p<0.05$), and this variable did not differ in LC, LS and LL groups (Fig. 3D). The liver ARG1 content was higher in the LS group than the LC group ($p<0.05$), and both were higher than the LL group ($p<0.05$). No difference was observed among the offspring from mothers that
were not protein restricted during pregnancy and lactation and those whose mothers were protein restricted during pregnancy and lactation (Fig. 3E).

The liver LDH activity did not differ in the LS, LC and CS groups, and these groups exhibited lower LDH activities than the CC group ($p<0.05$) (Fig. 4A). The liver ALT (Fig. 4B) and ARG1 (Fig. 4D) activities were higher in the LS and LC groups than in the LL group. There was no observed significant effect of nutritional status of mother and effect of diet of offspring after weaning or interaction between these effects with regard to the activities of both enzymes. In the LS and LC rats, the liver PEPCK activity was lower in relation to the CS and CC rats ($F_{1,12}=7.9$, $p<0.05$), which showed activity similar to the LL group (Fig. 4C).

**Discussion**

Protein restriction in early life followed by a soybean diet after weaning compromised body weight gain, EWAT weight and RWAT weight in adulthood, confirming previous findings (Cheim et al. 2009, Milanski et al. 2009, Paiva et al. 2012). The lean profile produced by a soybean diet and early protein restriction was accompanied by low basal serum glucose and FFA concentrations. We previously demonstrated that a soybean diet reduced liver glycogen content (de Arruda Oliveira et al. 2008), enhanced insulinemia and the insulin/glucose ratio, reduced liver IR and IRS-1 contents, decreased IRS-1/PI3-K association and phosphorylation of Akt insulin-stimulated (Feres et al. 2010).

It has been shown that the suppression of liver IR diminishes insulin clearance and increases insulin secretion, leading to hyperinsulinemia. Decreased concentrations of FFAs, due in part to the suppression of extrahepatic lipolysis,
reduce the liver glycogen content and enhance hepatic glucose production (Biddinger and Kahn 2006, Michael et al. 2000). Moreover, Akt mediates the effects of insulin on glycogen synthesis and the suppression of hepatic gluconeogenesis (Michael et al. 2000).

Despite the evidence reported above of increased hepatic glucose output, our rats fed a soybean diet exhibited low basal glucose concentrations. Beside to insulin, glucagon, is the key regulatory hormone for glucose homeostasis, and glucagon administration increases glucose concentrations in fasted animals as a result of increases in both glycogenolysis and gluconeogenesis (Beuers and Jungermann 1990, Ikeda et al. 1989, Young et al. 1993). We previously observed that animals fed a soybean diet did not show alterations in serum glucagon concentration (Feres et al. 2010); therefore, glucagonemia is not a persuasive explanation for the low glycemia observed in these animals. The reduced area under the glucose curves during the glucagon sensitivity test at 12 h of fasting suggests glucagon resistance in rats fed a soybean diet as well as in those subjected to protein restriction during early life.

To determine whether the soybean diet interfered with hepatic glucose production, we evaluated the mRNA expression, protein content and activity of several enzymes that promote gluconeogenesis.

