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EFFECTIVENESS OF DIFFERENT EXPERIMENTAL MODELS OF METABOLIC DISORDERS IN ANIMALS

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ABSTRACT

The objective of this study was to induce metabolic disorders in rats with changes in diet and environmental factors. The animals were divided in groups (n=10): control with standard diet; standard dietwith environmental change (change Light-dark cycle – 14h/10h); cafeteria diet 1 (richer in fat); and cafeteria diet 2 (richer in sugar). Animal weight and food intake were monitored weekly. A glucose tolerance test was performed monthly. After twelve weeks of treatment, the animals were euthanized. There were significant differences in weight gain especially after the third month of treatment. The groups that received cafeteria diet 1 and 2 had lower food intake compared to the control group and environmental change group. Higher values of cholesterol and HDL cholesterol occurred in the control group, showing significant differences compared to cafeteria diet 1 and 2 groups. After2 months, glucose levels inthe environmental alteration and cafeteria diet 2 groups showed a tendency to rise faster than in the control and cafeteria diet 1 groups. It was observed that even diets cited in the literature as effective in inducing metabolic disorders, did not result in the complete establishment of the disease in the animals tested.

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INTRODUCTION

The worldwide prevalence of obesity has increased significantly between 1980 and 2008. In 2008, 10% of men and 14% of women worldwide were obese, compared with 5% of men and 8% of women in 1980 (WHO, 2010). This increase is related to dietary changes that include changes in diet structure, moving to a diet with higher energy density with a greater focus on fat and added sugars in foods, increased intake of saturated fat (mainly from animal sources), and reduced consumption of complex carbohydrates, dietary fiber, and fruits and vegetables (Drewnowski and Popkin, 1997). These dietary changes are exacerbated by lifestyle changes that reflect reduced physical activity at work and leisure (Ferro-Luzzi and Martino, 1996).

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Thus, individuals who are overweight are likely to develop a metabolic disorder known as metabolic syndrome, characterized by the combination of at least three risk factors for cardiovascular disease, including obesity, increased triglycerides, decreased high-density lipoprotein (HDL) cholesterol, hypertension and glucose intolerance (NCEP, 2005). Some experimental models have been developed that allow the study and understanding of metabolic disorders, but there is no standardized protocol that shows 100% reproducibility in inducing models. High fat diets are used for induction of metabolic syndrome in animals and, in fact, alter successfully endpoints (Bezerra *et al*., 2001). Thus, it is important to standardize experimental models in which methabolicdisorders is induced in animals, so that studies can be conducted with a focus on alternative therapies for the control of this metabolic disorder. Thus, this study aimed to induce metabolic disorders in rats with changes in diet and

environmental factorslike temperature change and change in the circadian cycle, to determine which method is more efficient.

MATERIALS AND METHODS

The experimental study was conducted following the criteria established by Law No. 11.794, of October 8, 2008, and the Brazilian Guidelines for the Care and Use of Animals for Scientific and Didactic Purposes, 2013, National Council Controlling Animal Experimentation (CONCEA). The project was submitted to the Ethics Committee on Animal Use (CEUA) of the Federal University of Mato Grosso do Sul (UFMS), and approved in accordance Protocol 571/2013 of February 20, 2014. We used rats (*Rattus norvegicus*), 8 weeks old, purchased from the vivarium of UFMS/Campo Grande (MS). Before starting the experiments, the animals were acclimated to the test environment for 5 days and placed in polypropylene cages each with 4 animals, lined with wood shavings. The cages were kept in an environment with controlled light, humidity and temperature. The temperature was maintained at around 22 ± 2ºC, with a 12-12 h light-dark cycle, except for group 1, which had a 10-14 h light-dark cycle, maintained at 24 ± 2ºC, according to Rosini*et al*. (2012). All groups were given food and water*ad libitum*.

