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HEALTH SCIENCES

Effects of a low-protein, high-carbohydrate diet administered after weaning and the reversal of that diet in adult rats

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Abstract: To evaluate the effects in adults rats submitted of a low-protein, high-carbohydrate (LPHC; 6% protein, 74% carbohydrate) diet and reversion (R) to a balanced diet introduced after weaning. Research methods & procedures: Male rats weigting approximately 100g (30 to 32 d old) were treated with control (C; 17% protein, 63% carbohydrate) or LPHC diets for 120 days. The reverse group (R) was treated with the LPHC diet for 15 days, and changed to C diet for another 105 days. Results: The LPHC group showed an increase in serum fasting triglycerides (TAG). Serum adiponectin was increased only in the LPHC group. Lipoprotein lipase (LPL) activity was decreased in the extensor digitorum longus (EDL) and cardiac muscles. The adiponectin receptor 1 content is the same among groups in the cardiac muscle, but it is lower in the EDL muscle in the LPHC group. In animals from the R group, these parameters are the same as the LPHC group. Thus, the LPHC diet administered for a long period, it promotes an increase in TAG. It is possible that there is adiponectin resistance in the EDL muscle, due to the lower LPL activity. The reversal of the LPHC diet did not normalize these parameters.

Key words: Adiponectin, hypoproteic-hyperglicidic diet, hypertriglyceridemia, metabolic programming.

INTRODUCTION

The number of children and adults with obesity and metabolic syndrome has increased in the last decades globally (Steyn et al. 2013). There are over 20 million children, below the age of five years, who are overweight in the world (WHO - World Health Organization 2006). According to WHO, the increased intake of "energy-dense foods that are high in fat and sugars" can be one of the causes of increasing obesity in the world. When malnutrition occurs during a critical period of development, mainly in the foetal and perinatal phases, it increases the predisposition of living beings to future health damage such as hypertension, insulin resistance, hyperlipidemia, and obesity (Marti et al. 2005). In many countries, when people introduce solid food to the baby's diet, inadequate nutrition begins. In developing countries, the diet in this period is typically associated with a restriction in protein and an excess of carbohydrates.

For these reasons, we studied the effects of a low-protein, high-carbohydrate (LPHC - 6% protein and 74% carbohydrate) diet in rats introduced soon after weaning for short time (15 days) and a more prolonged (45 days), (França et al. 2009, Buzelle et al. 2010, Santos et al. 2012, Pereira et al. 2014, Ceolin et al. 2019).

The impact of low protein ingestion depends on many factors, such as the period when the protein restraint occurs, the length of time of protein restriction, and the grade of limitation compared to the recommendation for the phase of life.

The administration of the LPHC diet for a short time (15 days) increased the food and calorie intake, and the rats had an energy gain but lower body mass. Body energy increased, although lower body mass may be due to alterations in the body chemistry composition, with an increase in body lipids and a decrease in protein and water. Thus, the rats on the LPHC diet showed adiposity, and the level of TNFα increased about ten times, and two times for corticosterone and leptin (Santos et al. 2012). Leptin is an anorexigenic cytokine, and its increase in the blood of LPHC rats during higher food intake can indicate leptin resistance, presente in many cases of obesity in humans (Paz-Filho et al. 2012).

However, when we studied more prolonged exposure to an LPHC diet (45 days) (Ceolin et al. 2019), rats developed obesity or higher accumulation of lipids in a medium-term adaptation, and they showed similar energy gain during this period due to the increase in energy expenditure. Serum corticosterone, TNF-α and leptin were similar to rats on the C diet for 45 days. However, the level of adiponectin in the blood was higher in rats treated for 45 days when compared with rats that received the C diet for 45 days (Ceolin et al. 2019). The increase in glucose tolerance and similar homeostasis model assessment-insulin resistance (HOMA-IR) in the LPHC45 group suggests that the insulin resistance did not change in these animals. Rats treated for 15 days also showed higher tolerance to glucose overload (Pereira et al. 2014). Thus, the increase in adiponectin level may have contributed to a better response of LPHC45 rats to glucose overload (Kadowaki & Yamauchi 2005).

Thus, the objective of the present study was to evaluate the physiological and biochemical parameters, and hormonal and cytokine profiles in adults submitted to an LPHC diet for 120 days. Another point considered in our studies is whether the reversion to a balanced diet after a period on an LPHC diet can lead to healthy adults or not. The knowledge obtained in these studies can help guide proceedings which have the objective of reducing the impairment induced by malnutrition in childhood.

MATERIALS AND METHODS

Animals and treatment

Thirty male Wistar rats weighing approximately 100g (about 30 days), were randomly divided into three groups: 1) Control group (C), fed a diet of 17% protein and 63% carbohydrate (AIN-93G) (Reeves et al. 1993) for 120 days; 2) Low-Protein, High-Carbohydrate group (LPHC) fed a diet of 6% protein and 74% carbohydrate for 120 days; and 3) Reverse group (R), fed an LPHC diet for the first 15 days, which was then replaced by a control diet for the last 105 days. The diets were isocaloric (16.3kJ.g^{-1}) and the energy difference due to the reduction of dietary protein was compensated by carbohydrate. Rats were housed individually in metabolic cages in an environmentally controlled room (light from 6AM to 6PM, 22± 2° C) and had free access to food and water. The body weight, water and food intake of each rat were recorded daily. The animals were maintained according to the Colégio Brasileiro de Experimentação Animal and approved by the Ethics Committee of the Universidade Federal de Mato Grosso (protocol no. 23108.044442/14-6).

