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**ORIGINAL RESEARCH ARTICLE** **OPEN ACCESS**

# METABOLIC EVALUATION OF THE EFFECTS OF A HYPERLIPID DIET FOR OBESITY INDUCTION AND STANDARD NORMOLIPID DIET (AIN 93) CONSUMPTION IN WISTAR RATS

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**ABSTRACT**

Obesity is considered a multifactorial pathology that causes several metabolic changes. The objective is to evaluate the effects of the consumption of AIN-93 and high-fat (HF) diets on Wistar rats. We performed clinical, biochemical, histopathological, and nuclear magnetic resonance (NMR) -based metabolomic analyses. Among the diets studied, the visceral fat and adiposity index of animals fed with the HF diet were higher than those of animals fed with the AIN diet, but there were no differences in body weight between groups. By the glucose tolerance test, there were no differences between groups. However, the HF group had a lower decay rate of the glucose and was more intolerant to insulin. Severe steatosis was found in the HF group, and mild steatosis in the AIN group. The animals fed with AIN and HF diets showed significant metabolic changes, such as the increment of glucose, succinate, and lactate for HF group, and lipid derivatives for AIN ones. Thus, the comparative study of HF and AIN diets performed in this paper indicate the need to review the composition of healthy standard diets for obesity studies.



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# INTRODUCTION

Over the years, there has been an increase in the incidence of chronic diseases, such as obesity, diabetes, and cardiovascular diseases, all related to the nutritional status of individuals. Excess adiposity may cause several changes in the morphology and functionality of the adipose tissue, such as adipocyte hypertrophy and hyperplasia, inflammation and tissue fibrosis. It also favors the secretion of adipokines (EZQUERRO *et al.*, 2017) and an increase in inflammatory cytokines (PANIAGUA, 2016). For an investigation of obesity and its co-morbidities, experimental studies have been developed based on different models. Among them, there are those involving changes in the type of diet (LENQUISTE *et al.,* 2012; MARQUES *et al.,* 2016; RAMALHO *et al.*, 2017; WANKHADE *et al.*, 2017). Recent studies have focused on the use of hypercaloric diets (DOURMASHKIN *et al.,* 2005), hyperlipidic diets (FERREIRA *et al.*, 2015; RAMALHO *et al.*, 2017), and hyperglycogenic diets (FERREIRA *et al.*, 2015), whose formulations resemble a diet pattern observed in Western diets. Bhathena *et al.,* (2011) report that diets described by the literature present different nutritional compositions, since it is possible to vary the concentration and the source of macronutrients.

The experimental diets used to induce obesity are standardized diets, among which hyperlipidic diet, which presents 31% of lard and 4% of vegetable oil. Currently, parallel to experimental diets, the AIN-93 diet is used, a normolipid diet standardized according to Reeves *et al.*, (1993), as a control group. However, the effects of using AIN-93 over long periods on survival, weight maintenance, biochemical factors, and other pathological processes are not described. In addition, the global analysis of the metabolites present in animal serum via metabolomic analysis can provide an integrated perspective of the functional status of the organism. Metabolomics aid in the simultaneous understanding of multiple metabolic levels and systematic and temporal changes caused by environmental factors and genetic effects, among others (LINDON *et al.*, 2007). In this context, nuclear magnetic resonance (NMR) is a technique widely used in metabolomic analysis due to its versatility in the investigation of metabolic alterations. NMR associated with multivariate analysis has been useful in understanding metabolic variations caused by various conditions, including pathologies associated with eating disorders (JAMBOCUS *et al.*, 2016). In this study, NMR-based metabolomic approach and analysis of clinical, biochemical and histopathological parameters were applied aiming to compare the effects of consumption for 120 days of AIN 93 and hiperlipidic diets in Wistar rats.

# MATERIALS AND METHODS

### *Experimental Animals*

We used male Wistar rats, recently weaned, at 21 days of age, provided by the Central Bioterium of the Federal University of Mato Grosso do Sul (UFMS). The animals were housed in collective cages at 22 ± 2 °C, relative air humidity at 50–60%, light/dark cycle of 12 hours, and feed and water *ad libitum.* All procedures were approved by the Ethics Committee on Use of Animals/CEUA UFMS (Protocol No. 672/2015).

The animals were randomized into two groups according to the type of diet: (1) AIN: normolipid diet (n = 11) based on AIN 93 (REEVES *et al.,* 1993); (2) high-fat (HF): diet with 31% of lard and 4% of vegetable oil (n = 13).

### *Diets*

Table 1 shows the composition of the diets elaborated during the experimental period.

**Table 1. Composition of experimental diets (g/kg diet)**

| Components (g) | AIN-93M | HIGH-FAT (HF) |
|---|---|---|
| Corn Starch | 465.69 | 249.49 |
| Commercial casein | 140 | 157 |
| Sucrose | 100 | 63 |
| Maltodextrin | 155 | 80 |
| Soy oil | 40 | 40 |
| Lard | 0 | 310 |
| Cellulose | 50 | 50 |
| Mineral mix | 35 | 35 |
| Vitamin mix | 10 | 10 |
| L-cystine | 1.8 | 3 |
| Choline bitartrate | 2.5 | 2.5 |
| Tert-butylhydroquinone | 0.008 | 0.014 |

AIN-93G: standard diet; AIN-93 M: standard diet AIN-93M for maintenance of adult animals; high-fat diet (HF): Diet with 31% of lard and 4% of soybean oil.

The AIN-93G diet was used during the growth period of animals (up to eight weeks). After this period, the diet was replaced by AIN-93M, which comprises a diet with lower protein and lipid contents recommended for the maintenance of adult animals, as proposed by Reeves *et al.*, (1993). The composition of the diets was determined according to AOAC (1990) methods. Total carbohydrate content was obtained by theoretical calculation (difference) of the results of triplicates, according to the formula: %carbohydrates = 100–(%moisture + %proteins + %lipids + %ash). Total calories were calculated using the values of Atwater and Woods (1896).