The animals were divided into four groups, with 10 animals each. Environmental alteration group (Rosini*et al*., 2012): Standard diet Nuvital®, light-dark cycle: 10-14 h; temperature: 24 ± 2ºC; Control group (Souza *et al*., 2013): Standard diet Nuvital®, light-dark cycle: 12-12 h, temperature: 22 ± 2ºC; Cafeteria diet group 1 (Pastore *et al*., 2010): Standard diet (37.5%) + cornstarch wafer (12.5%) + roasted peanuts (25%) + granulated chocolate (25%), light-dark cycle: 12-12 h, temperature: 22 ± 2ºC; and Cafeteria diet group 2 (Naderali*et al*., 2001): Standard diet (42%) + condensed milk (42%) + sucrose (16%), light-dark cycle: 12-12 h, temperature: 22 ± 2ºC. The weight of the animals was measured twice a week, using a Toledo® digital scale with a precision of 1 g (Pastore *et al*., 2010). Dietary intake was measured every three days. Thefood efficiency ratio (FER) was determined monthly, using the equation: FER = monthly weight gain (g)/monthly food intake (g) (Freitas *et al*., 2012). One day before change in diet and at the 4th, 8th and 12th weeks, a glucose tolerance test (GTT) was performed as adapted from Nascimento *et al*. (2011). The animals were fasted for 8 h, and fasting blood glucose levels were then measured using a portable glucometer kit using the blood collected from the caudal artery. Afterwards, each animal was given2 g glucose per kg body weight, via gavage, and glucose levels were determined after 15, 30, 60, 90 and 120 min.

After twelve weeks of treatment and 12 h of fasting, the animals were anesthetized with ketamine and xylazine (15 mg/kg: 75 mg/kg) and euthanized by exsanguination by the inferior vena cava. The blood samples were centrifuged at 4000 rpm for 5 min, and the serum was separated and stored for further analysis. Uric acid, triglycerides, total cholesterol and HDL (high density lipoprotein) and VLDL (very low density lipoprotein) cholesterol, urea, AST (aspartate aminotransferase) and ALT (alanine aminotransferase) were determined in a Roche/Hitachi Cobas 6000® automated biochemical analyzer. For histological analysis, the liver tissue was fixed and stained with hematoxylin-eosin. Slide images of liver sections were captured with Leica Application Suite, version 4.0.0 [Build: 877], at 200x magnification, and analyzed for the presence of hepatic steatosis. Statistical analysis was performed in two ways. One considered all animals in the study, and other excluded 3 animals in each group, namely those that gained less weight after consuming the specific feed for 30 days, characterizing them as animals resistant to weight gain. The intention was to determine whether the selection of animals prone to weight gain would make the most efficient method in the development of metabolic disorders, according to Cabeço *et al*. (2010), with modifications. The results were expressed as mean ± standard deviation, when the numerical variables had normal distribution. Analysis of variance (ANOVA) was used to compare the groups. When a statistically significant difference was revealed, the analysis was complemented by the Tukey test, calculated with the use of the Jandel-Sigma Stat program, to reject the null hypothesis at the 5% level.

