Protocol of Insulin Therapy For Streptozotocin-Diabetic Rats Based on a Study of Food Ingestion and Glycemic Variation

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Summary

Aim: An extensive volume of scientific work uses diabetic rats treated with insulin, but the protocols of treatment are poorly described. The purpose of this study was to analyze the glycemia and food intake behavior to establish the protocol of insulin treatment for these diabetic rats. The efficiency of our methodology was tested by evaluating the biochemical profile of the animals.

Methods and results: We used male Wistar rats with diabetes mellitus (DM) induced by streptozotocin. We analyzed the food intake and glycemic level variations hourly throughout 24h to determine the schedules and doses of insulin to be administered. Following this, we tested the efficiency of different doses and fractionation of NPH insulin in keeping the glycemic levels close to normoglycemia. The best daily dose of insulin was 5 U/day, 1 U being applied at 13h and the remaining 4 U at 19h. The efficacy of the insulin therapy was evaluated by comparing body weight and biochemistry parameters among the experimental groups, as well as glycemia measurement. Glycemic levels, total cholesterol, c-LDL and c-VLDL were re-established in diabetic rats treated with insulin (ITD). DM did not change the levels of c-HDL, triglycerides and fructosamine. Body weight gain was similar between control and ITD rats.

Conclusion: We established an insulin therapy that consists of a daily insulin dose for all animals to maintain most of them at or near normoglycemia. Our results provide, what is to our knowledge, the most detailed schedule of insulin therapy for treating STZ-diabetic rats.

Introduction

Diabetes mellitus (DM) is currently a major public health concern, because its incidence and prevalence are elevated and increasing, reaching epidemic proportions (*Wild et al., 2004)*. The global mortality attributable to diabetes in the year 2000 was estimated to be 2.9 million deaths, equivalent to 5.2% of all deaths. Diabetes is likely to be the fifth leading cause of death worldwide (*Roglic et al., 2005)*. Nowadays, the USA spends annually more than $130 billion on problems resulting from diabetes, being one of the greatest expenses for health systems in the whole world (*Barceló et al.,2003; Martin et al., 2007*). Diabetes can be diagnosed by the presence of four classic signs that include polyuria, polyphagia, polydipsia and, foremost, hyperglycemia (*American Diabetes Association, 2008)*.

Because of the importance of diabetes, several research groups have studied whether diabetic compli-

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cations can be prevented, reduced or even reversed through appropriate control of blood glucose levels, using exogenous insulin or oral hypoglycemiant agents (*Parra et al., 2005; Taniguchi et al., 2006; Zagon et al., 2006*). For the development of these studies, different models and strains of diabetic rats have been used (*Ferreira et al., 2005*; *Yono et al., 2005*; *Inouye et al., 2006*).

But, despite the extensive volume of scientific papers making use of diabetic rats treated with insulin, the treatment protocols are poorly described. Most of the papers just report the insulin doses and type (*Shimonura et al., 1990; Rastelli et al., 2005; Izbéki et al., 2008*).

There is no doubt that a suitable insulin treatment is absolutely necessary to obtain reliable results. Therefore, a detailed and well-founded methodology is necessary.

To establish a protocol for treatment of diabetes mellitus by administration of exogenous insulin, it is necessary to know the pattern of change, throughout the 24h day, of the feeding behavior and glucose levels of the animal models . The lack of data on feeding behavior and glycemic variation undermines any attempt at treatment of diabetic rats and produces inconsistent results.

Therefore, the purpose of this study was to determine the feeding behavior and glucose level variations in normal and streptozotocin diabetic rats throughout the 24h day to establish the protocol of insulin treatment and to test the efficiency of our methodology by evaluating the biochemical profile of the animals.

Materials and Methods

Animals and housing

Male Wistar rats, 60 days old at the beginning of the experiment, were obtained from the Center of Reproduction Biology of the Federal University of Juiz de Fora. The microbiological status of the rats was not specified. All the animals were kept in cages, in groups of three, in an environmentally controlled room (23 ± 2ºC and 12:12 light/dark cycle with the light on 0700-1900 h). The rats had *ad libitum* access to water and complete commercial chow (Nuvital™, Colombo, PR, BR). The chow was composed of 55% carbohydrates, 22% proteins, 4.5% fats and 18.5% mineral residues. Our experimental model, together with the way of taking care of the rats, were performed according to the precepts of the Brazilian College of Animal Experimentation (COBEA).