Sample collection

The rats were killed by decapitation between 7 and 10 AM. Blood samples were collected, and plasma was obtained in test tubes with ethylenediaminetetraacetic acid (EDTA), to measure glucose and urea concentrations, and test tubes with EDTA and aprotinin for glucagon level determination. Serum was obtained by centrifuging, and samples were used to measure albumin, total protein, cholesterol, triglyceride, creatinine, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), insulin, adiponectin, and leptin. One group of rats was fasted for 12h for glycemia, insulin, triglyceride, HDL cholesterol, LDL cholesterol, total cholesterol and glucagon determination. The tissues were removed, weighed, and stored at -80°C for determinations of lipid and glycogen and lipoprotein lipase content and activity. The carcasses were eviscerated, weighed, and stored at -80°C for chemical composition and energetic balance component determination. Biochemical analyses were performed by enzymatic colorimetric methods using commercial kits (Labtest® and BioClin®). Insulin and glucagon (SIGMA-Aldrich Inc., St. Louis, MO, USA), leptin (ABCAM, England, UK) and adiponectin (R&D Systems Minneapolis, USA), in the blood were determined by ELISA assays using commercial kits.

Biometric parameters

The anus-nasal length (ANL) was measured on the last day for calculation of body mass index (Lee index) which was obtained by the ratio of the cubic root of body weight (in grams) and ANL (in centimeters) multiplied by 1000. This parameter is used as an index of obesity in rodents and a subjective assessment of the nutritive state of the animal (Bunyan et al. 1976).

Carcass composition and Energy intake

The procedure for determining the chemical composition of the carcass was performed as described by França et al. (2009). The water content of the carcasses was determined by the difference between the weight of the wet eviscerated carcass and dry weight of the carcass after maintaining it at 105°C, until reaching constant weight. Lipid content was calculated by subtracting the weight of the defatted carcass (obtained after extraction with petroleum ether continuous Soxhlet extractor) from the weight of the dry carcass. Ash content was estimated by the combustion of an aliquot of the defatted carcass to 550°C, until reaching constant weight. Protein determination was performed by subtracting the water, lipid and ash contents from the weight of the fresh carcass. For the baseline, which was needed to calculate the energy parameters, the chemical composition was determined in animals with about 90-100g before the introduction of the diets. For the calculations, a gram of protein and carbohydrate was considered to be equivalent to 23.8kJ.g^{-1}, whilst lipid to be equivalent to 39.6 kJ.g^{-1}. Energy gain was calculated as the difference between the carcass energy (kJ) and the energy baseline (kJ). Energy expenditure was calculated as the difference between energy intake (kJ) and the energy gain (kJ), as described by França et al. (2009).

Lipid and Glycogen determination

The method of Carroll et al. (1956) was used to determine liver and muscle glycogen content. The lipid content was determined by the gravimetric method after extraction with chloroform:methanol (2:1) according to Folch et al. (1957).

Western blot for protein analysis

Tissue samples were homogenized with 50 mM Tris-HCl buffer, pH 7.4, at 4°C, containing 1% Triton X-100, 150 mM sodium chloride, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM EDTA, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 5µg/mL aprotinin. Then, the homogenates were centrifuged at 11,000 rpm for 40min at 4°C. The total protein concentration was determined by the Bradford method (Bradford 1976). Samples containing 100µL of total extract from each experimental group were incubated for 5 min at 80°C with concentrated 4x Laemmli buffer (0.250 mM Tris-HCl buffer pH 6.8, 0.5% bromophenol blue, 50% glycerol, 10% sodium dodecyl sulfate, and 500 mM dithiothreitol) (4:1, v/v). Samples containing 50 µg of protein were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes and incubated with primary antibody lipoprotein lipase (LPL), AdopoR1 or α-tubulin, and followed by 2 hours incubation in secondary antibody. Specific bands were detected with Supersignal West Pico chemiluminescent substrate (Pierce) and the protein band intensity was normalized to the intensity of the band of α-tubulin. The intensity of the band was measured with the Scion Image program (version 4.03, Frederick, MD, USA) and the results were expressed as a relative ratio using an internal control as the baseline.

Lipoprotein lipase activity determination

Soleus, extensor digitorum longus (EDL) and cardiac muscles were homogenized in a buffer containing 250mM sucrose and 20U. ml^{-1} heparin, and pH 7.4, keeping the ratio 0.1:1 (m/v). The homogenate was centrifuged and the supernatant below the fat layer was used in the evaluation. Serum of rats fasted for 36h (source APO CII) was added to the supernatant. The LPL activity was determined by a commercially available kit from a business called Gold Analisa Diagnóstica, using the spectrophotometric method.