RESULTS

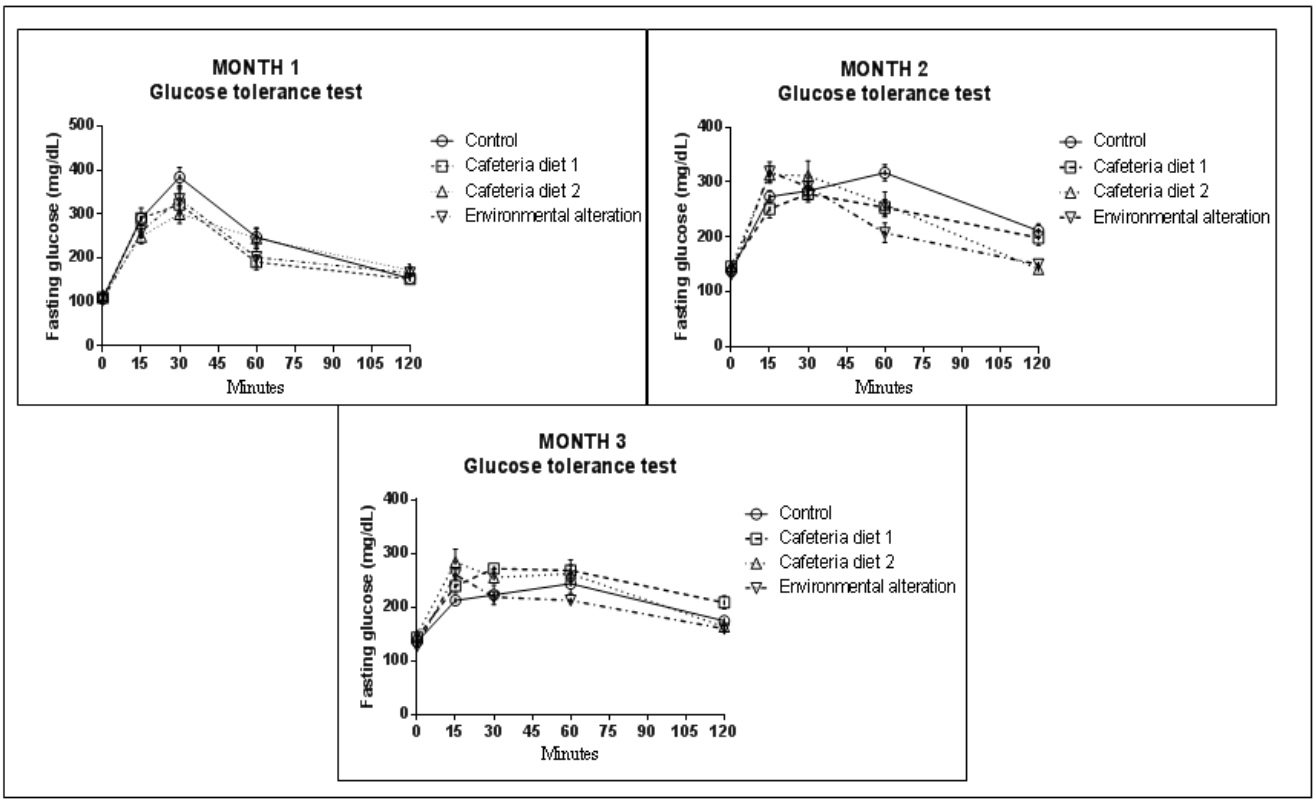
At the beginning of the experiment, the animals in thecontrol, environmental alteration, and cafeteria diet 1 and 2 groupsshowed respective body weights (g) or 112.5±46.26,111.2±39.10, and 105.0±28.15 112.0±14.75 ($p = 0.957$). The control, environmental alteration, and cafeteria diet 1 and 2 groups consumed diets with the following respective caloric values per kg of diet: 3495 kcal (carbohydrate: 60.5%, protein: 19% and lipid: 3.5%); 3495 kcal (60.5, 19 and 3.5%); 5550kcal (55.7,13.3 and 31%) and 4605kcal (73.6, 14.3 and 12.1%). Table 1 shows the monthly weight gain (MWG), monthly food intake (MFI) and monthly food efficiency ratio (FER), obtained for the different groups. There was a significant difference in weight gain especially after 3 months of treatment ($p \leq 0.001$) between the environmental alteration and cafeteria diet 1 groups and between the environmental alteration and cafeteria diet 2 groups, wherethe environmental alteration group had the lowest weight gain and where the cafeteria diet 1 group had a higher weight gain than did the control. Total weight gain was statistically higher in thecontrol group compared to the environmental alteration group ($p=0.036$). With regard to food intake, there was difference between the groups at month 2. In this case, the groups that received cafeteria diet 1 and 2 had lower food intake compared to the control and environmental alteration groups ($p \leq 0.001$), a trend that continued in other months, but with no significant difference.

Such behavior related to food intake and weight gain, determined a higher FER in the groups that received cafeteria diet 1 and 2 at all time points ($p<0.05$), while the group that received cafeteria diet 1 had a higher FER compared to the other groups, with significant difference after 2 and 3 months, and when considering the total. In relation toserumlipids in table 2, the highest values of TC and HDL were in thecontrol group, which were statistically significant from those in the cafeteria diet 1 and 2 groups ($p<0.001$). On the other hand, the last group mentioned showed higher values of VLDL, triglycerides and glucose, but without statistical significance in comparison with the other groups. In table 2, the control and environmental alteration groups displayed higher levels of urea, with significant difference in relation to the cafeteria diet 1 and 2groups ($p<0.001$).The environmental alteration group showed significantly lower creatinine levels compared to the groups ($p=0.047$).