Amino acids and lactate are the main substrates for gluconeogenesis in rats, and alanine is the predominant gluconeogenic amino acid (MacDonald et al. 1976). Alanine formed in extrahepatic tissues is transported to the liver where the amino nitrogen is metabolized to urea for excretion, and the carbon skeleton is converted to glucose (Mallet et al. 1969). The generation of alanine is coupled to the catabolism of branched-chain amino acids (O’Connel 2013) and
soybean used in the present study possessed higher concentration of leucine, isoleucine and valine than casein (de Arruda Oliveira et al. 2008). Alanine aminotransferase plays an important role in gluconeogenesis and amino acid metabolism by catalyzing the reversible transamination between alanine and 2-oxoglutarate to form pyruvate and glutamate (Jadhao et al. 2004). Arginase catalyzes the conversion of arginine to ornithine and urea in the liver (Ash 2010). Hepatic gluconeogenesis and ureagenesis share common intermediates if the glucose precursor is an amino acid and therefore must be evaluated simultaneously (Yang et al. 2000). In the present study, a soybean diet increased ALT mRNA expression but did not change the ALT protein content or activity. The ARG1 protein content was increased, but ARG1 activity was not modified in the rats recovered with a soybean diet. In addition, protein restriction in early life did not alter the mRNA expression, protein content or activity of these enzymes. These results suggest that gluconeogenesis from amino acids and ureagenesis were not altered by a protein restriction during early life or soybean diet despite the difference in amino acids profiles between casein and soybean diet. We also observed that a soybean diet and protein restriction in early life both reduced liver LDH mRNA levels and activity. The LDH system catalyzes the conversion of pyruvate to lactate in anaerobic glycolysis; lactate can be converted to glucose in gluconeogenic flux. Because this system reflects the NAD⁺/NADH ratio indicated by the lactate/pyruvate ratio in the hepatocyte cytosol (Williamson et al. 1967) it is reasonable to presume that low activity occurred due to regulation of the NAD⁺/NADH ratio by glucose oxidation. The reduced mRNA content and/or activity of ALT and ARG1 observed in our rats born from and suckled by mothers fed a low protein diet and subsequently
maintained on the same diet after weaning may have resulted from increased insulin sensitivity that was previously observed in these animals (Latorraca et al. 1998). Higher insulin sensitivity in the muscle of protein restricted rats appear to contribute to the lower proteolysis (dos Santos et al. 2016). The inhibitory effect of insulin on muscle proteolysis results in a further reduction in the supply of gluconeogenic precursors (Wahren and Ekberg 2017).

Glycerol, a product of adipose tissue lipolysis, is also a substrate for hepatic glucose synthesis and contributes significantly to hepatic gluconeogenesis during the fasting period (Lee et al. 2005). Glycerokinase phosphorylates glycerol to glycerol 3-phosphate (G-3P), which is a source for dihydroxyacetone phosphate (DHAP), glycerolipid and glucose production (McCabe 2001). Thus, GK is a critical link to glycolysis and gluconeogenesis. In the present study, the soybean diet did not interfere with GK mRNA expression in the liver but increased the GK protein content. However, an increased liver GK content does not necessarily indicate enhanced gluconeogenesis because the supply of the gluconeogenic substrate (glycerol) is not increased (Paiva et al. 2012) and because the amount of the gluconeogenic energy substrate (FFAs) was reduced in our rats fed a soybean diet.

The increase in the hepatic mRNA level of PEPCK, often observed in insulin resistance states (Taniguchi et al. 2006), was also found in our animals maintained with a soybean diet. However, the PEPCK protein content and activity were not affected by the soybean diet, indicating that the conversion of oxaloacetate into phosphoenolpyruvate, an early and rate-limiting step in the hepatic gluconeogenesis pathway, was not altered. This assumption was confirmed by unchanged area under the glucose curve measurements during
the pyruvate tolerance test. Pyruvate (together with citrate) is the main source of hepatic glucose production after a long fasting period. It is necessary to consider as a limitation to the present study the use of pyruvate tolerance test to evaluate gluconeogenesis. This test considers change in glucose in response to a bolus of pyruvate as a reflection of hepatic gluconeogenesis. However, pyruvate is utilized by many tissues and can possibly affect glucose concentrations by competition for oxidative processes in extrahepatic tissues. Nevertheless other data evaluated such as content and activity of enzymes minimized this limitation.

Thus, soybean diet impaired the response to glucagon, altered the transcription of genes, protein content and activity of enzymes involved in the hepatic glucose production, but did not alter the gluconeogenesis. Long-term these effects may adversely compromise glycemic homeostasis. It is noteworthy that these negative implications of soybean diet observed in the present study are contrary to epidemiological and clinical studies that have shown favorable effects of soy protein or its bioactive components on glucoregulation and insulin resistance in obese and diabetes patients.

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dissertation presented by Nelma Cristina Silva Pacheco as a partial requirement for the Master’s degree in Biosciences at the College of Nutrition, UFMT.

**Conflict of interest**

The authors have no conflicts of interest to report.