Induction of diabetes mellitus and animal sacrifice

The DM was induced in the rats, after 12h fasting, by intravenous injection of streptozotocin (STZ) diluted in 0.05M citrate buffer (50 mg/kg body weight). The control group received the citrate buffer injection without STZ. Two days afterwards, all the control group rats presented fasting glycemia of 60-100 mg/dL. The animals chosen as diabetic were those which presented fasting glycemia above 250 mg/dL; the animals that were not above this established standard were discarded from the study. Fasting glycemia was measured by the glucose oxidase method (*Bergmeyer and Bernt, 1974*) using a clinical glucometer (Roche). At the end of the experiments, the animals were weighed, sacrificed and then they had their blood collected.

Treatment of diabetic rats with human NPH insulin

To decide on the insulin treatment, food intake and glycemic level variation of the diabetic and control rats were analyzed hourly throughout 24h. This information was needed to determine the schedules and the doses of insulin to be administered to the animals. The food ingestion and the glycemic levels were measured by weighing the chow and collecting blood, respectively.

Glycemic level control of diabetic rats was made with subcutaneous injections of exogenous human NPH insulin. The objective of the insulin therapy was to keep the glycemia of these animals as close as possible to the normoglycemia (from 60 to 150 mg/dL) throughout the 24h day. Initially, the administration chosen was 4 U/day of NPH insulin. One unit of insulin was injected at 10h, another at 13h and the 2 remaining units at 19h. The insulin

was administrated when the rats presented high glycemic levels and food ingestion (*see results and discussion*). The control group received saline solution. The glycemia was measured every day at 13h and 19h.

Throughout the treatment this daily dose of insulin was adjusted on average every 3 days according to the glycemia of each animal. After testing the efficiency of different doses and fractionation of NPH insulin in keeping the glycemic levels close to normoglycemia, the best daily dose of insulin was considered to be 5 U/day, 1 U being applied at 13h and the remaining 4 U at 19h. To ratify the efficiency of the treatment, an experiment was carried out in which the animals had their glycemic levels monitored for 15 days. The glycemia of diabetic-STZ rats was measured 4 times during the treatment (day 2, 5, 10 and 15). At days 2, 5 and 10 the glycemia was measured at 19h and at day 15, the glycemic level was evaluated after 10h fasting, at 0800h .

Evaluation of the effectiveness of the treatment of diabetic rats with NPH insulin

The efficacy of treatment was evaluated by comparison of body weight, the fasting capillary glycemia and the serum levels of fructosamine, triglycerides, total cholesterol, HDL cholesterol (c-HDL), LDL cholesterol (c-LDL) and VLDL cholesterol (c-VLDL) among all three groups: controls (C), diabetic rats treated with insulin (ITD) and diabetic rats receiving saline solution (NITD). The experiment was carried out for 15 days. Amongst the animals of group ITD, the rats that presented fasting glycemia between 60 and 150 mg/dL at the end of the experiment were used. Immediately after the sacrifice, blood was collected from the aorta. The serum was used to quantify fructosamine, triglycerides, total cholesterol and c-HDL by colorimetric methods (*Fossati and Prencipe, 1982*; *Svensson et al., 1982; Baker et al., 1985*; *Kimberly et al., 1999*). The serum levels of c-VLDL and c-LDL had been calculated by the formula of Friedewald (*Friedewald et al., 1972*).

Statistical analysis

All results are expressed as mean ± SEM. The groups were compared using Student`s t-test and the results were considered statistically significant when $p < 0.05$.

Results

Feeding behavior and glycemic level variations of control and diabetic rats

Two days after STZ injection, diabetic-STZ rats did not increase chow consumption compared to controls. While the diabetic rats ate in mean 22.38 ± 0.84 g/day, the control rats ate 20.93 ± 0.99 g/day. Control rats ingested 54.8% of daily chow consumption during the dark phase; while diabetic rats ingested 54.4%. Therefore, the food intake between the dark and light phase of the photoperiod was the same in both groups.

The feeding behavior and glycemic level variations displayed by control and diabetic-STZ rats are shown in Figure 1. The peaks of food intake occurred at 19h and 21h in both groups. Additionally, a significant increase in food consumption was observed from 11h to 12h. Along with this, during the dark phase, the pattern of variation of chow consumption was similar in both groups. During the dark phase, 19h until 7h, the normal and diabetic rats showed two peaks in the food intake. The first was at 21h in both groups, and the second at 1h in the normal animals and at 2h in the diabetic animals. In both groups, these peaks were separated by a strong fall of the food intake at midnight.