Table 1. Monthly weight gain (MWG), total adipose tissue weight (TAT), epididymal fat (EF), liver weight, monthly food intake (MFI) and monthly food efficiency rate (FER), in rats treated for 90 days with standard feed (control group), environmental alteration and standard feed, or cafeteria diet (type 1 and 2)

		Control (n=10)	Environmental alteration (n=10)	Cafeteria diet 1 (n=10)	Cafeteria diet 2 (n=10)	p
MWG (g)	1 Month	116.80± 15.93[a]	108.70± 10.87[a]	98.40± 11.02[b]	109.20± 7.36[a]	0.012
	2 Months	105.30± 10.24	91.30± 10.65	104.30± 12.99	105.50± 16.40	0.049
	3 Months	38.30± 7.13[ac]	29.20± 8.26[a]	58.70± 15.09[b]	42.70± 15.29[bc]	≤0.001
	Total	282.40 ±24.71[a]	248.20 ±21.70[b]	280.10± 30.53[ab]	276.00± 34.03[ab]	0.036
TAT		18.96 ± 7.62	16.57 ± 4.93	19.03 ± 4.78	16.79 ± 4.99	0.650
EF		6.83 ± 2.59	6.07 ± 1.42	6.79 ± 1.66	6.06 ± 1.92	0.695
Liver		12.01 ± 2.14	10.90 ± 1.75	11.23 ± 1.29	11.99± 1.05	0.328
MFI (g)	1 Month	1427.75± 286.70	1424.00± 280.24	993.50± 146.25	1192.50± 269.57	0.104
	2 Months	1728.40± 103.02[a]	1716.00± 189.71[a]	1106.60± 64.82[b]	1349.40 ± 91.68[c]	≤0.001
	3 Months	1932.75± 434.17	1595.00± 145.40	1238.25± 338.37	1519.00± 379.82	0.071
	Total	5521.0 ± 627.5[a]	5164.0 ± 418.42[ac]	3615.00 ± 391.58[b]	4398.25±555.45[bc]	<0.001
FER`	1 Month	0.25± 0.03[bc]	0.23± 0.02[b]	0.30± 0.03[a]	0.27± 0.02[ac]	≤0.001
	2 Months	0.14± 0.01[a]	0.11± 0.01[a]	0.20± 0.02[b]	0.18± 0.03[c]	≤0.001
	3 Months	0.04± 0.01[a]	0.02± 0.01[a]	0.10± 0.03[b]	0.06± 0.02[c]	≤0.001
	Total	0.13 ±0.01[a]	0.12 ±0.01[a]	0.20 ± 0.02[b]	0.16 ±0.02[c]	<0.001

*Values expressed as mean ± standard deviation. ANOVA followed by Tukey's test. Different letters in the same row signify a statistically significant difference between groups. n=number of animals.



*Data expressed as mean and standard error of the mean (SEM). After 1 month, the control group significantly differed from the other groups. At 30 min, there was a difference between the control group and the cafeteria diet 1 ($p<0.05$) and environmental alteration ($p<0.001$) groups. At 60 min, the control group differed from the cafeteria diet 1 ($p<0.05$) and cafeteria diet 2 ($p<0.001$) groups. At 120 min, control group showed a significant difference compared to the cafeteria diet group 2 ($p<0.001$). After 2 months, there were differences starting at 60 min, at which time the group control showed a significant difference compared to the cafeteria diet 1 ($p<0.05$), cafeteria diet 2 ($p<0.001$) and environmental alteration ($p<0.01$) groups. At 120 min, the control group significantly differed from the cafeteria diet 2 ($p<0.01$) and environmental alteration ($p<0.05$) groups. After 3 months, the differences were seen only in the first 30 min. At 15 min, the control group showed a significant difference in relation to the cafeteria diet 2 ($p<0.001$) and environmental alteration ($p<0.05$) groups. At 30 min, the control group differed from the cafeteria diet 1 group ($p<0.05$).

Figure 1. Glucose tolerance test (GTT) curves obtained in rats fasting, fed standard feed (group control), standard feed with environmental alteration, or cafeteria diet (type 1 and 2), before and after oral administration of glucose