### *Experimental Design*

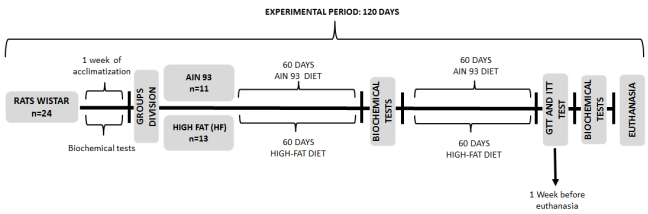



**Figure 1. Experimental design of 120 days; AIN Diet; high-fat diet (HF); glucose tolerance test (GTT); insulin tolerance test (ITT)**

### *Measurements*

Dietary intake control was performed three times a week (every two days), considering the difference in grams between the amount offered and the remaining amount. The energy intake (EI) (Kcal/day) and feed efficiency (FE) (%) were calculated according to the equations proposed by Novelli *et al.,* (2007). The animals' body weight was verified twice weekly using a semi-analytical scale (Científica and Instrumentação Ltda, L S2), with a maximum capacity of 2.0110 kg and a sensitivity of 0.5 g.

### *Experimental Procedure*

At the end of the 120-day experimental period, all animals were anesthetized with Ketamine (80 mg/kg) and Xylazine (10

mg/kg), submitted to euthanasia (cardiac puncture exsanguination) and confirmation of death in a $CO_2$ chamber. The blood was immediately centrifuged and the serum aliquots were stored in a freezer at –18°C for further analysis. The liver, epididymal, omental, retroperitoneal, perirenal, and mesenteric adipose tissues were removed, weighed, and stored for subsequent analysis.

### *Assessment of Liver Fat Content*

The liver of the animals was extracted and weighed on a semi-analytical scale (Bel Diagnóstica®), and then stored in a conventional freezer at –18°C. Lipid contents in the liver were determined according to Folch *et al.* (1957).

### *Biochemical Analyses*

The analyses were carried out at time intervals of 0, 60, and 120 days of experiment. Parameters such as glucose, triglycerides, total cholesterol, and HDL-cholesterol, AST (aspartate aminotransferase), and ALT (alanine aminotransferase) were evaluated by colorimetric kits (Labtest Diagnostics SA). Fractions of VLDL and LDL cholesterol were determined by the following calculations: VLDL (triglycerides / 5) and LDL (total cholesterol – (HDL + VLDL)).

### *Glucose Tolerance (GTT) and Insulin (ITT) Tests*

GTT and ITT tests were performed one week before the end of the experiment. The animals were fasted for six hours, weighed, and then fasting blood glucose was determined by flow rate (time 0). Soon after, the animals received orally (gavage) glucose at a concentration of 2 g/kg body weight. Glycaemia was determined at 15, 30, 60, and 120 minutes after glucose administration. The results were expressed as mg/dL and used to calculate the area under the curve (AUC). For ITT, the animals remained in fed state; they were weighed, and then the glycaemia was determined at the time 0. Afterwards, 1.5 U/kg insulin (NovoRapid®Penfill®) was applied via intraperitoneal (ip), and the glycaemia was determined at 3, 6, 9, and 12 minutes according to Lenquiste *et al.,* (2012). The results were expressed as mg/dL and used to calculate the glucose decay rate (KITT). Blood glucose (GTT and ITT) was evaluated using a portable glycosometer (Injex Sens II®).

### *Histopathological Analysis of the Liver and the Adipose Tissue*

Fragments of the liver and epididymal adipose tissue were fixed in 10% formalin for 12 hours. Thereafter, the samples were processed up to paraffin inclusion. Cuttings 5 µm thick were obtained and stained with hematoxylin/eosin. For the morphological analysis of the liver, the presence or not of the following histopathological parameters was verified: steatosis (fatty degeneration), necrosis, vasodilation, and leukocyte infiltrate. We used a scored evaluation system: 2: absence of injury; 4: injury to a mild degree; 6: lesion to moderate degree; and 8: lesion to intense degree (adapted) (Pokorska-Śpiewak *et al.*, 2017). The volume density of hepatic steatosis (Vv [steatosis]) was estimated as the ratio between the points of the screen that touched the fat vesicles (Pp) and the number of total points (PT, in this case 36 points). Data were expressed as percentage (AGUILA *et al.,* 2003).

For the morphometric analysis of epididymal fat, the area ($\mu m^2$) and the perimeter (µm) of 100 adipocytes of each animal were estimated using the Image J 1.44o software, USA. The mean of the measurements of each group was calculated, according to the methodology adapted from Fonseca Junior *et al.* (2016).

### *$^1$H NMR Analysis of Serum*

Serum samples were thawed and centrifuged at 2500 rpm for five minutes. Three-hundred microliters of the supernatant was mixed with 200 µL of phosphate buffer containing 0.2% TSP-d4 (2,2,3,3-d4-3-(trimethylsilyl)propionic acid sodium salt) and transferred into 5 mm NMR tubes. Spectra were acquired on a Bruker DPX 300 (7.05 T) spectrometer, operating at 300.13 MHz for $^1$H frequency. A standard one-dimensional (1D) NOESY-presat pulse sequence was used for suppression of the water peak. For each sample, 128 scans (NS) were recorded with an acquisition time (AQ) of 1.82 s, relaxation delay (d1) of 2.0 s, time domain of 32k, and spectral width (SW) of 30 ppm. In addition to the NOESY-presat experiments, water suppressed Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse was performed to suppress broad signals from macromolecules. The CPMG spectra were acquired with the same parameters described above and number of loops of $n = 80$. Data processing was performed with 32,000 points in the Fourier transformation, manual phase and baseline corrections and exponential multiplication of 0.30 Hz. In both evaluated extraction systems, the TSP-$d_4$ signal was used as a reference for calibration.

### *Statistical and Multivariate Data Analysis*

For the results of the physical-chemical analysis of the experimental diets and biological data, the Student *t*-test was used, considering $p < 0.05$ as the minimum probability between means. For the statistical analysis, the software SigmaStat 3.5 was used, and the software Graph Pad Prism 6.0 was used to treat biological data and plot graphs. Qualitative analyses were expressed as medians. We used the Kruskal-Wallis test with Mann-Whitney post test, considering $p < 0.05$. Prior to chemometric analysis, the $^1$H NMR spectra from serum samples were binned and Pareto scaled in the AMIX 3.8 Bruker software. The buckets were built with a simple rectangular form and 0.03 ppm of width, integrated by the sum of absolute intensities, and scaled by the total intensity. The evaluated spectral region was 0.60–5.60 ppm, excluding the noises above 5.60 ppm and the region between 4.30 and 5.15 ppm for the residual water signal. For primary visualization, distribution, and clustering, the principal component analysis (PCA) was applied. Orthogonal projections to latent structures-discriminant analysis (OPSL-DA) was further performed as a supervised pattern recognition method, which maximizes the variation between the different groups and identifies variables responsible for the separation. The uncorrelated variation was eliminated using with one orthogonal correction. OPLS-DA was carried out in the Pirouette 4.0 Infometrix softwarewith a three-fold cross validation procedure (CV, 1/7 of the samples being excluded from calculations in each round) to determine the variation between datasets.