There were no significant glycemic variations in diabetic and control animals during the day (Figure 1 A and B). As expected, non-diabetic rats presented smaller glycemic variations during the dark and light phases than diabetic rats. The control rats showed variation of 24.22 mg/dL during the light phase of the photoperiod and 22.45 mg/dL during the dark phase. The diabetic rats showed variation of 77.5 mg/dL during the light phase and 68.5 mg/dL during the dark phase of the photoperiod.

Figure 2 shows the chow ingestion variation during 24 hours. We observed that diabetic-STZ rats

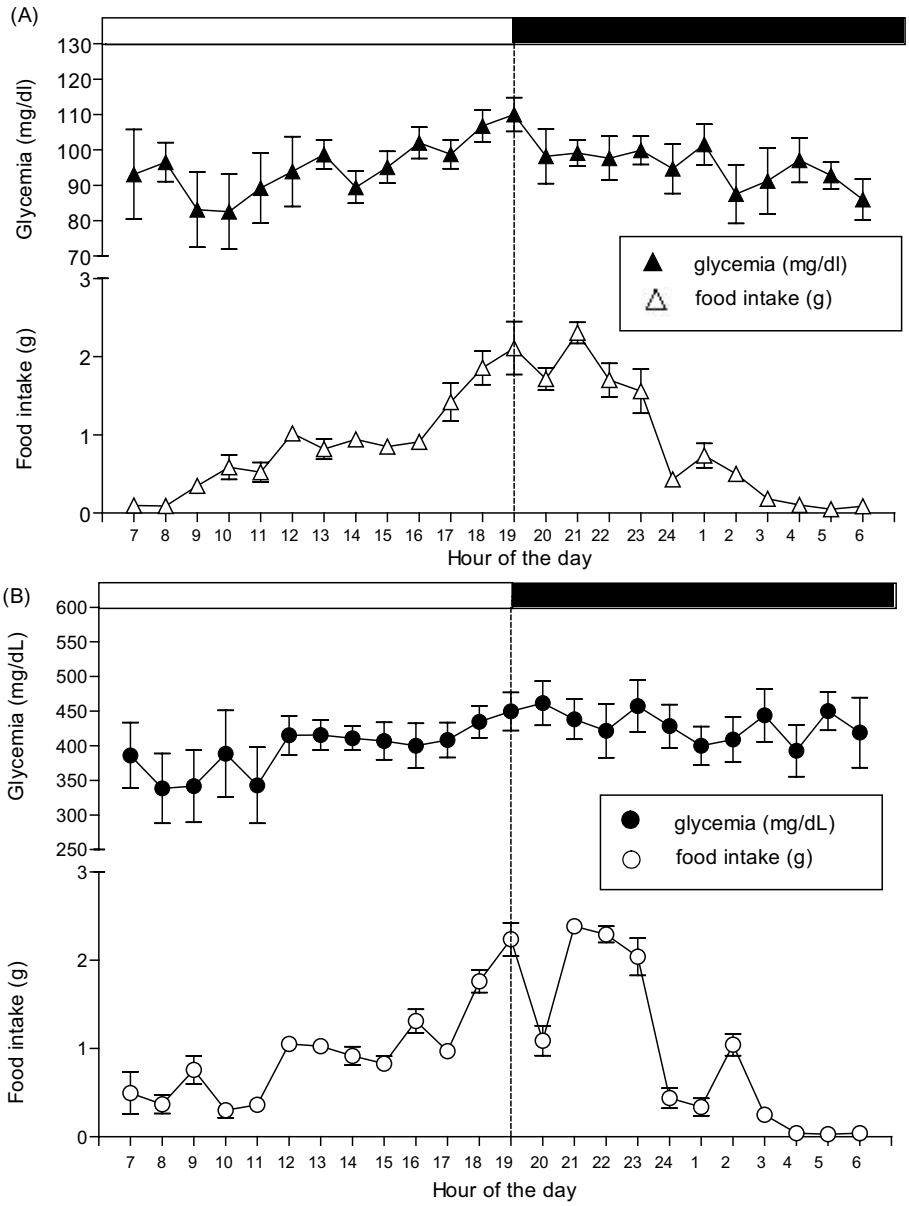

Figure 1. Glycemic level variations and feeding behavior of control and NITD rats during 24 hours. In (A) the glycemic level variations and feeding behavior of the control rats (n = 9) are shown. In (B) the glycemic variations (n = 10) and feeding behavior (n = 12) of NITD rats are shown. The photoperiod is indicated at the top of the figure: white bar represents the light phase and the black bar represents the dark phase. Vertical dashed line indicates the switch of photoperiod from light to dark phase. Vertical lines represent the SEM.