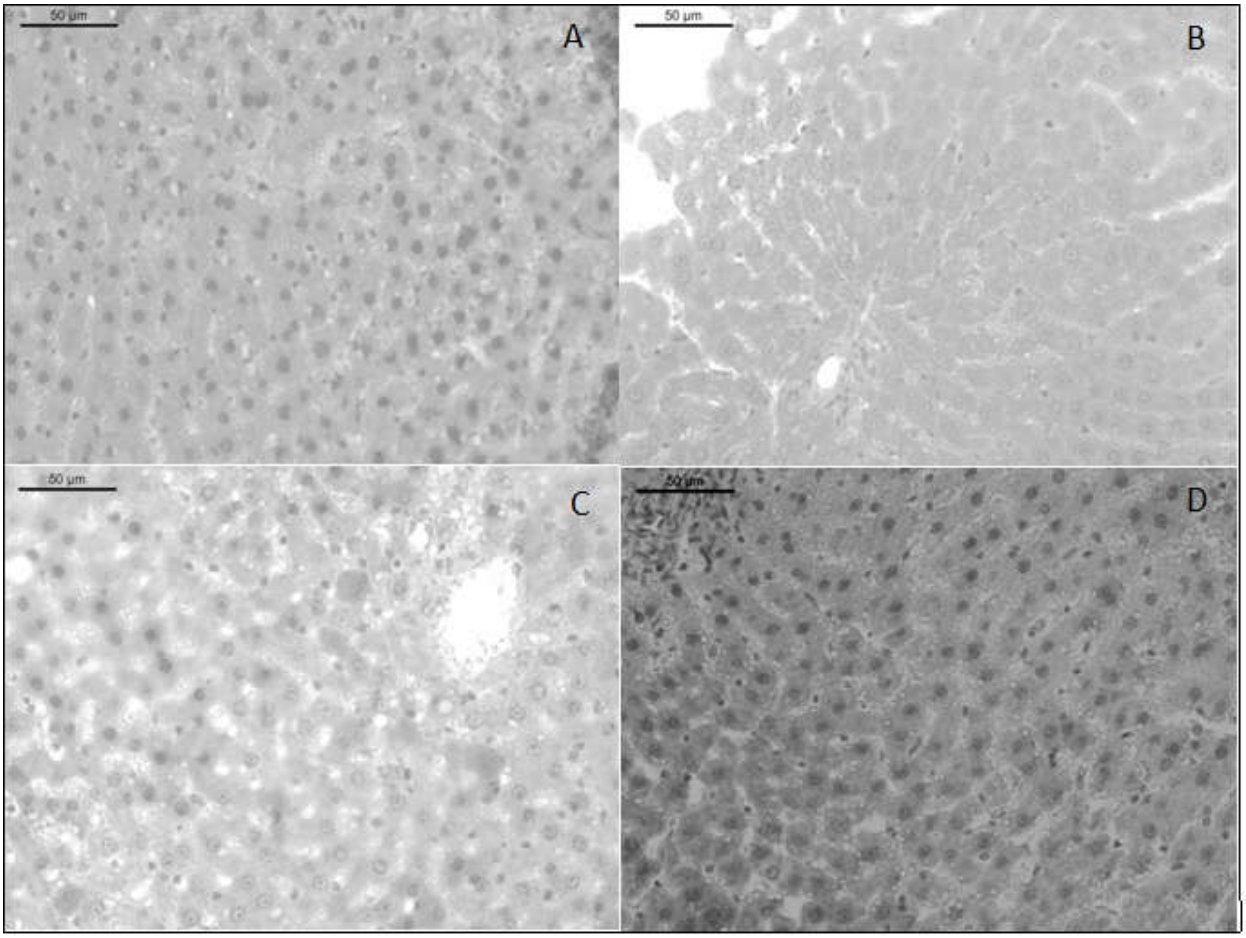
There were no statistically significant differences in AST, ALT and uric acid levels between groups. The glucose curves obtained after 1, 2 and 3 months are described in Figure 1. Significant differences were observed between groups at some evaluation times. The results from the statistical analysis excluding 30% of animals that gained less weight in each group after a month of consuming the diet of the respective group did not reveal a different interpretation with respect to those described here (data not shown).

In liver histology, differences were observed between the groups. The control group showed normal hepatocytes, without accumulation of fat, like the environmental alteration group as well. The cafeteria diet 1 group showed hepatocytes withlipid microdroplets. This histological picture was compatible with mild steatosis. The cafeteria diet 2 group showed lipid micro- and macrodroplets, compatible with moderate steatosis.