Statistical analysis

Initially the data were analyzed for normality using the Kolmogorov-Smirnov test to check whether the data collected met the prerequisite for a parametric analysis of variance. Statistical analyses were obtained using one-way analysis of variance (ANOVA) followed by Tukey's post-test. When necessary, the data were transformed into log to correct the heterogeneity of variance or abnormality. When there was uncorrected homogeneity, Kruskal Wallis nonparametric analysis, followed by the Dunn post-test were used for analysis. In all tests, a significant level of 5% ($p<0.05$) was adopted. The results were analyzed by the Statistic program for Windows, version 4.3 (StatSoft, Tulsa, OK, USA). All data were expressed as mean and standard error of the mean (SEM) for the indicated number of rats.

RESULTS

The animals treated with the LPHC diet for 120 days (LPHC group) increased by approximately 12% and 14% on the daily relative food intake compared to the C and R groups, respectively (Table I). When the diet of the R group was reverted to the C diet, the animals showed daily relative food intake similar to the C group. The daily absolute water ingestion of the LPHC group was 30% lower than C and the daily relative water consumption in the LPHC group was lower than the C and R groups (Table I). The R group in water intake was similar to the C group (Table I), but they drank more water (absolute) than the LPHC group. The protein ingestion in the LPHC group was 63% lower than the C group. The reversal of the diet was sufficient to equate

Table I. Consumption of food, water, and protein intake in the control group (C), low-protein, high-carbohydrate group (LPHC) and reverse group (R).

Variable	Groups		
	C (n: 10)	LPHC (n: 10)	R (n: 10)
Daily food intake (g)	16.71 ± 0.41	17.53 ± 0.60	16.66 ± 0.67
Total food intake (g)	2004.50 ± 49.41	2103.71 ± 72.35	1998.84 ± 79.97
Daily food intake ($g.100g^{-1}$ body weight)	4.00 ± 0.12^{a}	4.47 ± 0.14^{b}	3.90 ± 0.10^{a}
Daily water intake (mL)	20.27 ± 0.58^{a}	14.52 ± 0.53^{b}	18.49 ± 0.67^{a}
Daily water intake ($mL.100g^{-1}$ body weight)	4.93 ± 0.35^{a}	3.78 ± 0.30^{b}	$4.42 \pm 0.32^{a,b}$
Total protein intake (g)	340.80 ± 8.40^{a}	126.20 ± 4.34^{b}	312.6 ± 13.51^{a}

Values represent as the means ± SEM of number of animals in parentheses. Different letters represent statistical differences ($P<0.05$), as determined by ANOVA one way.

the intake of proteins in the R group compared to the C group.

Despite the LPHC group showing higher relative food intake, at the end of 120 days, there was no difference in body weight gain and Lee index among the groups (Table II).

The relative weight of IBAT was 125% higher in the LPHC group, when compared to the C and R groups (Table III). The relative weight of the EDL muscle was 11% lower in the LPHC group compared to the C and R groups. There was no difference between the relative weight of the EDL muscle and IBAT of the R and C groups. There was also no difference in the relative weight of the liver, retroperitoneal, perirenal, epididymal white adipose tissues and soleus muscle in the groups.

Rats in the LPHC group had lipid content in IBAT 133% and 100% higher than the C and R groups, respectively (Table IV). No difference was observed in lipid content in the other tissues evaluated among the groups. The glycogen content in the soleus and EDL muscles in the LPHC group was 40% and 52% lower than the C group, respectively (Table IV). There was no difference in the glycogen content of the muscles between the R and C groups. The liver glycogen content was approximately 49% lower in the R group compared to the C and LPHC groups. There was no difference between the LPHC and C groups in liver glycogen content (Table IV).

The carcass weight was similar between groups (Table V), although there was a reduction of approximately 17% in protein weight in the LPHC group compared to the C and R groups. In relation to the energy balance components, the LPHC group intake had about 17% more energy and showed an increase in energy expenditure (20%) compared to the C and R groups. No difference was observed between the C and R groups. The energy gains were similar among groups (Table V).

In the LPHC group, total cholesterol (CT) and TAG in the fed state were lower than the C and R groups. These parameters are similar between the C and R groups. However during the fasted state, the serum levels of TAG between the LPHC and R groups were similar and higher than the C group. Urea levels were reduced in the LPHC group compared to the C and R groups (Table VI).

The LPHC group had lower serum levels of insulin in the fed period compared to the C group (Table VII). The animals of the LPHC group showed increased serum adiponectin compared to the C and R groups. Serum insulin and

adiponectin in the C and R groups were similar (Table VII).

There was no difference in the HOMA index among groups (Figure 1a). However, the insulin-glucagon ratio in the fed state was 80% lower in the LPHC group when compared to the C group. There was no difference in insulin-glucagon ratio between the R and C group, nor the R and LPHC group (Figure 1b).