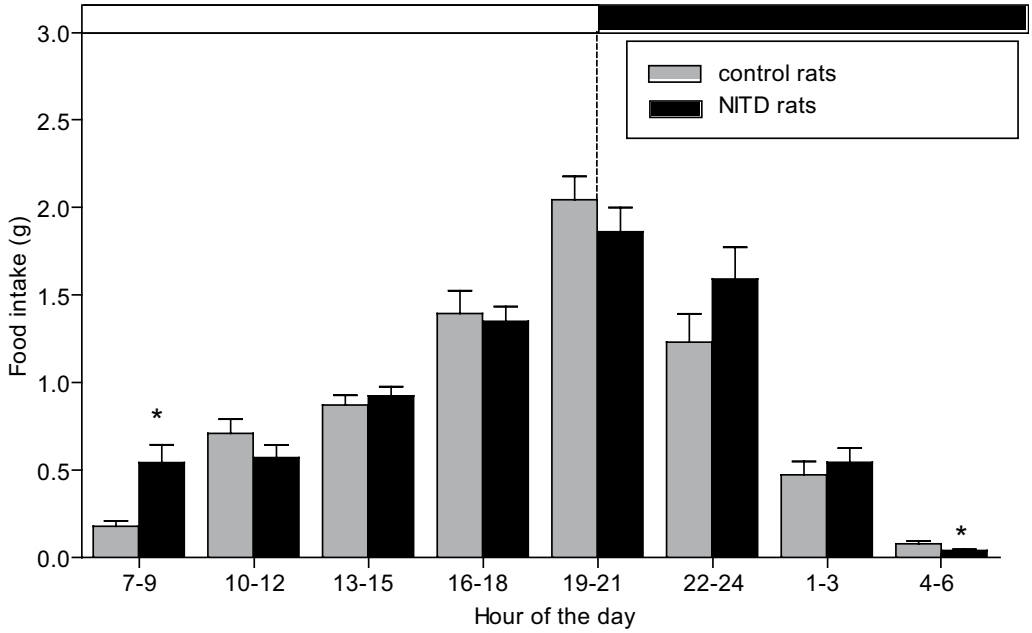

Figure 2. Variation of food intake by control (n= 9) and NITD (n= 12) rats during 24 hours. Data are arranged in intervals of 3 hours. The photoperiod is indicated at the top of the figure: white bar represents the light phase and the black bar represents the dark phase. Vertical dashed line indicates the switch of photoperiod from light to dark phase. All values represent mean ± SEM. *$p<0.05$ when compared with the control rats.

increased food intake for the first three hours of the light phase (7-9h) compared with controls. During the last hours of the dark phase there is also a statistical difference in the food intake, however control rats presented higher food intake.

Insulin treatment of diabetic-STZ rats

Initially, we investigated the best dose and schedule to inject insulin into animals. We started injecting insulin at 13h and 19h, according to the increased chow consumption observed. The insulin doses were adjusted until we obtained the desired results (see Material and Methods). After testing the efficacy of different daily doses of NPH human insulin in different fragmentations, we proposed that the best glycemic control was obtained with the dose of 5 U of insulin/day (1 U injected at 13h and 4 U at 19h). The glycemia was evaluated at 2, 5, 10, 14 and 15 days after the diabetes induction. The ITD rats presented glycemia of 438 ± 43 mg/dL on day 2, 132 ± 30 mg/dL on day 5, 124 ± 29 mg/dL on day 10 and 92.60 ± 7.15 mg/dL on day 15 (Figure 3).