Table 2. Concentrations of plasma lipids and fasting glucose, and serum liver enzymes, creatinine, urea and uric acid in rats treated for 90 days, with standard diet (group control), standard diet and environmental alteration, or cafeteria diet (type 1 and 2)

	Control (n=10)	Environmental Alteration (n=10)	Cafeteria diet 1 (n=10)	Cafeteria diet 2 (n=10)	p
TC (mg/dL)	78.70± 14.95[a]	68.00± 16.01[ac]	57.70± 8.46[bc]	48.70± 8.12[b]	<0.001
HDL (mg/dL)	65.61± 10.20[b]	57.41± 13.71[bc]	50.43± 6.92[ac]	39.98± 7.61[a]	<0.001
VLDL (mg/dL)	7.26±1.95	6.27± 1.82	5.72± 1.85	7.45± 2.29	0.183
Triglycerides(mg/dL)	36.30± 9.63	31.30± 9.17	28.70± 9.13	37.20± 11.38	0.187
Glucose (mg/dL)	259.70 ± 43.80	245.80± 73.27	305.40± 57.48	315.60± 125.02	0.170
AST (IU/L)	82.70± 16.17	93.10± 32.96	86.30± 20.87	82.40± 18.05	0.705
ALT (IU/L)	31.80± 3.52	36.00± 14.26	32.80± 12.38	24.60± 6.27	0.097
Urea (mg/dL)	43.60± 3.92b	48.10± 4.80b	32.10± 5.53a	35.90± 3.31a	<0.001
Creatinine (mg/dL)	0.55± 0.053a	0.52± 0.06b	0.61± 0.09a	0.55± 0.07a	0.047
Uricacid (mg/dL)	1.97± 1.28	3.14± 2.21	2.70 ± 1.84	2.82± 2.26	0.588

* Values expressed as mean ± standard deviation. ANOVA followed by Tukey's test. Different letters in the same row signify a statistically significant difference between groups. n=number of animals.



*Identification: (A) Histological analysis of hepatocytes of control group. (B) Histological analysis of hepatocytes of environmental alteration group. (C) Histological analysis of hepatocytes of cafeteria diet type 1 group. (D) Histological analysis of hepatocytes of cafeteria diet type 2 group.

Figure 2. Histological analysis of liver tissue of the animals fed standard feed (control group), standard feed with environmental alteration, or cafeteria diet (type 1 and 2)

DISCUSSION

Given the high incidence of obesity and related diseases, scientific studies seek to reproduce the condition experimentally to evaluate the effectiveness of different treatments. Accordingly, diets are manipulated in an attempt to achieve excess weight, and resultant altered blood levels of glucose and lipids and other changes that characterize metabolic disorders (Monteiro *et al*., 1995; Monteiro *et al*., 2000). However, in experiments conducted in rats, it was observed that even the use of a highly palatable and high calorie diet does not guarantee that all fed animals become obese, since many of them gain weight like animals fed a standard diet (Levin *et al*., 1989; Levin *et al*., 1998; York *et al*., 1992; Shen *et al*., 2004). Increased ambient temperature decreases energy expenditure that the animal would have to maintain their body temperature in the case of colder environments and, therefore, a positive energy balance would be generated. Furthermore, the rodents have nocturnal and thus an increase in dark period of the cycle would provide more time for food intake, particularly if the diet is highly palatable (Tschöp, 2001). The greater weight gain observed in the cafeteria diet 1 group after 3 months of the study was not sufficient to generate higher total weight gain and increased

adipose tissue in isolated sites in a significant way. Duarte *et al*. (2006) was able to induce obesity in Wistar rats treated with hypercaloriccafeteria diet for a long period. They subjected 21-day-old Wistar rats to 15 weeks of treatment. Their method differed from the present study, which involved adult animals (8 weeks) and 12 weeks of treatment. The different results found in the literature are due to the fact that the treatments are for different times and in animals of different ages (Kusunoki, 1993; Kim, 2000). With regard to food intake,after 1 month, the different groups showed no significant difference. After 2 months,there was a significantly lower food intake in the cafeteria diet 1 and 2groups compared to control and environmental alteration groups ($p<0.001$). This pattern of food intake was maintained when assessed over the whole experimental period. These results show that hypercaloric diets are not always characterized by hyperphagia. The results of this study were similar to those of Kretschmer *et al*. (2005), who reported a reduction in food intake in animals subjected to a diet rich in fat. It was expected that the environmental alteration group would consume larger amount of food, but the increased period of darknessmight have caused a change in eating habits, which led to a decrease in food intake (Tallett *et al*., 2009). This is explained by the fact that therest period was shorter, causing anorectic factors to disturb the natural sequence and to inhibit food intake through appetite regulation mechanisms (Halford *et al*., 1998;Rodgers *et al*., 2001;Tallett*et al*., 2008).