LPL activity was decreased in the EDL muscle of the LPHC group when compared to the C group. The enzyme activity in the R group was similar to the C and LPHC groups (Figure 2a). There was no difference in the LPL activity in the soleus muscle among groups (Figure 2b). LPL activity was decreased in the cardiac muscle of the LPHC and R group when compared to the C group. LPL activity in the LPHC and R groups was similar (Figure 2c). LPL protein content in retroperitoneal and epididymal white adipose tissues was similar among the groups (Figure 2d-e).

There was no difference in AdipoR1 content among the groups in the cardiac muscle (Figure 3a). However, it was about 36% lower in the EDL muscle of the LPHC and R groups compared to the C group (Figure 3b). The protein content in EDL muscle was similar to the LPHC and R groups.

Table II. Initial and final body weight, body weight gain and Lee index in the control group (C), low-protein, high-carbohydrate group (LPHC) and reverse group (R).

Variable	Groups		
	C (n: 10)	LPHC (n: 10)	R (n: 10)
Initial body weight (g)	95.35 ± 1.17	93.97 ± 1.33	95.55 ± 1.63
Final body weight (g)	422.74 ± 19.50	397.35 ± 21.52	432.96 ± 26.01
Gain in body weight (g)	327.40 ± 19.89	303.38 ± 21.50	337.41 ± 25.73
Lee index	302.52 ± 3.56	304.76 ± 2.52	316.01 ± 5.06

Values represent as the means ± SEM of number of animals in parentheses. (P<0.05), as determined by ANOVA one way.

Table III. Relative weight of tissues in the control group (C), low-protein, high-carbohydrate group (LPHC) and reverse group (R).

Variable	Relative weight ($g.100g^{-1}$ body weight)		
	C (n: 10)	LPHC (n: 10)	R (n: 10)
Liver	3.41 ± 0.14	3.31 ± 0.18	3.29 ± 0.13
Interescapular brown adipose tissue	0.08 ± 0.007^{a}	0.18 ± 0.012^{b}	0.08 ± 0.006^{a}
Soleus muslce	0.09 ± 0.004	0.09 ± 0.002	0.11 ± 0.007
Extensor digitorum longus muscle	0.09 ± 0.001^{a}	0.08 ± 0.003^{b}	0.10 ± 0.007^{a}
Retroperitoneal white adipose tissue	1.89 ± 0.24	2.35 ± 0.16	1.72 ± 0.28
Epididymal white adipose tissue	1.91 ± 0.20	2.00 ± 0.09	1.80 ± 0.23
Perirenal white adipose tissue	0.31 ± 0,06	0.31 ± 0.03	0.30 ± 0.06

Values represent as the means ± SEM of number of animals in parentheses. Different letters represent statistical differences (P<0.05), as determined by ANOVA one way.

Table IV. Lipid contents of the liver, retroperitoneal and epididymal white adipose tissues, interscapular brown adipose tissues, gastrocnemius and extensor digitorum longus muscles and glycogen content in liver and muscles in the control group (C), low-protein, high-carbohydrate group (LPHC) and reverse group (R).

Variable	Lipid (g.tissue^{-1})			Glycogen (mg.tissue^{-1})		
	C (n: 10)	LPHC (n: 10)	R (n: 10)	C (n: 10)	LPHC (n: 10)	R (n: 10)
Liver	0.60 ± 0.05	0.58 ± 0.05	0.61 ± 0.10	1255.27 ± 191.40^{a}	1274.50 ± 178.15^{a}	635.52 ± 136.29^{b}
Retroperitoneal white adipose tissue	5.48 ± 0.62	6.13 ± 0.68	5.13 ± 1.11			
Epididymal white adipose tissue	4.89 ± 0.73	4.84 ± 0.41	4.82 ± 0.79			
Interescapular brown adipose tissue	0.12 ± 0.0^{2}a	0.28 ± 0.04^{b}	0.14 ± 0.03^{a}			
Soleus muscle				1.48 ± 0.14^{a}	0.89 ± 0.09^{b}	1.31 ± 0.12^{a}
Extensor digitorum longus muscle				0.21 ± 0.04^{a}	0.10 ± 0.02^{b}	0.16 ± 0.01^{a,b}

Values represent as the means ± SEM of number of animals in parentheses. Different letters represent statistical differences (P<0.05), as determined by ANOVA one way.

Table V. Carcass composition and energetic balance in the control group (C), low-protein, high-carbohydrate group (LPHC) and reverse group (R).