Evaluation of the effectiveness of the treatment with NPH insulin on diabetic rats

To test the efficacy of the insulin treatment, the body weight and biochemistry parameters were evaluated. These results showed that body weight decreased significantly in NITD-STZ rats, which was reversed by the insulin treatment, reaching a value similar to the C group (Table 1). Glycemia of ITD rats was reduced significantly when compared with NITD-STZ rats. Total cholesterol, and LDL-c were improved by insulin treatment, ($p<0.05$), while the VLDL-c was restored by the insulin therapy. However, the fructosamine in ITD rats decreased by

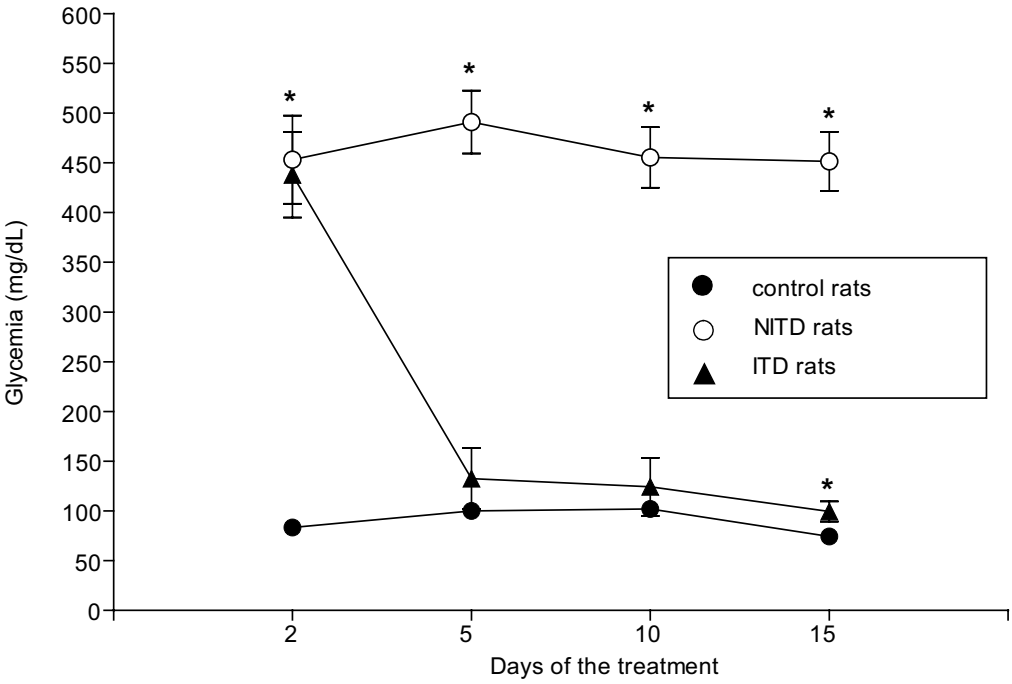

Figure 3. Effect of treatment of diabetic rats using 5IU of NPH insulin per day, 1U being injected at 13 hours and 4IU at 19 hours. All the values represent means ± SEM. Control rats (n= 6), NITD rats (n= 6) and ITD rats (n= 5) for days 2, 5 and 10. Control rats (n= 20), NITD rats (n= 15) and ITD rats (n= 23) for day 15. *p<0.05 when compared with the control rats.

21.67% compared with the control group (p<0.05). The NITD rats showed a decrease of total cholesterol and LDL-c levels, which is different to what happens in diabetic humans.

Discussion

Currently, the two most widely used chemicals for inducing experimental diabetes are alloxan (2, 4, 5, 6 tetraoxohexahydropyrimidine, CAS Nº 50-71-5) and streptozotocin (2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose, CAS Nº 18883-66-4) (*Lenze, 2008*). Alloxan and streptozotocin are toxic glucose analogues that preferentially accumulate in pancreatic ß cells via the GLUT2 glucose transporter (*Lenze, 2008*). Their diabetogenic action is due to the ability to destroy pancreatic ß cells (*Szkudelski et al., 2001*).

Pancreatic ß cell toxicity and the resultant diabetogenicity of alloxan are due to the generation of reactive oxygen species (ROS) in a cyclic redox reaction with its reduction product, dialuric acid. Autoxidation of dialuric acid generates superoxide radicals, hydrogen peroxide and, in a final iron-catalyzed reaction step, hydroxyl radicals. These hydroxyl radicals are ultimately responsible for the death of the beta cells (*Lenze, 2008*). The toxicity of STZ is related to the inhibition of the enzyme 0-GlcNAcase (N-acetyl-D-glicosaminidase) which removes protein linked GlcNAc (*Liu et al., 2000; Konrad et al., 2001; Szkudelski et al., 2001*). The increase in intracellular levels of proteins modified by GlcNAc results in cell death by apoptosis (*Konrad et al., 2001)*. Other diabetogenic actions of STZ are the production of reactive oxygen species (ROS)

Table 1. Effect of insulin treatment on plasma glycemia, variation of body weight and biochemical profile in rats.