## RESULTS

In this study, the experimental diets incorporated into the animal feed were analyzed as for their physical-chemical composition. All components showed a significant difference ($p < 0.05$) between diets (Table 2).

**Table 2. Physical-chemical analysis of experimental diets**

| % (g/100 g) | AIN-93M | HIGH-FAT (HF) |
|---|---|---|
| Moisture | 30.41 ± 0.16[a] | 9.33 ± 0.18[b] |
| Ash | 1.93 ± 0.054[b] | 2.65 ± 0.048[a] |
| Proteins | 9.67 ± 0.085[b] | 13.65 ± 0.15[a] |
| Carbohydrates | 55.07 ± 0.13[a] | 43.29 ± 0.23[b] |
| Lipids | 2.92 ± 0.23[b] | 31.08 ± 0.22[a] |
| Energy value* | 2.92 ± 0.02[b] | 5.11 ± 0.01[a] |

Data presented in mean ± standard deviation; different letters on lines indicate a significant difference ($p < 0.05$); AIN-93 (Reeves *et al*., 1993); high-fat (HF): Diet with 31% of lard and 4% of soybean oil. *Energy value expressed in Kcal/g of diet determined from values of Atwater and Woods (1896).

Experimental diets were offered to animals in creamy form. For the AIN diet to acquire a creamy consistency, similar to the high-fat diet, water was added up to the point of rolling, which may have contributed to the increase ($p < 0.05$) in the moisture content of this diet (Table 2). Ash and protein contents were higher in the high-fat diet ($p < 0.05$) than in the AIN diet. As for the carbohydrate content, the AIN-93 diet presented a higher value ($p < 0.05$) than the high-fat diet. The AIN diet had 55.07% of carbohydrates, corroborating with the protocol of Reeves *et al.,* (1993). The high-fat diet had a lower carbohydrate content (43.29%), as corn starch was replaced by lard, a source of saturated fat (Table 1). Animals receiving the AIN diet showed a higher dietary intake ($p < 0.001$) throughout the experimental period (Figure 2a). We expected the increase in AIN diet consumption when compared to the high-fat diet, since the AIN diet presented a higher content of simple carbohydrates and a lower lipid content, which provides less satiety. On the other hand, the animals that received a high-fat diet had a lower daily consumption ($p < 0.001$), which is related to the slow digestion and satiety that lipids provide.

Water intake was higher ($p < 0.05$) in the group that received the AIN diet (Figure 2b) compared to the group that received a high-fat diet. Thus, it is suggested that the higher the diet intake, the greater the water intake, and the higher the lipid content, the lower the water consumption. In this study, feed efficiency was higher ($p < 0.05$) in animals that received a high-fat diet in all periods evaluated (Figure 2c). Energy intake was higher ($p < 0.05$) up to 60 days of the experimental period in animals receiving the AIN diet (Figure 2d). This higher energy intake may be justified by the higher feed intake in this group (AIN) (Figure 2a). In this study, dietary intake was directly related to dietary energy density (Table 1). The body weight of animals that received the AIN and the high-fat diets did not present a difference ($p > 0.05$) in the experimental period (Figure 3a,b). The AIN diet provided a weight gain equal to animals that received a high-fat diet, standardized for induction of obesity. Thus, this behavior caused us concern about the use of the AIN diet as a "control" diet, because, at 120 days, it was possible to verify a weight gain similar to that of a high-fat diet standardized for obesity induction.

In this study, body weight did not differ between experimental diets (Figure 3a,b), but there was an increase in visceral fat (Figure 3c) and an increase in adiposity index (Figure 3d). Visceral fat corresponds to the sum of fat sites (epidydimal, omental, peritoneal, retroperitoneal, and mesenteric). In this study, visceral fat (Figure 3c) and adiposity index (Figure 3d) of animals that received a high-fat diet were higher ($p < 0.01$) than of animals that received the AIN diet. No differences ($p > 0.05$) were observed between AIN and high-fat diets in relation to liver weight (Figure 3e) and liver fat content (Figure 3f). In the glucose tolerance test (GTT), there was a difference in fasting glycaemia of experimental groups ($p < 0.01$) (Figure 4b).