Variable	Absolute weight (g)			Relative weight (g.100g of body weigh^{-1})		
	C (5)	LPHC (5)	R (5)	C (5)	LPHC (5)	R (5)
Carcass weight	361.1 ± 20.3	302.6 ± 20.9	360.1 ± 30.0	-	-	-
Water	236.2 ± 10.9^{a,b}	196.9 ± 12.3^{a}	239.2 ± 12.3^{b}	62.2 ± 0.9	61.8 ± 1.2	62.4 ± 1.3
Fat / Lipid	35.1 ± 3.3	34.7 ± 3.9	38.8 ± 9.2	24.5 ± 2.4	28.5 ± 2.1	25.7 ± 3.9
Protein	100.5 ± 3.6^{a}	79.8 ± 4.6^{b}	99.2 ± 4.2^{a}	26.5 ± 0.3	25.1 ± 0.5	25.9 ± 0.5
Ash	7.8 ± 0.5	6.8 ± 0.3	7.3 ± 0.4	7.2 ± 0.5	7.9 ± 0.3	6.8 ± 0.2
	Energetic balance (Kj.100g^{-1})					
	C (5)	LPHC (5)	R (5)			
Carcass energy	811 ± 20	844 ± 28	830 ± 44	-	-	-
Energy intake	7304 ± 169^{a}	8560 ± 152^{b}	7213 ± 224^{a}	-	-	-
Energy gain	678 ± 19	683 ± 28	696 ± 52	-	-	-
Energy expenditure	6626 ± 167^{a}	7877 ± 175^{b}	6518 ± 271^{a}	-	-	-

Values represent as the means ± SEM of number of animals in parentheses. Different letters represent statistical differences (P<0.05), as determined by ANOVA one way.

DISCUSSION

We had previously studied the effects of the LPHC diet administered for short and medium length periods, introduced in the early phase of life. Rats that received the LPHC diet showed a higher food intake and lower protein ingestion, but no difference in body weight gain. It is known that the administration of a hypoproteic diet leads to higher food intake as an attempt by the organism to obtain more amino acids during development (White et al. 2003). The amount of protein ingested was less than the required amount, resulting in animals treated with the LPHC diet for 15 and 45 days presenting lower protein content in carcass and muscle mass, and low blood urea (França et al. 2009, Ceolin et al. 2019). Our results for 120 days also demonstrated the same.

Table VI. Biochemical analysis in fed and fasted states in the control group (C), low-protein, high-carbohydrate group (LPHC) and reverse group (R).

Biochemical parameters (fed state)	Groups		
	C (10)	LPHC (10)	R (10)
Glycemia (mg.dL^{-1})	121 ± 5	125 ± 4	111 ± 3
Albumin (g.dL^{-1})	3.13 ± 0.12	2.83 ± 0.07	3.09 ± 0.09
Total protein (g.dL^{-1})	6.99 ± 0.29	6.59 ± 0.13	7.33 ± 0.22
Total cholesterol (mg.dL^{-1})	124 ± 9^{a,b}	109 ± 5^{b}	132 ± 10^{a}
HDL-cholesterol (mg.dL^{-1})	69 ± 9	60 ± 7	64 ± 4
Triglycerides (mg.dL^{-1})	124 ± 9^{a,b}	109 ± 5^{b}	141 ± 8^{a}
Urea (mg.dL^{-1})	35.25 ± 1.67^{a}	15.15 ± 1.27^{b}	38.42 ± 3.82^{a}
Creatinine (mg.dL^{-1})	1.05 ± 0.18	0.92 ± 0.06	0.94 ± 0.04
Aspartate aminotransferase (U.dL^{-1})	128.14 ± 5.57	140.37 ± 16.73	108.80 ± 13.83
Alanine aminotransferase (U.dL^{-1})	36.90 ± 4.76	39.49 ± 3.64	43.40 ± 6.92
Biochemical parameters (fasted state)	**Groups**		
	C (10)	**LPHC (10)**	**R (10)**
Glycemia (mg.dL^{-1})	92 ± 5	80 ± 10	96 ± 6
Total Cholesterol (mg.dL^{-1})	132 ± 14	117 ± 8	146 ± 18
HDL-cholesterol (mg.dL^{-1})	75.24 ± 3.16	71.77 ± 3.28	79.37 ± 2.82
LDL-cholesterol (mg.dL^{-1})	14.91 ± 1.23	14.78 ± 1.68	15.79 ± 2.00
Triglycerides (mg.dL^{-1})	58 ± 9^{a}	114 ± 10^{b}	139 ± 17^{b}

Values represent as the means ± SEM of number of animals in parentheses. Different letters represent statistical differences (P<0.05), as determined by ANOVA one way.

Animals fed the LPHC diet for 15 and 45 days showed higher calorie intake and lower body mass. Lower body weight is probably due to lipid increase as well as protein and water reduction in the carcasses (França et al. 2009, Ceolin et al. 2019). Feeding the LPHC diet for 45 days did not change energy gain and adipose tissues depots, which could be due to diet adaptation leading to lower food efficiency (Ceolin et al. 2019). When the animals were submitted to the LPHC diet for 120 days, the same was observed.

Leptin serum levels are the same among groups after 120 days. This can be explained by the not change in adipose tissues depots, since the relation between increased adipose tissue and leptin is well known (Considine et al. 1996).