	Control rats	ITD rats	NITD-STZ rats
Plasma glycemia (mg/ dL)	74.35 ± 1.37	99.43 ± 10.16*§	451.5 ± 29.63* #
Change in body weight (g) [a]	37.4 ± 3.4	43.4 ± 3.24	- 9.3 ± 6.62*#
Fructosamine (µmol/L)	299.5 ± 20.33	234.63 ± 20.77*§	307.38 ± 20.92
Triglycerides (mg/dL)	95.88± 6.93	97.75 ± 3.10	115.38 ±7.91
Total Cholesterol (mg/dL)	73.13 ±3.35	70.76 ± 4.09	60.13 ± 3.62*
HDL-c (mg/dL)	29.5 ± 2.24	34.13 ± 3.36	24.50 ± 1.64#
LDL-c (mg/dL)	24.75 ± 2.13	17.50 ± 4.30	11.63 ± 1.90*
VLDL-c (mg/dL)	18.88 ± 1.38	19.13 ± 1.17	24.00 ± 1.17* #

All values represent means ± SEM. For plasma glycemia and variation of body weight the number in each group was: Control rats (n= 20), NITD rats (n= 15) and ITD rats (n= 23). For all the other biochemical parameters evaluated, the number in each group was: Control rats (n= 8), NITD rats (n= 5) and ITD rats (n= 8).

[a] represents variation of body mass during the experimental procedure, 15 days.

* represents significant differences with $p<0.05$ compared to control rats;

§ $p<0.05$ compared with NITD rats

# $p<0.05$ compared with ITD rats.

and, perhaps, nitric oxide (NO) (*Szkudelski et al., 2001; Bolzán and Bianchi, 2002*). The pancreatic ß cells are particularly susceptible to the STZ action because the enzyme GlcNAc transferase (OGT), responsible for transfering 0-GlcNAc to proteins, is expressed at higher concentrations in the pancreatic ß cells than in any other cell (*Liu et al., 2000*). Also, the ß cell 0-GlcNAcase is more sensitive to inhibition by STZ than 0-GlcNAcase from other tissues. These two factors, high OGT expression and an STZ-sensitive O-GlcNAcase, appear to render ß cells selectively sensitive to this toxin (*Konrad et al.,2001*). Thus, an advantage of STZ to alloxan is its greater specificity to pancreatic ß cells (*Konrad et al.,2001*).

To establish the protocol of insulin therapy of STZ-

diabetic rats it was necessary to study the food consumption pattern and the fluctuation of glycemic levels in normal and diabetic rats throughout the entire day. Two days after STZ injection, diabetic rats did not present an increase in food intake compared to controls. Sprague Dawley rats injected with STZ 50 mg/kg or more showed moderate food intake depression at the first day after injection of STZ and then the ingestion stabilized at the pre-injection levels on the second and third days after injection (*Booth, 1972*). Food ingestion over the pre-injection levels was detected only from the fourth day after the treatment of animals with STZ (*Booth, 1972*). Our experiments were performed on the second day after DM induction. Therefore, our results corroborate these results. Several studies show that STZ-diabetic rats present a significant increase in food intake when compared to non-diabetic rats (*Castro and Balaguara, 1975; Shimonura et al., 1990*; *Plaza et al., 1993*). These investigations assessed this parameter several days after the induction of DM. We suggest that in our studies the period of only 2 days between the DM induction and the food intake measurement is too short to cause significant alteration in food ingestion.

Our control rats showed predominance of food consumption during the dark period. This information was revealed by analysis of area under curve comparison between the diurnal and nocturnal periods (data not shown). Others investigators have reported that healthy Wistar and Sprague-Dawley rats show predominantly nocturnal food intake (*Plaza et al., 1993; Demaria-Pesce and Nicolaidis, 1998*). Our STZ-diabetic rats did not maintain the same food intake pattern. Unlike the control group, the diabetic rats did not present a significant difference between diurnal and nocturnal food consumption, despite the smaller intake in the light phase. Our results show that the food intake behavior of diabetic animals is more heterogeneous and, even presenting similar food consumption to control group, when comparing the dark and light phase there are not significant differences. Plaza *et al.* (1993) also showed that diabetic rats present a reduction in the difference between the diurnal and nocturnal food ingestion, but this difference was still significant.