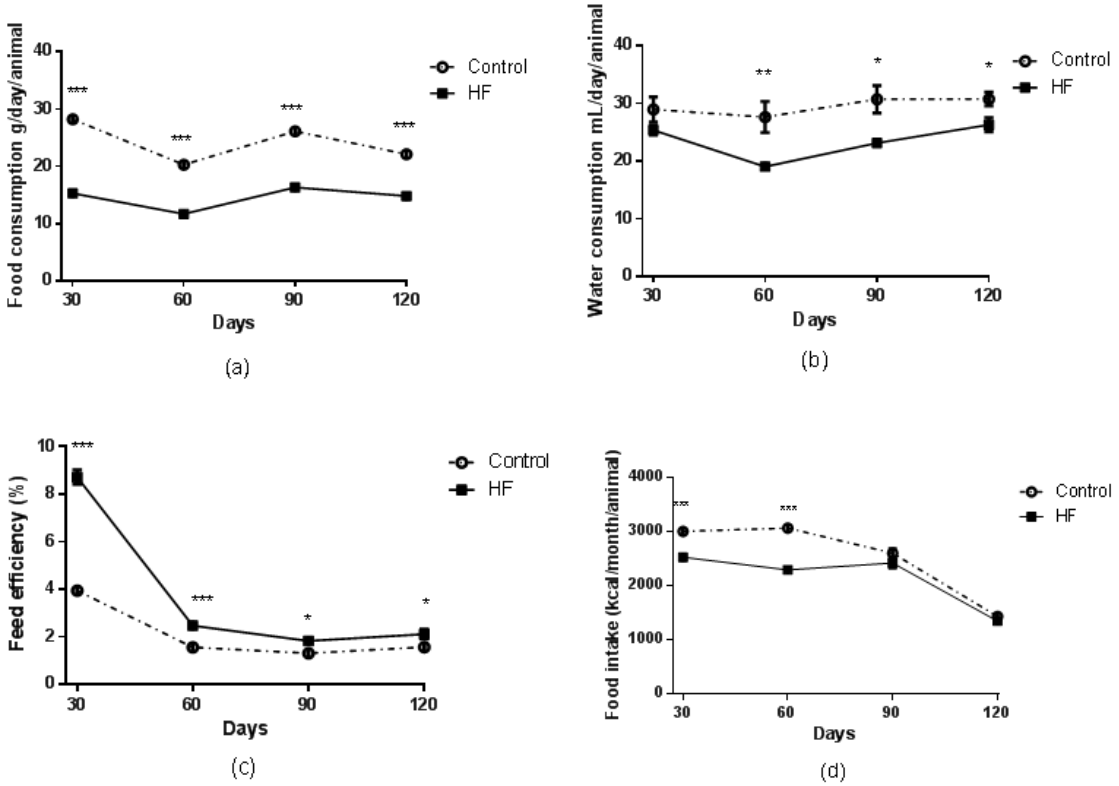



**Figure 2. Comparison between food consumption (kg/day/animal) (a), water consumption (kg/day/animal) (b), feed efficiency (%) (c) and food intake (kg/day/animal), (d) for 120 days between groups of animals fed with AIN diet and HF diet; AIN (Reeves *et al*. 1993); HF (high-fat diet with 31% of lard and 4% of soybean oil). Results expressed as mean ± SD. Data statistically analyzed by Student's *t*-test ($p < 0.05$). Values of means are significantly different between the AIN-93 and HF groups: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$**

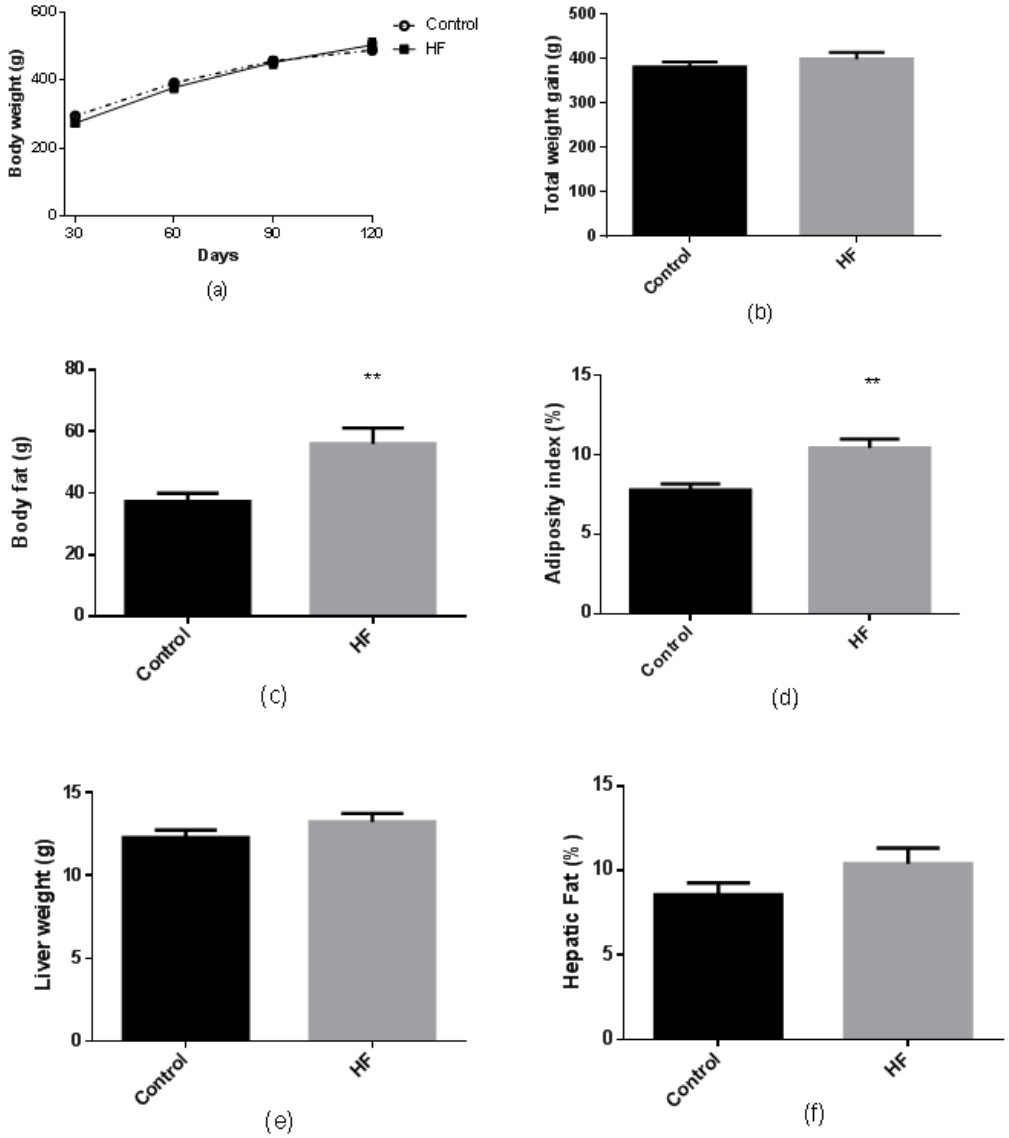



**Figure 3. Comparison between body weight (g) (a) for 120 days demonstrated at 30, 60, 90, and 120 days, total weight gain (g) (b), visceral fat (g) (c), adiposity index (%) (d), liver weight (g) (e), and liver fat (%) (f) between groups of animals fed with AIN and HF diets. AIN (Reeves *et al*., 1993); HF (High-fat diet with 31% of lard and 4% of soybean oil). Results expressed as mean ± SD. Data statistically analyzed by Student's *t*-test ($p < 0.05$). Values of means are significantly different between the AIN-93 and HF groups: **$p < 0.01$**

The animals that received a high-fat diet had higher levels ($p < 0.01$) of fasting glycaemia. However, when evaluating the area under the glucose tolerance test curve, no difference ($p > 0.05$) was observed between experimental groups (Figure 4c). The glucose decay rate (KITT) corresponds to the decrease in glucose expressed as %/minute. The lower the KITT, the greater the resistance to insulin. In this study, the glucose decay rate presented a difference ($p < 0.05$) between experimental groups. The high-fat diet group had a lower decay rate, being more intolerant to insulin (Figure 4e) and, thus, more effective in promoting a changed insulin sensitivity. In this study, the glycaemia of the animals on a high-fat diet was higher ($p < 0.01$) in the evaluated period (Figure 5a) when compared to that of the AIN diet group. In this study, total cholesterol levels (Figure 5b) were higher ($p < 0.001$) in animals receiving AIN diet at 60 days, leading to an increased concern about a prolonged use of the AIN diet. On the other hand, HDL-cholesterol levels (Figure 5d) were higher ($p < 0.001$) in the AIN group at 60 days of the experiment. The other fractions, LDL-cholesterol, VLDL-cholesterol, and triglycerides did not differ between experimental groups ($p > 0.05$) (Figure 5c,e,f).