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**Figure legends**

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Table 1. Composition of the control, low-protein and soy flour diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control (AIN-93G) (170 g protein/kg)</th>
<th>Soybean flour (170 g protein/kg)</th>
<th>Low protein (60 g protein/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean flour¹</td>
<td>–</td>
<td>415.0</td>
<td>–</td>
</tr>
<tr>
<td>Casein (850 g protein/kg)</td>
<td>202.0</td>
<td>–</td>
<td>71.5</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>397.0</td>
<td>312.2</td>
<td>480.0</td>
</tr>
<tr>
<td>Dextrinized cornstarch</td>
<td>130.5</td>
<td>103.7</td>
<td>159.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.0</td>
<td>78.6</td>
<td>121.0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>70.0</td>
<td>70.0</td>
<td>70.0</td>
</tr>
<tr>
<td>Fiber</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Mineral mix (AIN93G-MX)²</td>
<td>35.0</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Vitamin mix (AIN93G-VX)²</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3.0</td>
<td>3.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Choline chlorhydrate</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

¹Composition of soybean flour (100 g): 455 Kcal, 41 g carbohydrate, 25.5 g protein, 21 g total fat, 2 g fiber, 6 g humidity, 4.5 g ash, 100 mg isoflavone. (genistein: 1.9 mg/100 g; daidzein: 2.6 mg/100 g). ²Reeves et al. (1993)
Table 2 – Somatic and biochemical parameters from adult offspring from mothers fed a control diet during pregnancy and lactation and maintained with casein (CC) or soybean (CS) diets after weaning, adult offspring from mothers fed a low protein diet during pregnancy and lactation and maintained on a casein (LC) or soybean (LS) diets after weaning, or offspring of mothers fed an low protein diet and subsequently fed the same diet after weaning (LL).

<table>
<thead>
<tr>
<th>Variables</th>
<th>CC</th>
<th>CS</th>
<th>LC</th>
<th>LS</th>
<th>LL</th>
<th>Two-way ANOVA</th>
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<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>NS effect</td>
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<td></td>
<td></td>
<td></td>
<td>D effect</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NSxD</td>
</tr>
<tr>
<td>Food intake (g)</td>
<td>1396±41</td>
<td>1301±14</td>
<td>1001±2</td>
<td>933±21</td>
<td>766±23</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>(13)</td>
<td>(13)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
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</tr>
<tr>
<td>Body weight gain (g)</td>
<td>393±28</td>
<td>353±15</td>
<td>318±27</td>
<td>284±17</td>
<td>166±17</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>(17)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
<td></td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>16±3</td>
<td>15±2</td>
<td>12±3</td>
<td>11±2</td>
<td>8±2</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>(17)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
<td></td>
</tr>
<tr>
<td>Muscle weight (g)</td>
<td>1.1±0.2</td>
<td>1.0±0.3</td>
<td>1.1±0.3</td>
<td>0.9±0.2</td>
<td>0.6±0.2</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>(17)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
<td></td>
</tr>
<tr>
<td>EWAT weight (g)</td>
<td>11.5±2.3</td>
<td>8.4±1.3</td>
<td>6.6±1.9</td>
<td>4.7±1.1</td>
<td>2.3±0.5</td>
<td>&lt;.0001</td>
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<tr>
<td></td>
<td>(16)</td>
<td>(17)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
<td></td>
</tr>
<tr>
<td>RWAT weight (g)</td>
<td>12.3±2.4</td>
<td>8.7±1.5</td>
<td>6.3±2.0</td>
<td>4.3±1.3</td>
<td>3.4±0.7</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>(17)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
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<tr>
<td>Serum glucose (mmol/L)</td>
<td>6.6±0.5</td>
<td>5.7±0.7</td>
<td>5.8±0.4</td>
<td>5.4±0.6</td>
<td>5.6±0.4</td>
<td>&lt;.05</td>
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<td></td>
<td>(16)</td>
<td>(17)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
<td></td>
</tr>
<tr>
<td>FFA (mmol/L)</td>
<td>1.7±0.2</td>
<td>1.0±0.3</td>
<td>0.8±0.2</td>
<td>1.2±0.2</td>
<td>0.5±0.2</td>
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<tr>
<td></td>
<td>(6)</td>
<td>(6)</td>
<td>(6)</td>
<td>(6)</td>
<td>(6)</td>
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</tr>
</tbody>
</table>

The values are the mean ± SD of the number of rats indicated in parentheses. The means with different superscript capital letters are significantly different by a two-way ANOVA, and the means with superscript minuscule letters are significantly different by a one-way ANOVA followed by the LSD test (p<0.05). NS= nutritional status; D= diet; NSxD= interaction effects.
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266x349mm (300 x 300 DPI)
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221x244mm (300 x 300 DPI)