In regard to the serum lipid results, there were significant changes only in total and HDL cholesterol levels, which were higher in the control group compared to the cafeteria diet 1 and 2 groups ($p <0.001$). These lipids were high in the group control, but levels were similar to values reported in other works for rats that consumed standard feed, and were thus in the normal range (Dantas*et al*., 2006;Spinelli *et al*., 2013;Melo *et al*., 2012), indicating that the diets did not cause hypercholesterolemia in groups studied. This result may also be related to the composition of lipids contained in the different diets offered, and it should be considered that the cafeteria diet 1 and 2 groups had lower food intake, i.e., lower caloric intake. It can be extrapolated, considering the evolution of the results throughout the experiment, that more study time could have led to a change in serum lipid levels, producing dyslipidemia in cafeteria diet 1 and 2 groups, since the alteration in weight gain became apparent after 3 months, the time of euthanasia (Schrauwen *et al*., 2000; West and York, 1998; Santos *et al*., 2006). Regarding glucose, high values were observed, albeit not statistically different compared to the literature (Dantas*et al*., 2006;Spinelli *et al*., 2013;Melo *et al*., 2012). The glucosecurve after 1 monthshowed that the control group had a glycemic peak greater than in the other three groups, followed by a decrease in blood glucose levels as in the other groups. After 2 months, the glucose levels of the environmental alteration and cafeteria diet 2groups had a tendency to rise faster than in the control and cafeteria diet 1 groups. Similarly, the fall in glucose levels was sharp in theenvironmental alteration and cafeteria diet 1 groups. These results tended to be repeated at three months and might have been related to possible defects in insulin secretion or action by lower number of receptors or their affinity, or to a decrease in the amount of GLUT-4 transporter, which is responsible for glucose uptake by the cell. This causes large amount of glucose to remain in the external environment, and thus increase blood glucose levels (Santos *et al*., 2006). AST and ALT values were similar in all groups and to normal reference values (Dantas*et al*., 2006; Spinelli *et al*., 2013; Melo *et al*., 2012). High values are indicators of liver injury (Zamin*et al*., 2002), which was not identified in this study. Urea was highest in the environmental alteration group followed by the control group, with significant differences in relation to the other two groups.An increase in urea levels can be related to the presence of renal disorders, since a high value indicates that urea is not being excreted by the body, indicating dehydration, excess protein in the body or increased protein catabolism (Lima and Reis, 2012). The cafeteria diet 1 group had a low concentration of protein in its diet, and could be classified as a hypoproteic diet, contributing to the lowurea levels. Moreover, the literature shows controversy regarding the urea values in animals. Spinelli *et al*. (2013) reported values similar to those found in the control group of this study. However, other studies (Dantas *et al*., 2006; Melo *et al*., 2012) found much lower values. The latter cited authors obtained results showing excess urea and, therefore, likely development of kidney disease. Increased urea levels occurred in the control and environmental alteration groups because the diet of both groups had higher protein percentage. A diet rich in protein causes the body to use energy from gluconeogenesis releasing urea (Lima, 2003). Creatinine values in environmental alteration group was significantly lower compared to the other groups. Creatinine production is proportional to muscle mass, since it occurs mainly in skeletal muscle (Riehl *et al*., 2004; Burtis and Ashwood,1994). The fact that the environmental alteration group was subjected to environmental stress might have contributed to muscle reduction. Among the causes of an abnormal, low ratio of serum urea and creatinine levels, as observed in this study, are low protein intake and severe liver disease (Marshall, 2013). A high content of fat in the diet is an important factor for the development of hepatic steatosis picture in groups fed hyperlipidemic diets (Meijera *et al*., 2010, Osei-Hyiaman *et al*., 2008). This study proved the effectiveness of the development of non-alcoholic hepatic steatosis in animals fed diets with high lipid content. In the present study, it was observed that even diets described in the literature as effective in inducing metabolic disorders, did not completely result in the establishment of the disease in the animals tested. We conclude that further studies should be performed to develop an experimental feed which is able to generate the metabolic disorders in non-genetically modified rats equivalent to a metabolic disorders model similar to the development of this disease in humans currently, where it is also important to pay attention to the time required for disease induction in rats.

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