In this study, HE staining indicated the presence of hepatic steatosis for the two experimental groups (Figure 6A,B). However, no fibrosis, cirrhosis, and apoptosis were found in liver tissues. The animals had non-alcoholic fatty liver disease, an initial stage of liver changes caused by obesity. The animals of the high-fat diet group presented an intense steatosis with a greater extension throughout the tissue, evaluated as microvesicular and macrovesicular changes located in the perilobular and intermediate regions (zones 1 and 2, respectively) (Figure 6B). However, the surprising result we found in this research was the presence of microvesicularhepatic steatosis in the perilobular region (called zone 1) in the AIN group (Figure 6A), which, according to the evaluation using scores, corresponds to light steatosis. The AIN diet should not cause hepatic steatosis, as it is used as a control diet (standard) in studies using experimental animals. Morphometric analyses of adipocyte phenotypes were evaluated (Figure 6C,D). The quantitative results of the adipocyte area and the perimeter had a difference ($p < 0.001$) between experimental groups (Figure 6C,D). The AIN control group presented low values (3861.59 ± 160.87 $\mu m^2$ and 245.04 ± 5.67 $\mu m^2$), whereas the high-fat diet group

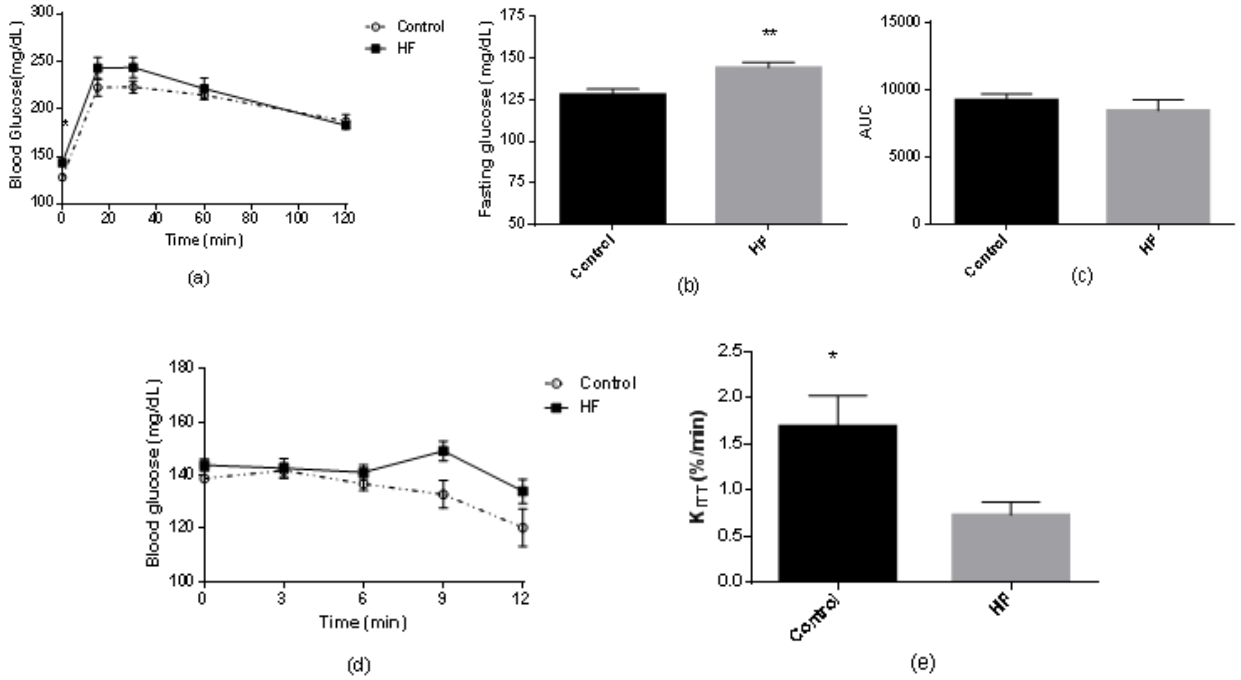



**Figure 4. Glucose tolerance test (GTT) analyzed at 0, 15, 30, 60, and 120 minutes (a); fasting glucose (b); Area under the curve of the glucose tolerance test (AUC) (c); Insulin tolerance test (ITT) analyzed at 0, 3, 6, 9, and 12 minutes (d); decay rate (%/min) during insulin tolerance test (e); AIN (Reeves *et al*., 1993); HF (high-fat diet with 31% of lard and 4% of soybean oil). Results expressed as mean ± SD. Data was statistically analyzed by Student's *t*-test ($p < 0.05$). Values of means are significantly different between the AIN-93 and HF groups: *$p < 0.05$, **$p < 0.01$**