The increase in interscapular brown adipose tissue (iBAT) mass and in the lipid content in animals for 15, 45, and 120 days in the LPHC diet suggests that thermogenesis in IBAT could be an adaptive mechanism. In 15 days of the LPHC diet, increased sympathetic activity was observed that activated thermogenesis and increased UCP1 expression via p39/MAPK and ATF2 (França et al. 2016). Therefore, this could contribute, at least partially, to the increase in energy expenditure not only in animals submitted to a LPHC diet for 15 days, but also for 45 and now in 120 days. In this work, energy expenditure was calculated as the difference between energy intake (kJ) and the energy gain (kJ), and is know that classic procedure is calorimeter. We believe, that this increase in energy expenditure in iBAT can contribute to the same lipid content in adipose tissues of animals from 120 days on the LPHC diet. Moreover, we have no experimental data that might support this suggestion, as UCP1 expression, substrate

Table VII. Hormonal analysis in the control group (C), low-protein, high-carbohydrate group (LPHC) and reverse group (R).

Hormonal parameters	Groups		
	C (10)	LPHC (10)	R (10)
Insulin fed state (ng.mL^{-1})	3.53 ± 0.63^{a}	1.04 ± 0.16^{b}	2.45 ± 0.52^{a,b}
Fasting insulin (ng.mL^{-1})	0.56 ± 0.02	0.53 ± 0.01	0.54 ± 0.04
Glucagon fed state (ng.mL^{-1})	0.03 ± 0.007	0.03 ± 0.005	0.03 ± 0.014
Fasting glucagon (ng.mL^{-1})	0.12 ± 0.02	0.10 ± 0.01	0.10 ± 0.02
Leptin fed state (ng.mL^{-1})	2.79 ± 0.26	2.99 ± 0.23	2.50 ± 0.47
Fasting Leptin (ng.mL^{-1})	1.89 ± 0.26	2.21 ± 0.14	1.88 ± 0.27
Adiponectin fed state (µg.mL^{-1})	7.10 ± 0.40^{a}	10.16 ± 0.51^{b}	6.55 ± 0.36^{a}

Values represent as the means ± SEM of number of animals in parentheses. Different letters represent statistical differences (P<0.05), as determined by ANOVA one way.

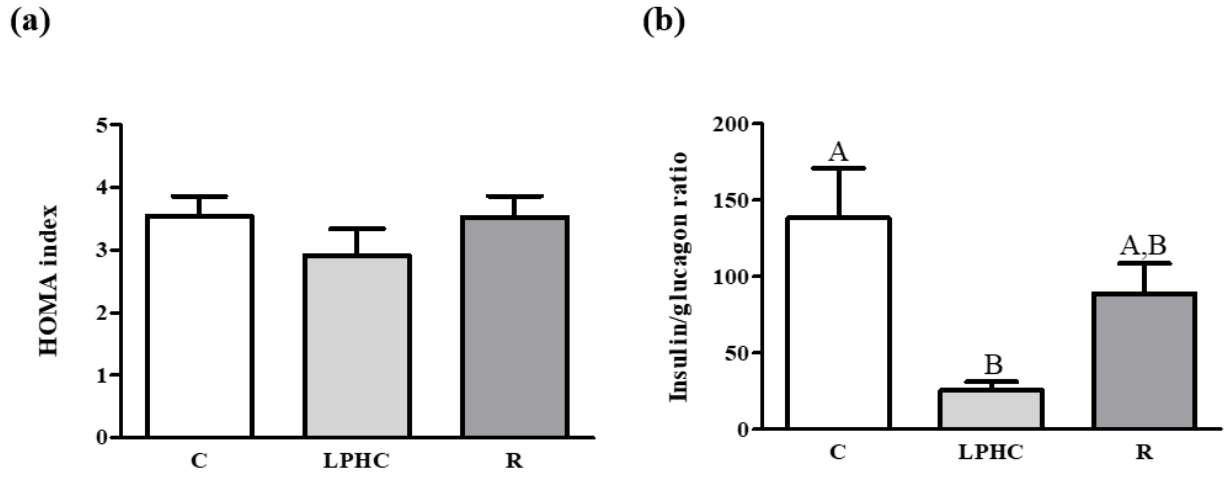

Figure 1. HOMA index (a) and the insulin/glucagon ratio (b) in the control group (C), low-protein, high-carbohydrate group (LPHC) and reverse group (R). Values represent the mean ± SEM of the number of animals (7-9). Different letters represent statistical differences (P <0.05), as determined by ANOVA one way.

uptake, oxygen consumption). Furthermore, other energy expenditure mechanisms may emerge as a response to metabolic programming in defense of increased adipose deposits since the LPHC animals showed high food intake. An example would be the browning process in adipose tissue, which was previously observed in the perirenal white adipose tissue of animals fed the LPHC diet for 15 days (Pereira et al. 2017).

In previous studies we confirmed that LPHC rats showed an increase in plasma adiponectin on the 15th and 45th day of treatment (Ceolin et al. 2019) . In this study we have shown the same. Despite high serum levels, AdipoR1 content in EDL muscles was the same in all groups, suggesting an adiponectin resistance in this tissue. Adiponectin resistance was seen before in two tissues: liver and muscle (Larter et al. 2008, Sente et al. 2015). Other authors suggested that adiponectin resistance in muscle and adipose tissue of insulin-resistant *ob/ob* mice may be due to decreased expression levels of AdipoR1 and AdipoR2 (Tsuchida et al. 2004)

The LPHC diet, when offered for 15 or 45 days, did not cause changes in the serum lipid profile (França et al. 2009, Ceolin et al. 2019).