The schedule of insulin injections was determined in accordance with the food intake increase of diabetic rats. At 12h there was a new glycemic plateau which then rose to a peak at 19h. However it was decided to inject insulin at 13h due to proximity to that period of the day (8 until 11h) which presented a tendency to a reduction of food intake and glycemia. The injection of insulin at 12h could cause episodes of hypoglycemia in the rats throughout the treatment.

We established an insulin therapy that consists of a daily insulin dose for all animals to maintain most of the rats at or near normoglycemia.

The efficacy of the insulin therapy was evaluated by comparison of body weight and biochemistry parameters among the experimental groups, in addition to the glycemia measurement.

Diabetic rats display lower gain of body weight when compared to healthy animals (*Patel, 1983*; *Wilke and Hillard, 1994*). Our results corroborated these studies. Our NITD rats were emaciated. The control and ITD rats showed gain of body weight, which was similar between the two groups.

Table 1 shows that the diabetes significantly altered three of the six biochemistry parameters measured. Soltani *et al.* (2007) compared the lipid profile among control rats and acute (10 days) and chronic (8 weeks) STZ-diabetic rats. DM was induced using STZ 40 mg/kg body weight. Significant differences between control and acute diabetic rats were not observed, but all the parameters analyzed changed when compared with chronic diabetic animals (*Soltani et al., 2007*). Others studies did not observe changes in the lipidic profile of rats after 30 days from the STZ diabetes induction (*Pepato et al., 2002*; *Taniguchi et al., 2007*).

Our NITD-STZ rats showed decreased total cholesterol and c-LDL when compared with the control group, the contrary of what happens with humans. Although the STZ-diabetic rat is the most utilized model to study lipoprotein metabolism in diabetes, there are some physiological differences between

rats and humans that limit the application of the results in rats to humans (*Ebara et al., 1994*). In spite of this, it was reported that STZ-diabetic rats showed changes in lipid profile as happens in humans. Liptáková *et al.* (2002) observed these changes in Wistar STZ-diabetic rats (60 mg STZ/kg body weight) 8 weeks after the induction of the disease . Mahmud *et al*. (2004) observed similar results in Long Evans STZ-diabetic rats (90 mg/kg body weight) 100 days after the induction.

The level of fructosamine (a glycated serum protein) did not change in the NITD rats with regard to control rats. Shima *et al.* (1991) reported, following administration of streptozotocin to rats, that the average time to reach steady levels of glycated serum albumin was 3 days and, after starting the insulin therapy, the average time for this index returning to baseline values was 4.5 days. In this work high-performance liquid chromatography was used to determine rat serum glycated albumin. The duration of the experiment reported here, just 15 days, may not have been enough to increase significantly the concentration of glycated proteins in the plasma, because the analysis done by reduction of nitro blue tetrazolium refers to the glycemic variation of the last 20 days. On the other hand, in the ITD group a decrease in the level of fructosamine was observed. These decreases can be an indication of the occurrence of episodes of hypoglycemia. Haughton *et al.* (1999) reported that the attempted normalization of glucose values of diabetic Sprague Dawley was limited by hypoglycemia. In this investigation diabetes was induced by single intravenous injection of 110 mg STZ/kg body weight and the insulin dosage administered was determined by the glucose value for each individual rat. When the animals were treated with NPH insulin given twice daily they showed 4.9 episodes of moderate hypoglycemia (<70 mg/dl) and 2.7 episodes of severe hypoglycemia (<50 mg/dl) over a five day period.

Conclusion

In conclusion, these results provide what is, to our knowledge, the most detailed schedule of insulin therapy for treating STZ-diabetic rats, a critical parameter for achieving reproducible and comparable results. Also, we show that a single insulin treatment is not enough to make the whole diabetic group normoglycemic. There will always be some hypoglycemic and hyperglycemic animals. Considering that the insulin, *per se*, could be an important factor affecting the results, and since in most cases it may not be feasible to treat animals individually, studies will require larger groups of rats. Finally, we reassert that, as expected, the animal model does not reflect all the characteristics of the human disease but, even with this limitation, could be a useful model for studying several aspects of diabetes mellitus.

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