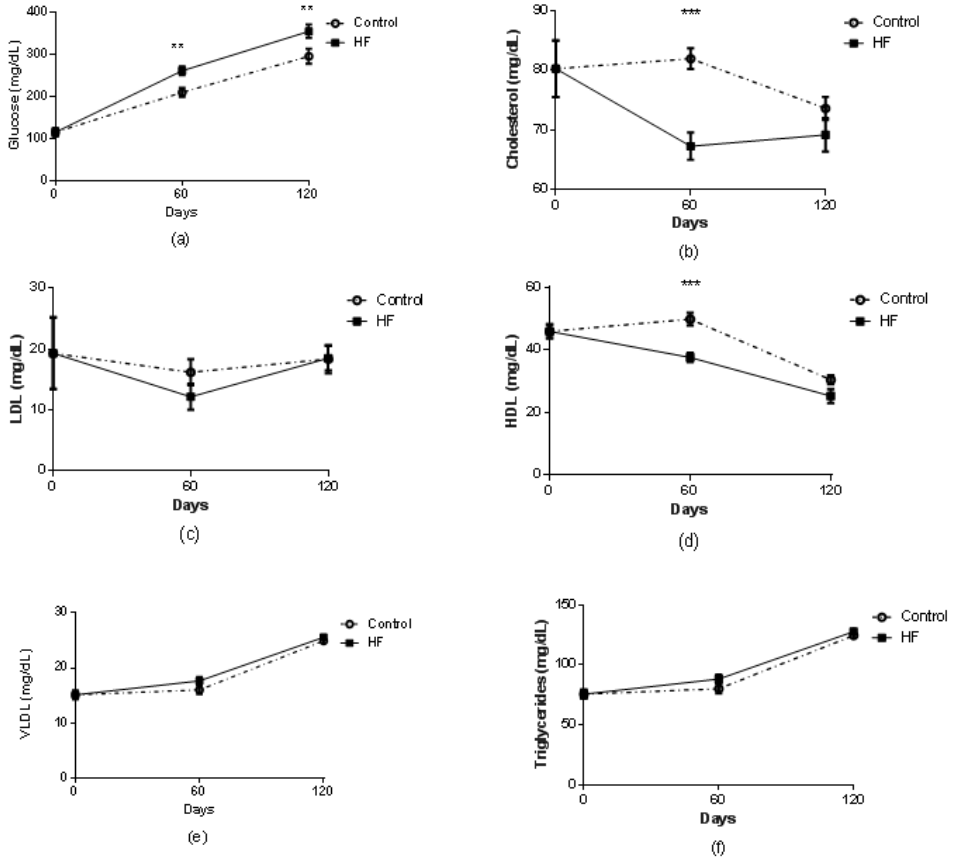



**Figure 5. Values of serum glucose (mg/dL) (a), total cholesterol (mg/dl) (b), LDL-cholesterol (mg/dl) (c), HDL-cholesterol (mg/dl) (d), VLDL (mg/dL) (e), and triglycerides (mg/dL) (f) for 0, 60, and 120 days between groups of animals fed with AIN and HL. AIN (Reeves *et al*., 1993); HF (high-fat diet with 31% of lard and 4% of soybean oil). Results expressed as mean ± SD. Data statistically analyzed by Student's *t*-test ($p < 0.05$). Values of means are significantly different between the AIN-93 and HF groups: **$p < 0.01$; ***$p < 0.001$**

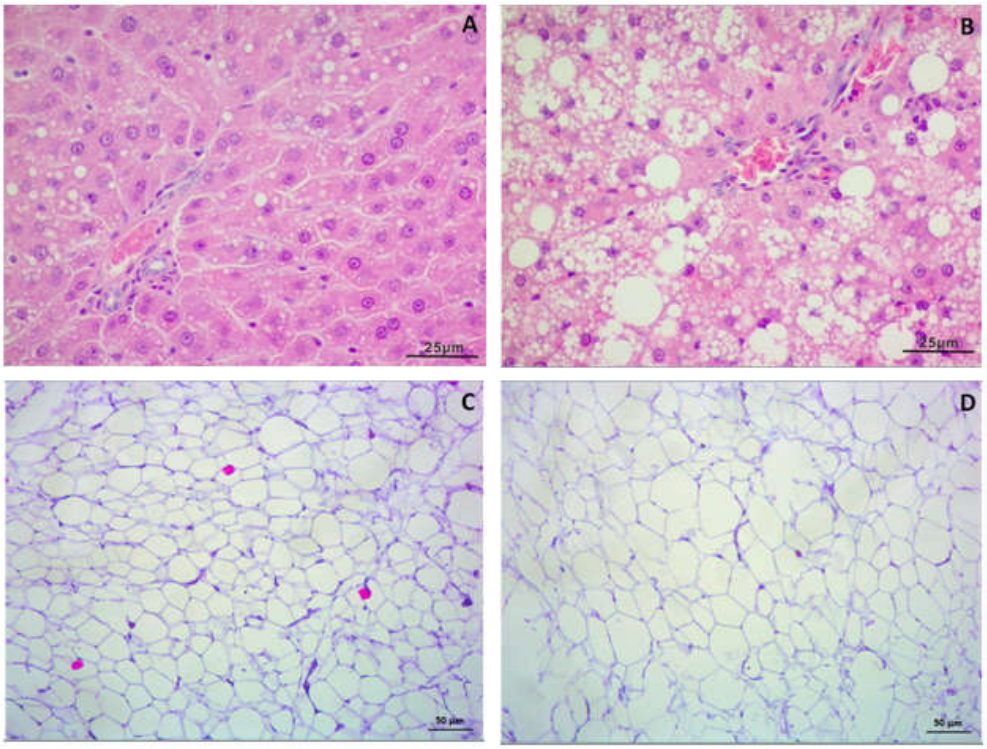



**Figure 6. Histological sections of the liver (40×) and epididymal adipose tissue (10×) stained with H and E. (A) Histological section of the liver of the AIN diet group; (B) histological cut of the liver of the high-fat diet group (HF group); (C) histological section of the adipose tissue of the AIN group; and (D) histological cut of the adipose tissue of the high-fat diet group**

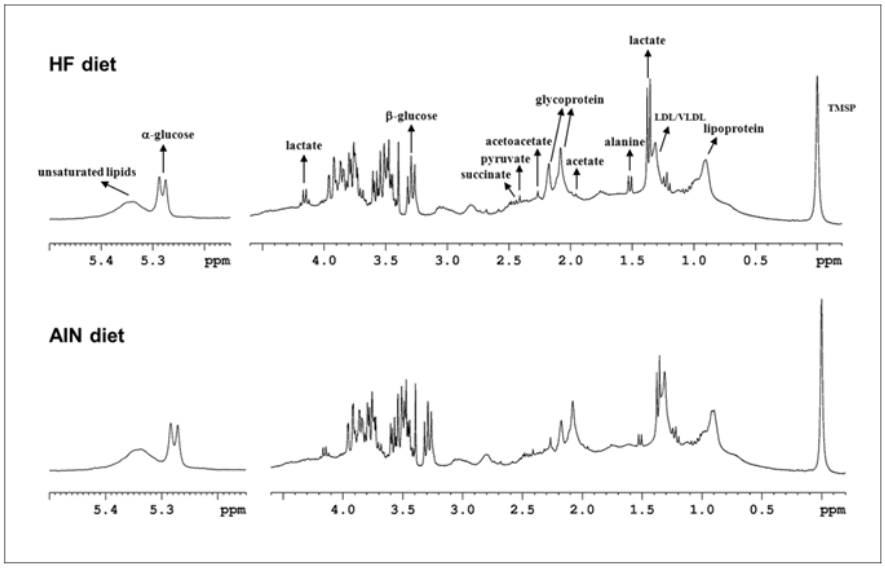



**Figure 7. Representative $^{1}$H NMR spectral profile of serum obtained from Wistar rats fed the HF and AIN diets**

(HF) was high (6258.09 ± 430.72 µm$^2$ and 317.11 ± 12.38 µm). Thus, the high-fat diet favored adipocyte hypertrophy, corroborating body fat findings (Figure 3c,d) and observing that the AIN group presented a lower body fat and a lower adiposity index. In metabolomic analysis of the serum samples, $^{1}$H NMR spectra assembled additional information to the study of the effect of AIN and HF diets on the metabolism of animals. The spectral profile from NOESY experiments emphasized the metabolites (small molecules), while the CPMG data showed the broad spectral profile due to the presence of macromolecules (Figure S1, Supplementary material).