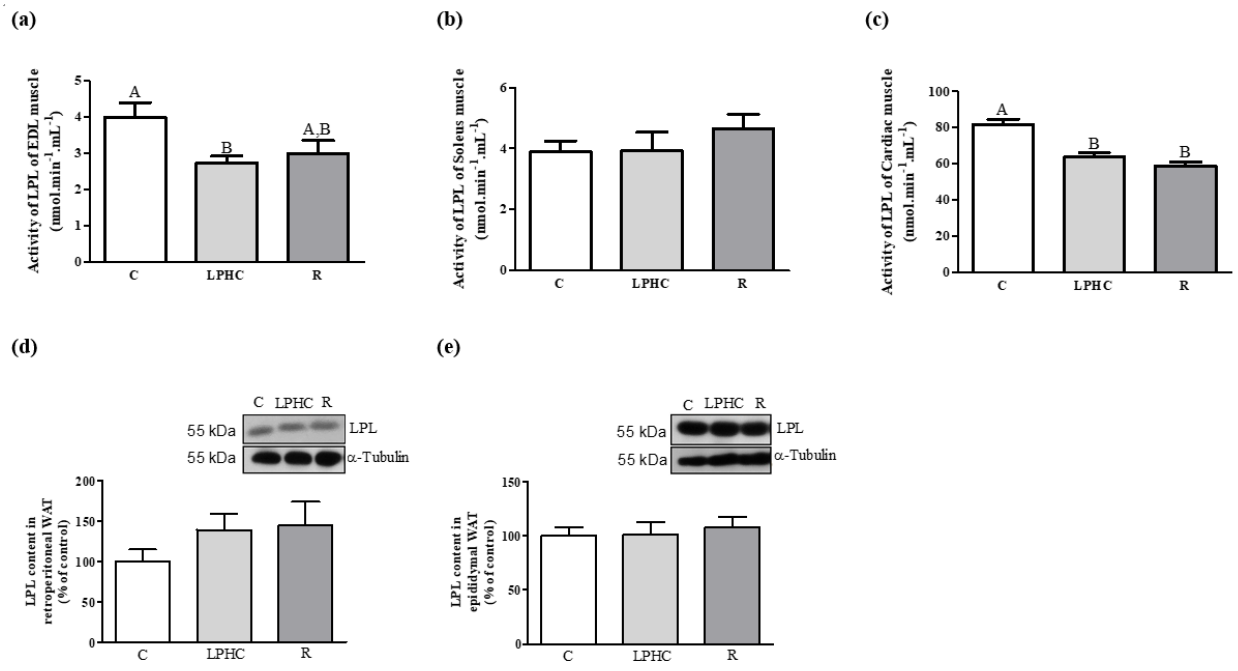

Figure 2. LPL activity in the EDL (a), soleus (b) and cardiac muscles (c) and LPL content in retroperitoneal (d) and epididymal (e) in the control group (C), low-protein, high-carbohydrate group (LPHC) and reverse group (R). Different letters represent statistical differences (P <0.05), as determined by ANOVA one way.

However, in animals treated by a diet for 120 days, the serum TAG of the fasting period was higher, when compared to the C groups. This is due to the importance of the activity of LPL in the hydrolysis of triglycerides into lipoproteins in the skeletal muscle under fasting conditions (Kersten 2014) . The LPL activity is regulated by apolipoproteins, hormones and other factors (Paglialunga et al. 2009). Its activity is altered in a way which is specific to the tissue, which directly affects the use of fatty acid (FA). This is greater in tissues that oxidize FA, such as the heart and skeletal muscle (Botion 2001). After 120 days of the LPHC diet, the animals showed lower LPL activity in the EDL and cardiac muscles. It is well studied that LPL activity is important in the removal of circulating TAG to tissues, and we believe that the lower LPL activity is contributing to the increase in serum TAG levels in the LPHC group.

Research has shown that adiponectin levels are inversely correlated with VLDL-TAG, suggesting that adiponectin may influence lipid metabolism (Yanai & Yoshida 2019), especially triglyceride, independent of insulin signaling (Lee & Shao 2012). Kubota et al. (2002) observed hypertriglyceridemia in adiponectin-deficient mice. The adiponectin increases the LPL activity in skeletal muscle to provide FA as substrates for oxidation (Qiao et al. 2012). Furthermore, adiponectin promotes higher mitochondrial biogenesis and oxidative capacity in skeletal muscle by activated AMPK and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1 α) (Qiao et al. 2008). Iwabu et al. (2010) showed that muscle-specific AdipoR1 *knockout* mice demonstrated insulin resistance due to a reduction in mitochondrial content and activity in skeletal muscle, suggesting that adiponectin and/or adiponectin signaling plays an indispensable role in regulating mitochondrial biogenesis and function in mice. Therefore, we conclude

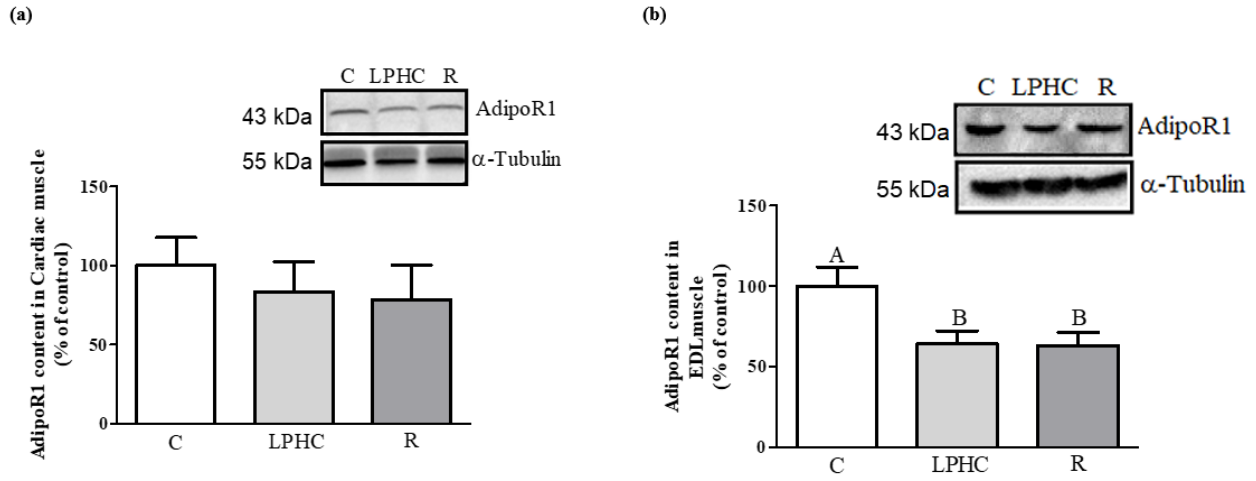

Figure 3. AdipoR1 content protein in the cardiac (a) and EDL (b) muscles in the control group (C), low-protein, high-carbohydrate group (LPHC) and reverse group (R). Values represent the mean ± SEM of the number of animals (5-9). Different letters represent statistical differences (P <0.05), as determined by ANOVA one way.

that lower LPL activity is due to adiponectin resistance, at least in the EDL muscle.

It is interesting to note the glycogen deposition in the EDL and soleus muscles, and the liver. The levels of serum insulin in the fed state are lower in the LPHC group. Moreover, the insulin:glucagon ratio is lower in the LPHC group, suggesting a predominance of glucagon effects and greater glycogenolysis. Kalra & Gupta (2016) showed that stimulating mobilization of stored nutrients, such as glycogen by glycogenolysis occurs when the insulin:glucagon ratio is lower. This fact explained the lower glycogen content in the EDL muscle of animals treated with the LPHC diet for 45 days (Ceolin et al. 2019), and also at 120 days, even with higher carbohydrate consumption. The LPHC group showed the same hepatic glycogen content as the C group. This can be explained by the affinity to glucose of the glycogen synthase enzyme isoform present in the liver being higher than in muscle (Ceolin et al. 2019). This too can contribute to the same glycemia even with lower insulinemia.

Evaluating the effects of the reversion for a long time (15 days in the LPHC diet and more than 105 days in the C diet, totalling 120 days) also revealed the normalization of several parameters, such as food consumption, weight, and lipid content of IBAT, protein content in the carcass, as well as serum adiponectin, as in the 45 days regimen (Ceolin et al. 2019). However, the AdipoR1 in the EDL muscle and serum TAG in the fasting period was similar to the difference between the LPHC group and the C group. Therefore, in the same way as in the LPHC group, adiponectin resistance and the lower activity of LPL in the EDL and heart muscle may be contributing to the increase in serum TAG levels. The serum TAG levels in the fed state were higher in the R group than in the LPHC group, however they were similar to the values found in animals in the C group. Furthermore, the higher depletion of liver glycogen in the R group may be for maintenance of glycaemia in this group, due to diet reversal. However, experiments that aim to study the hepatic metabolism of glycogen and liver insulin resistance or sensitivity need to be evaluated.

We conclude the following facts:

1) Independent of the protein restriction time, the animals eat more in a frustrated attempt to meet the protein requirement.
2) Long-term protein restriction causes an increase in serum TAG levels.

3) Protein restriction increases serum levels of adiponectin and at 120 days of treatment, animals show resistance to this adipokine in the EDL muscle (due lower AdipoR1 receptor content), which may be related to the decrease in LPL activity resulting in a higher level of serum TAG.
4) The reversal of the diet is efficient in restoring some parameters altered by protein restriction for 15 days. However, it was not enough to improve adiponectin resistance and the effects of this on lipid metabolism, and it promoted greater depletion of hepatic glycogen.

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DATA AVAILABLE ON REQUEST FROM THE AUTHORS

The data that support the findings of this study are available from the corresponding author [Pereira MP], upon reasonable request.

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