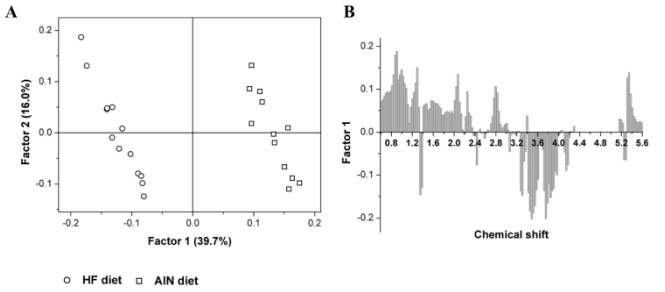
Metabolites were assigned based on two-dimensional NMR experiments, previous studies (HE *et al.*, 2012; JAMBOCUS *et al.*, 2016; TIAN *et al.*, 2013) and the Human Metabolome Database Wishart *et al.*, (2013). The identification of metabolites is shown in Figure 7 and Table 3. Representative $^{1}$H NMR spectra of AIN and HF serum samples (Figure 7) showed a high spectral similarity between both groups highlighting the importance of multivariate analysis to extract the metabolomic information. Considering PCA does not contribute to evaluated differences between samples (data not shown), OPLS-DA algorithm was applied to identify discriminating metabolites between the two groups fed the

both diets. According to OPLS-DA results (Figure 8), rats fed the HF diet were allocated on the negative Factor 1 axis due to increment of lactate, succinate and glucose compounds, while those fed the AIN diet were observed on the positive Factor 1 axis due to increment of lipid derivatives (lipoproteins, LDL/VLDL, and unsaturated lipids) and glycoproteins.

**Table 3. $^{1}$H NMR chemical shift assignments of the metabolites identified in rat serum**

| Metabolites | Assignments | Chemical shifts[a] |
|---|---|---|
| Lipoprotein | $CH_3(CH_2)n$ | 0.90 (m) |
| LDL/VLDL | $CH_3CH_2CH_2$= | 1.25–1.34 (m) |
| Lactate | α-CH | 4.15 (q) |
| | β-$CH_3$ | 1.36 (d) |
| Alanine | β-$CH_3$ | 1.51 (d) |
| Acetate | $CH_3$ | 1.95 (s) |
| Pyruvate | $CH_3$ | 2.40 (s) |
| Succinate | αβ-$CH_2$ | 2.43 (s) |
| Acetoacetate | $CH_3$ | 2.26 (s) |
| β-glucose | 1-CH 2-CH | 4.69 (d) 3.29 (t) |
| α-glucose | 1-CH | 5.28 (d) |
| Unsaturated lipids | -CH=CH- | 5.33 (m) |
| Glycoprotein | $CH_3$-C=O | 2.08 (bs), 2.18 (bs) |

[a] s, singlet; sb, broad singlet; d, doublet, d; t, triplet; q, quartet; m, multiplet




## DISCUSSION

The results suggest the need to review the composition of diets used for experimental animals. Gulati and Misra (2017) report that protein intake may improve blood glucose, serum insulin, serum lipids, inflammatory markers, and liver fats. Ferreira *et al.,* (2015) evaluated the composition of elaborated AIN-93M diets, a high-fat, and a commercial diet (Nuvital®). They found a composition of 81% carbohydrates, 10% protein, and 18% lipids for the AIN-93M diet. Meanwhile, the high-fat diet had 67% carbohydrates, 17% protein, and 32% lipids. The authors reported that the AIN-93 diet has a high carbohydrate content and a low protein content, while the commercial diet has a higher protein content (23%), a lower carbohydrate content (56%), and a lower lipid content (4.5%). Therefore, it is necessary to review the constituents of the diets as recommended for laboratory animals. Picchi*et al.,* (2011) report that, in addition to the nutritional composition of the diet, the period of exposure to this di*et al*so strongly influences weight gain and metabolic syndrome development, since this syndrome is associated with diets with a simultaneous increase of lipids and carbohydrates, thereby favoring the increase in adipose tissue. These long-term changes imply the development of resistance to insulin. As for the dietary intake, this behavior corroborates that found by Ramalho *et al.*, (2017), whose study shows that the animals that received a high-fat diet had a lower dietary intake, maintaining weight and energy intake similar to that of the commercial diet group (Nuvilab). Furthermore, Ramalho *et al.*, (2017) also found a lower ($p < 0.05$) water intake by Wistar rats fed with a high-fat diet.

Food efficiency verifies the animal's ability to lose or gain weight when subjected to a dietary intake (NERY *et al.*, 2011). This means that an increased feed efficiency provides a better performance and feed utilization by the animal. Hariri and Thibault (2010) report that the high-fat diet presents a high dietary efficiency, because it favors weight gain and body fat gain. Thus, a diet with a higher energetic density led to a lower intake (Figure 2 (a)), which leads to a reduction in the intake of other nutrients, such as vitamins and minerals (FUHRMAN *et al.*, 2010). This physiological behavior of the animals may trigger nutritional deficiencies and cause a greater metabolic imbalance. The increase in adiposity index and visceral fat are an important risk factor for cardiovascular diseases (BI*et al.*,2016). Therefore, using body weight alone as a nutritional assessment tool does not allow us to diagnose the true nutritional status of the animals. Bruder-Nascimento *et al.,* (2013) stated that the high-fat diet induced an increase in body fat gain and an increase in adiposity index, corroborating with the results found in this study.

Obesity is closely correlated with insulin resistance and hyperglycemia (Greenberg *et al.*, 2006). Thus, Bruder-Nascimento*et al.,* (2013) also reported that animals that received a high-fat diet remained tolerant to glucose throughout the test, corroborating the findings of this study. However, the results are conflicting in the literature, as studies conducted by Winzell and Ahrén (2004) and Lenquiste *et al.,* (2012) have recognized that the use of high-fat diets may be considered an efficient model to induce tolerance to glucose and type 2 diabetes. In normal individuals, the decay rate should be fast, since it indicates that they are tolerant to insulin. When this decay rate is slowed or decreased, it can be stated that insulin is failing to perform its endocrine function of intracellular glucose carrier (GELONEZE *et al.*, 2006). The glycemic value at the end of the experiment (120 days) was high in both diets (above 200 mg/dL); values were above those found for normal male rats (79–144 mg/dL) (MELO *et al.*, 2012). Ramalho *et al.*, *(*2017) found a significant difference in glycaemia at the 15th week of the experiment using high-fat diets (22.7% lard and 22.5% vegetable oil) and a commercial diet (Nuvilab). Previously, Ferreira *et al.*, (2015) reported that the diet with high carbohydrate and lipid contents (AIN and HF) increased blood glucose levels when compared to animals receiving a commercial diet, whose glycemic level remained within the physiological limit. Probably, the prognosis of this imbalance in glycaemia comes from the composition of the diet, since the AIN diet has a higher content of carbohydrates compared to the commercial diet, while the high-fat diet elaborated in this study had a higher content of saturated fat.

As for the lipid profile, the cholesterol is one of the risk factors for metabolic syndrome (BHATHENA *et al*., 2011). Similar results were also observed by Ramalho *et al.*, (2017), who found an increase in the HDL-cholesterol content in animals that received the Nuvilab diet in relation to the high-fat diet group (49.66 ± 2.19 and 40.40 ± 2.22, respectively) at 15 weeks of the experiment. However, studies have described that the high-fat diet resulted in dyslipidemic changes due to increased levels of triglycerides and total cholesterol, and a reduction in the level of HDL-cholesterol (YANG *et al.*, 2005; NOEMAN *et al.*, 2011). In addition, consumption of a low-fat diet and a high carbohydrate content may be associated with a reduction in LDL and HDL cholesterol and increased triglyceride levels when compared to high-fat diets with a saturated source (GRUNDY, 1986).

Non-alcoholic fatty liver disease and hepatic steatosis are important co-morbidities associated with obesity (WANKHADE *et al.*, 2017), since dietary changes and changes in the concentration of inadequate nutrients may promote metabolic changes that compromise the nutritional state, triggering the appearance of diseases such as non-alcoholic fatty liver steatosis (LEONARDI *et al.*, 2010). According to some authors, diets deficient in choline and methionine are considered diets for induction of liver diseases, since both cause non-alcoholic fatty liver steatosis (VETELÄINEN *et al.*, 2007; BHATHENA *et al*., 2011). Choline is considered a substance that prevents the accumulation of fat in the liver, also called lipotropic substance. It is very important because it is essential for the maintenance of health (ZEISEL, 2012). Thus, one of the hypotheses is that the AIN diet presents an insufficient amount of choline in its composition in the form of choline bitartrate, as recommended by Reeves *et al.*, (1993).

Raubenheimer, Nyirenda, and Walker (2006) stated that the accumulation of fat in the liver may be responsible for the appearance of resistance to insulin during the consumption of a high-fat diet. In addition, choline deficiency promotes accumulation of triglycerides in the hepatic tissue, thus favoring the incidence of liver diseases. This event corroborates with the results of the ITT (Figure 4e), because the high-fat diet group was more resistant to insulin. The combination of diets rich in fat and carbohydrates favors the development of metabolic changes that, when associated, are termed "metabolic syndrome". However, the high-fat diet induces the accumulation of liver tissue fat and concomitantly progresses to more advanced damage, such as non-alcoholic hepatic steatosis, which promotes inflammation in hepatocytes (PICCHI *et al.*, 2011). This effect of the consumption of high-fat diet corroborates this study, evidencing its veracity under the impacts of a diet rich in lipids on the health of animals.
Marques *et al.*, (2016) verified that animals that received a high-fat diet increased energy intake, weight gain and body fat gain, and showed hypertrophy of mesenteric adipocytes. This behavior occurred in this study. The animal in the high-fat group presented a higher energetic intake and a greater accumulation of adipose tissue, a higher adiposity index and, consequently, a greater adipocyte hypertrophy. However, body weight did not differ in relation to the AIN group.

Considering the HF group showed a marked resistance to insulin, the results from NMR-based metabolomic analysis corroborate this observation, since the carbohydrate levels in the serum of the animals were higher for the HF group. Therefore, the increase of carbohydrates in the serum is due to the indirect consequences of a fat-rich diet (HF diet). In addition, the lactate increase observed by NMR for HF group is directly related to lipid synthesis, which triggers the increase of body fat, adipocyte hypertrophy, and intense hepatic steatosis observed in the biochemical and histopathological analyses (Figures 5 and 6), such as reported by Moon *et al.*, (2016). For the animals that consumed the AIN diet for 120 days, the highest changes observed by NMR occurred in the lipid and glycoprotein contents. These results are consistent with changes in serum lipid content (Figure 5) and mild steatosis in hepatic tissue (Figure 6A and 6B). Lima *et al.* (2016) report that carbohydrate-rich diets favor the development of metabolic and hepatic disorders, such as hepatic steatosis, hyperglycemia, hypertriglyceridemia and elevated levels of very low density lipoprotein (VLDL). Thus, animals that consumed the AIN diet, considered as a healthy control diet in the literature, have shown lipid alterations due to high starch intake, which may also be related to the body weight gain similar to the HF diet. The mild steatosis and increased serum lipids detected by both biochemical and metabolomic analyses in animals fed the AIN diet suggest that the use of the AIN diet as a healthy control may be detrimental in comparative studies on obesity, since the animals that consumed this di*et al*ready have important metabolic alterations related to unbalanced diet.

## Conclusion

The animals receiving the AIN and HF diets showed significant metabolic changes observed by clinical, biochemical, and NMR-based metabolomic analyses. The group that received the high-fat diet was resistant to insulin, had increased body fat and adipocyte hypertrophy, had an intense hepatic steatosis, and highlighted the glucose, succinate, and lactate metabolites. The animals receiving the AIN diet showed changes in serum lipids and had mild steatosis in liver tissue. In addition to this, animals from both AIN and HF groups showed a similar weight gain. Our findings suggest the need to review the composition of "standard" diets used for experimental animals aiming to establish a diet for a "healthy" control group with reliable data for future nutritional studies.

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